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CagY is an Immune-Sensitive Regulator of the *Helicobacter pylori* Type IV Secretion System

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Abstract

Background & Aims—Peptic ulcer disease and gastric cancer are most often caused by *Helicobacter pylori* strains that harbor the cag pathogenicity island (*cag*PAI), which encodes a type IV secretion system (T4SS) that injects the CagA oncoprotein into host cells. *cagY* is an essential gene in the T4SS and has an unusual DNA repeat structure that predicts in-frame insertions and deletions. These *cagY* recombination events typically lead to a reduction in T4SS function in mouse and primate models. We examined the role of the immune response in *cagY*-dependent modulation of T4SS function.

Methods—*H pylori* T4SS function was assessed by measuring CagA translocation and the capacity to induce interleukin-8 (IL8) in gastric epithelial cells. *cagY* recombination was determined by changes in PCR restriction fragment-length polymorphisms. T4SS function and

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cagY in *H pylori* from C57BL/6 mice were compared to strains recovered from *Rag1*−/− mice, T and B cell deficient mice, mice with deletion of IFNGR or IL10, and *Rag1*−/− mice that received adoptive transfer of control or *Ifng*−/− CD4+ T cells. To assess relevance to humans, T4SS function and *cagY* recombination were assessed in strains obtained sequentially from a patient after 7.4 years of infection.

Results—*H pylori* infection of T-cell deficient and *Ifngr1*−/− mice, and transfer of CD4+ T cells to *Rag1*−/− mice, demonstrated that *cagY*-mediated loss of T4SS function requires a T-helper 1-mediated immune response. Loss of T4SS function and *cagY* recombination were more pronounced in *Il10*−/− mice, and in control mice infected with *H pylori* that expressed a more inflammatory form of *cagY*. Complementation analysis of *H pylori* strains isolated from a patient over time demonstrated changes in T4SS function that were dependent on recombination in *cagY*.

Conclusions—Analysis of *H pylori* strains from mice and from a chronically infected patient showed that CagY functions as an immune-sensitive regulator of T4SS function. We propose that this is a bacterial adaptation to maximize persistent infection and transmission to a new host under conditions of a robust inflammatory response.

Keywords

IL8; bacteria; adaptation; stomach

INTRODUCTION

Approximately 10% of those infected with *Helicobacter pylori* will develop peptic ulcer disease and 1–3% will progress to gastric adenocarcinoma[1], the third most common cause of cancer death worldwide. The bacterial genetic locus most closely associated with development of peptic ulcer and gastric cancer is the *H pylori cag* pathogenicity island (*cag*PAI), a 40kb DNA segment that encodes a type IV secretion system (T4SS) that is essential for translocation of the CagA oncoprotein into host gastric epithelial cells[2]. A series of complex, T4SS-dependent changes in host cell signaling lead to actin cytoskeletal rearrangements, disruption of tight junctions, alterations in cell polarity, and the induction of proinflammatory cytokines, including interleukin-8 (IL8)[3].

A functional T4SS that translocates CagA and induces IL8 requires 18 genes on the *cag*PAI, including *cagY*[4]. CagY is an orthologue of VirB10, an essential component in the canonical T4SS of *Agrobacterium tumefaciens* and closely related systems in *Escherichia coli* and other Gram-negative bacteria. Protein-protein interaction studies[5] and negative stain electron microscopy[6] in *H pylori* suggest that CagY also forms part of a 41 nm core complex, which is substantially larger than in *E. coli* or *A. tumefaciens*[5]. CagY is also much larger than VirB10, ~220 kDa depending on the *H pylori* strain, and it is encoded by a gene that contains an extraordinary number of direct DNA repeats. *In silico* predictions suggest that these DNA repeats would generate in-frame insertions or deletions via homologous recombination, yielding numerous theoretical variants of the *cagY* allele[7]. Immunogold labeling of CagY demonstrates that this repeat region is localized to the bacterial surface[8]. Thus, CagY has several features that distinguish it from other VirB10 orthologs, which suggests that it may be functionally unique.

It has been known for many years that passage of *H pylori* in mice leads to loss of T4SS function[9], though the mechanism was unknown. We recently demonstrated that recombination in the *cagY* repeat region during colonization of mice often yields *cagY* variants that form a non-functional T4SS pilus that does not translocate CagA or induce IL8, though the CagY protein is expressed[8]. Similar observations were made in the rhesus macaque model, where we could also demonstrate CagY-mediated gain of T4SS function. Loss of T4SS function and recombination of *cagY* did not occur in *Rag1*−/− mice, which do not have functional B or T cells, suggesting that CagY-mediated modulation of T4SS function occurs in response to selective pressure by the adaptive immune system[8].

H pylori infection of the gastric mucosa triggers a predominantly CD4+ T cell response that differentiates towards a Th1 phenotype, with expression of interferon gamma (IFNγ) and other proinflammatory cytokines that are essential for development of *H pylori* induced gastritis and control of bacterial burden[10, 11]. Here we used the mouse model to test the hypothesis that this Th1-biased immune response is also required for selection of *cagY* variants that have lost T4SS function during persistent *H pylori* infection. Using knockout mice and adoptive transfer experiments, we demonstrate that IFNγ and CD4+ T cells are essential for selection of *cagY*-mediated loss of T4SS function. Moreover, we show that *cagY* recombination and loss of T4SS function rescues *H pylori* colonization in *Il10*−/− mice, which have an exaggerated inflammatory response to *H pylori* infection. Analysis of paired patient isolates collected over many years demonstrates that *cagY* recombination can modulate T4SS function during chronic *H pylori* infection in humans. These results suggest that CagY functions as an immune-sensitive molecular regulator that modulates T4SS function.

METHODS

H pylori strains and culture

H pylori strains (Table S1) were cultured on brucella agar (BBL/Becton Dickinson, Sparks, MD) supplemented with 5% heat-inactivated newborn calf serum (NCS, Invitrogen, Carlsbad, CA) and ABPNV antibiotics (amphotericin B, 20 μg/ml; bacitracin, 200 μg/ml; polymyxin B, 3.3 μg/ml; nalidixic acid, 10.7 μg/ml; vancomycin, 100 μg/ml), unless otherwise indicated. Cultures were incubated at 37°C under microaerophilic conditions generated by a 5% CO2 incubator or by a fixed 5% O_2 concentration (Anoxomat, Advanced Instruments, Norwood, MA).

Animals and experimental challenge

Specific-pathogen free female mice (Table S3) from Jackson Laboratories were housed in microisolator cages and provided with irradiated food and autoclaved water *ad libitum*. At 10 to 12 weeks of age mice were challenged with 2.5×10^9 CFU of *H pylori* suspended in 0.25 ml of brucella broth administered by oral gavage. Mice were euthanized between 2 and 16 weeks PI with pentobarbital sodium injection (50 mg/ml IP). Stomachs were cut longitudinally, and half was homogenized and plated by serial dilution on brucella agar supplemented with 5% NCS and ABPNV. Multiple single colony isolates (3-6/mouse) were

characterized by *cagY* PCR-RFLP and for their capacity to induce IL8 in AGS gastric epithelial cells.

Study Approval

Experiments were carried out at the University of California, Davis under protocols approved by U.C. Davis Institutional Animal Care and Use Committee, which has been accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. All animal experiments were performed in accordance with NIH guidelines, the Animal Welfare Act, and U.S. federal law.

IL8 ELISA

H pylori induction of IL8 was measured as described previously[12]. WT *H pylori* PMSS1, its isogenic *cagY* deletion, and brucella broth were included on every plate as positive and negative controls. IL8 values were normalized to WT *H pylori*, arbitrarily set to 1.0.

Adoptive Transfer

Rag1−/−mice were reconstituted with 1×10^6 CD4+ T cells isolated from WT or *Ifnγ*−/− C57BL/6 mice, which had been infected for 8 weeks with PMSS1. Mice were euthanized with pentobarbital sodium injection (50 mg/ml IP) and single cell suspensions were obtained by passing spleens through a 40μm cell mesh into PBS. Cells were pelleted by centrifugation and erythrocytes lysed for 2 min at room temperature with AKC buffer (0.15M NH_4Cl, 10mM $KHCO_3$, 0.1mM EDTA, pH7.35). Cells were washed with PBS, pooled, and CD4+ T cells were isolated by using anti-CD4+ magnetic beads (Miltenyi, San Diego, CA) over a magnetic column, resulting in > 90% purity of CD3+CD4+ cells demonstrated by flow cytometry. CD4+ T cells were resuspended in PBS at 5×10^6 cells/ml per 200μl and injected into uninfected *Rag1*−/− mice via tail vein.

Flow Cytometry

Following RBC lysis, splenocytes were resuspended and washed once in FACS buffer (1XPBS, 0.5% BSA). Cells were stained with anti-CD3 FITC (clone 17A2, BD Biosciences) and anti-CD4 PE (clone RM4-5, BD Biosciences) in FACS stain buffer (FACS buffer, 20% mouse serum) for 15 minutes at room temperature. Cells were washed two times in FACS buffer and fixed with 2% PFA. Data were collected on a BD FACSCalibur and analyzed using FlowJo software 8.8.7 (Treestar).

Immunoblots and CagA translocation

Immunoblots of CagA and CagA translocation were performed as described previously using an MOI of 100:1 and 22 hours of culture at 37°C in 5% CO_2[8]. CagY expression was detected by electrophoresis of sonicated bacterial proteins on a 7.5% polyacrylamide gel, incubating with rabbit antiserum (1:10,000) to CagY[7] as primary antibody and HRP-conjugated anti-rabbit IgG (1:20,000) as secondary antibody.

cagY PCR-RFLP

cagY genotyping was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) using primers in Table S4 as previously described[8]. Changes in PCR-RFLP patterns are referred to as recombination based on DNA sequence analysis from prior experiments[8], although this was not formally demonstrated.

Contraselection for genetic exchange of *cagY*

Alleles of *cagY* were exchanged between *H pylori* strains using contraselectable streptomycin susceptibility as described previously[8] using primers in Table S4 and plasmid constructs in Table S2.

Competition experiment

cagY was deleted from PMSS1 and replaced with either wild type PMSS1 *cagY* (PMSS1Δ*cagY*[PMSS1]) or SS1 *cagY* (PMSS1Δ*cagY*[SS1]) using contraselection. Strains expressing *cagY* from PMSS1 or SS1 were also marked by replacing bases 343–360 of the *rdxA* locus with an antibiotic resistance gene encoding either kanamycin or chloramphenicol resistance, respectively. Briefly, plasmid pJ318 (Table S2) was constructed in pBluescript SK- by amplifying fragments 1194 bp upstream and 904 bp downstream of the *rdxA* deletion site in PMSS1, and ligating to a kanamycin resistance cassette[13]. pJ318 was then used to naturally transform PMSS1Δ*cagY*[PMSS1] with selection on 25 μg/ml kanamycin. pJ319 (Table S2), which was created in the same fashion but with a chloramphenicol resistance gene, was similarly used to naturally transform PMSS1Δ*cagY*[SS1], with selection on 5 μg/ml chloramphenicol. Gastric tissue from mice challenged with a 1:1 mixture of the marked PMSS1Δ*cagY*[PMSS1] and PMSS1Δ*cagY*[SS1] strains was plated separately on brucella agar with ABPNV plus either kanamycin or chloramphenicol to enumerate the relative abundance of each strain. This was expressed as a log10 competition index calculated as CFU of PMSS1Δ*cagY* [SS1]/[PMSS1], normalized to the abundance of each strain in the input inoculum.

Histology Scoring

Sections of the glandular stomach were formalin fixed and stained with hematoxylin and eosin. Each microscopic field was scored separately for the presence or absence of neutrophilic infiltration (polymorphonuclear leukocytes), gastritis, and epithelial metaplasia using a system previously validated in mice[14]. The results were reported as the mean percentage of fields affected for each mouse averaged over the three histologic criteria (minimum10 fields/sample).

Statistics

H pylori colonization (CFU/g) was analyzed using a 2-tailed Student's *t* test (Prism 6.0) after log10 transformation. Normalized IL8 levels were compared between groups using Wilcoxon rank-sum tests. Analysis of gastritis and proportions of samples with changed *cagY* were compared between groups using chi-square tests. In experiments with more than two groups, logistic regression was used to evaluate pairwise differences in proportion of

output strains with recombination in *cagY*. A P value ≤ 0.05 was considered statistically significant.

RESULTS

CD4+ T cells are required for *in vivo* selection of *cagY* recombination and loss of T4SS function

H pylori SS1 is a mouse-passaged strain that was proposed as the standard for *H pylori* studies in mice[15], and was later found to have a defective T4SS that we showed was a result of *cagY* recombination[8]. PMSS1, which has a functional T4SS and readily colonizes mice, is the original *H pylori* human isolate that gave rise to the SS1 strain after serial passage mice[16]. Unlike in wild type (WT) mice, PMSS1 does not undergo *cagY* recombination or lose T4SS function when recovered from *Rag1*−/− mice[8], which do not have functional B or T cells. To identify which arm of the adaptive immune response is responsible for loss of T4SS function, knockout (KO) mice lacking functional B cells (μMT) or T cells (TCR β/δ−/−) were challenged with PMSS1 and sacrificed 8 weeks post infection (PI). WT and *Rag1*−/− mice were challenged simultaneously as controls. The *H pylori* bacterial burden in *Rag1*−/− and T cell KO mice was approximately 10-fold higher than in WT mice and in B cell KO mice (Figure 1A). Loss of T4SS function (reduced IL8 induction) and recombination in *cagY* (defined as a change in PCR-RFLP) occurred commonly during infection of WT and B cell KO mice, but never in RAG−/− mice and only occasionally in T cell KO mice (Figure 1B, C).

Since *H pylori* infection in mice primarily triggers a CD4+ Th1 immune response[10, 11], we also asked if CD4+ T cells alone could select for *H pylori* with *cagY* alleles that encode a nonfunctional T4SS that is no longer capable of inducing IL8. To examine this possibility, we performed adoptive transfer experiments in which CD4+ T cells were isolated from WT mice that were infected with *H pylori* for 8 weeks, and then transferred into *Rag1*−/− mice (*Rag1*−/−$^{\text{WT CD4+}}$) 24 hours before *H pylori* challenge. Flow cytometry on splenocytes from adoptively transferred mice demonstrated engraftment, with a mean of 8.9% (± 1.1% SEM) CD3+CD4+ cells in the lymphocyte gate. The bacterial burden 8 weeks PI was significantly lower in *Rag1*−/−$^{\text{WT CD4+}}$ mice than in *Rag1*−/− mice (Figure 1A). It was also lower than in WT mice, which has been observed previously and likely reflects a relative failure of Treg engraftment[17]. Loss of T4SS function (Figure 1B) and recombination in *cagY* (Figure 1C) were also more common in *Rag1*−/−$^{\text{WT CD4+}}$ mice than in RAG−/− mice, though the difference in IL8 induction did not reach statistical significance. Together, these data suggest that CD4+ T cells are essential for control of bacterial burden and for selection of *H pylori* with CagY variants that form a non-functional T4SS.

Selection of *cagY* variants and loss of T4SS function requires IFNγ signaling

The development of gastritis and control of *H pylori* bacterial burden are mediated by CD4+ T cells[18], which also drive CagY-mediated loss of T4SS function (Figure 1). Since CD4+ T cells are a major source of IFNγ, we next asked if loss of T4SS function is mediated downstream of IFNγ. WT mice and mice lacking the IFNγ receptor (*Ifnγ R*−/−) were infected with *H pylori* PMSS1 and sacrificed 4 or 8 weeks PI. Similar to the bacterial burden

in *Rag1*−/− mice, *Ifnγ R*−/− mice were colonized at approximately 10-fold higher levels than WT mice (Figure 2A). *H pylori* isolated from WT mice 4 and 8 weeks PI showed gradual loss of T4SS function associated with recombination in *cagY*. In contrast, *H pylori* from *Ifnγ R*−/− mice retained T4SS function (Figure 2B) and showed no *cagY* recombination (Figure 2C). To determine if IFNγ from CD4+ T cells alone is sufficient for selection of *cagY* variants and loss of T4SS function, we performed adoptive transfer. CD4+ T cells from *Ifnγ*−/− mice infected with PMSS1 for 8 weeks were adoptively transferred into *Rag1*−/−mice (*Rag1*−/−$^{\text{Ifn}\gamma^{-/-}\ \text{CD4+}}$), which were then infected with *H pylori* PMSS1 and sacrificed 8 weeks PI. Flow cytometry on splenocytes from adoptively transferred mice demonstrated engraftment, with a mean of 9.3% (± 0.7% SEM) CD3+CD4+ cells in the lymphocyte gate. Adoptive transfer of *Ifnγ*−/− CD4+ T cells was sufficient to control bacterial load (Figure 2D), but did not select *H pylori* variants with loss of T4SS function (Figure 2E) or recombination in *cagY* (Figure 2F). These results indicate that signaling downstream of IFNγ derived from CD4+ T cells is essential for CagY-mediated loss of T4SS function, but is not strictly required for control of *H pylori* bacterial load.

Variation in *cagY* functions as a molecular rheostat to alter the inflammatory capacity of *H pylori*

Most *H pylori* strains that recombined *cagY* showed a markedly reduced capacity to induce IL8, though we occasionally identified strains with changes in *cagY* but intermediate levels of IL8 induction (e.g., WT infected mice 8 weeks PI, Figure 2B). This suggests that recombination in *cagY* may function to modulate T4SS function rather than eliminate it. In other words, *cagY* may function more like a rheostat than a switch. To test this hypothesis, we first identified mouse output strains with unique *cagY* RFLP patterns (Figure 3A) that reproducibly showed high (Out1, *cagY* PCR-RFLP equivalent to WT PMSS1), intermediate (Out2), or low (Out3) induction of IL8 (Figure 3B, grey bars). We next used contraselection[8] to replace the *cagY* gene in PMSS1 with that from each output, which was confirmed by PCR-RFLP (Figure 3A). Transformants complemented with *cagY* from output strains (ΔY[Out1], ΔY[Out2], ΔY[Out3]) restored the capacity to induce IL8 (Figure 3B, white bars) and translocate CagA (Figure 3C) to levels similar to that of the respective output strain. These results suggest that different *cagY* alleles vary in the extent to which they enable the bacterial cell to induce IL8 and translocate CagA, and that recombination in *cagY* functions as a molecular rheostat to modulate *H pylori* T4SS function.

cagY recombination and loss of T4SS function are strain-dependent and associated with the capacity of *H. pylori* to induce inflammation

H pylori strains encoding a T4SS can differ markedly in their capacity to induce IL8, despite having an intact *cag*PAI[19]. Since loss of T4SS function and *cagY* recombination are immune driven, we hypothesized that the capacity of *H pylori* to induce IL8 would be inversely related to *cagY* recombination and loss of T4SS function during *in vivo* infection. To test this hypothesis, we first compared the *in vitro* response of AGS gastric epithelial cells to *H pylori* strains J166 and PMSS1, which show relatively low and high induction of IL8, respectively (Figure 4A). Mice infected with PMSS1 had significantly more inflammation in the gastric mucosa compared to J166 (Figure 4B, Figure S1), though bacterial loads were similar (data not shown). Consistent with the greater capacity of *H pylori* PMSS1 to induce

IL8 *in vitro*, and induce inflammation *in vivo*, PMSS1 infected mice also showed more rapid and more complete loss of T4SS function that was associated with *cagY* recombination (Figure 4C,D).

To examine this more systematically using isogenic strains, we next infected WT C57BL/6 mice with PMSS1 bearing the *cagY* from Out1 or Out3, which have high and low T4SS function (Figure 3), respectively, and sacrificed them 8 weeks PI. Colonization density was significantly greater in mice challenged with ΔY[Out3], which has poor T4SS function, compared to ΔY[Out1] (Figure 4E). Recombination in *cagY* occurred only in output colonies from mice infected with ΔY[Out1] (Figure 4F), though the frequency in this experiment was lower than observed previously. Similarly, complete elimination of T4SS function by deletion of *cagE*[16], which encodes an ATPase that is essential for T4SS function, increased bacterial load and eliminated recombination in *cagY* 8 weeks PI (Figure S2). These data suggest that control of bacterial load and selection of *H pylori* with a nonfunctional T4SS are enhanced in *H pylori* strains that induce a more robust host immune response.

Competitive advantage of CagY-mediated loss of T4SS function increases progressively during *H pylori* infection

Recombination in *cagY* and loss of T4SS function increase over time during infection of WT mice, beginning around 4 weeks PI. Since loss of T4SS function is immune-mediated, this may simply reflect the time required for development of adaptive immunity. On the other hand, we previously reported that early during infection of rhesus macaques we could detect *cagY*-mediated gain of T4SS function[8], suggesting the possibility that there may be selection for a functional T4SS very early during infection. To address this question, we used contraselection to construct isogenic strains of PMSS1 bearing either the WT *cagY* (PMSS1Δ*cagY*[PMSS1]) or the non-functional *cagY* from SS1 (PMSS1Δ*cagY*[SS1]), which were marked respectively in the neutral *rdxA* locus with antibiotic resistance to kanamycin or chloramphenicol. We then performed a competition experiment in which WT C57BL/6 mice were inoculated with a 1:1 mixture of both strains and sacrificed between 1 and 8 weeks PI. Gastric contents were plated on kanamycin and chloramphenicol to permit calculation of a competition index. The results demonstrated progressive selection for loss of T4SS function beginning 4 weeks PI, with > 300-fold competitive advantage by 8 weeks PI (Figure 5A). As expected, competition index showed a strong inverse correlation (R^2=0.64, $P \leq 0.0001$) with IL8 induction performed on colony sweeps from each mouse (Figure 5B), which confirms that loss of T4SS function was due to selection for PMSS1Δ*cagY*[SS1], and not to a mutation in PMSS1Δ*cagY*[PMSS1]. These results are consistent with progressive loss of T4SS function that results from development of adaptive immunity, with no fitness advantage to a functional T4SS early during infection in mice.

CagY-mediated loss of T4SS function promotes bacterial persistence in the setting of increased inflammation

CagY-mediated loss of T4SS function occurs less commonly in mice with impaired immunity (Figures 1, 2). This suggests that mice with an enhanced immune response might have a greater selection for *H pylori* strains with loss of T4SS function, which might be a

bacterial strategy to persist in the face of inflammation. To test this hypothesis, we infected *Il10*−/− mice with *H pylori*, which triggers a robust inflammatory response with severe gastritis[20, 21] and increased levels of IFNγ and other Th1 cytokines[22] compared to WT mice. Because we anticipated that the aggressive inflammatory response would clear the infection at later time points, mice were sacrificed at 2 and 4 weeks PI, rather than 8 weeks PI as usual. *Il10*−/− mice infected with *H pylori* PMSS1 showed more inflammation (Figure 6A) and a lower bacterial burden (Figure 6B) compared to WT mice. Colonization in *Il10*−/− mice was significantly lower than in WT—often near the limit of detection (~100 CFU/g) 4 weeks PI (Figure 6B)—and was undetectable in 3 mice. At 4 weeks PI, recombination in *cagY* occurred in 22 of 82 colonies recovered from *Il10*−/− mice but only 10 of 75 colonies from WT mice (chi-square=4.23, $P \leq 0.05$, Figure 6C). All colonies recovered 4 weeks PI from the 3 mice that showed the highest colonization levels (similar to WT mice), also induced a low level of IL8 and showed recombination in *cagY* (Figure 6B,C; bracketed data points). Moreover, the average IL8 induction of *H pylori* isolates from *Il10*−/− mice showed a highly significant inverse correlation with the bacterial burden (Figure 6D). These data suggest that CagY-mediated loss of T4SS function allows for increased *H pylori* colonization in the face of a robust immune response.

Recombination in *cagY* modulates T4SS function during chronic infection in humans

T4SS function (IL8 induction) is highly variable among *H pylori* clinical isolates, even when the *cag*PAI is fully intact[19]. The explanation for this is unknown, but it is intriguing that *cagY* is under strong diversifying selective pressure—second only to *cagA* among genes on the *cag*PAI[19]. To examine the possible role of *cagY* recombination in modulating T4SS function during chronic infection of humans, we examined paired isolates that were previously collected from 14 patients over intervals ranging from 3.0 to 10.2 yrs (mean=6.1 yrs). Multilocus sequencing typing analysis demonstrated that each pair was clonal, but showed microevolution during prolonged infection[23]. Of the 14 pairs, one showed changes in *cagY* PCR-RFLP (Figure 7A) together with a significant decrease in the capacity to induce IL8 between the A and B isolates, which were collected 7.4 years apart (Figure 7B). Deletion of *cagY* completely eliminated IL8 induction in both the A and B isolates (Figure 7B), which demonstrated that the T4SS was intact in both. To determine if recombination in *cagY* was responsible for the change in T4SS function, we used contraselection to exchange *cagY* genes between the A and B isolates, and confirmed it by PCR RFLP. Exchange of *cagY* genes demonstrated that change in *cagY* was sufficient to explain the differences in IL8 induction of the A and B isolates (Figure 7B). Control experiments in which *cagY* was deleted from the A and B strains, and then reinserted by contraselection, recovered the IL8 induction of the parent strain (data not shown). These results demonstrate that recombination in *cagY* during chronic human infection can modulate T4SS function.

DISCUSSION

The T4SS system encoded on the *cag*PAI is the key bacterial virulence factor associated with progression to peptic ulcer disease or gastric cancer, rather than asymptomatic gastritis. Analysis of the PAI *in vivo* has been hampered by the observation that T4SS function is lost during experimental infection of mice[9], which was initially viewed as an artifact of infecting

mice with a bacterium that is naturally found only in humans and some non-human primates. The mechanism was unknown. We recently demonstrated that loss of T4SS in mice is typically due to in-frame recombination in the middle repeat region of the *cagY* gene, which encodes an essential component of the *H pylori* T4SS[4]. Loss of T4SS does not occur in RAG−/− mice, which lack functional B or T cells[8]. While indels or SNPs in any of the essential genes can result in loss of T4SS function, *cagY* seems specifically designed for recombinatorial variation, suggesting that it is a bacterial contingency locus[24] that modulates or "tunes" the host inflammatory response.

Here we have further investigated the immunologic basis and functional significance for loss of T4SS function during *H pylori* infection of mice and humans. Previous investigators speculated that recombination in *cagY* was a form of antigenic variation to avoid antibody responses directed against a surface-exposed component of the *H pylori* T4SS pilus[7]. However, this was difficult to reconcile with the general lack of human antibody response to CagY[7] and the evidence that humoral immunity is generally not thought to play an important role in control of *H pylori* infection[25]. Our results suggest that loss of T4SS function and recombination in *cagY* are largely independent of B cells, but instead require CD4+ T cells expressing IFNγ (Figures 1, 2). The modest loss of T4SS function we observed in B cell KO mice (Figure 1B) may actually reflect a decrease in B cell-mediated immunoregulation[26], rather than B cell control of infection. Together, these observations are consistent with seminal vaccine studies demonstrating that MHC class II-restricted, Th1-polarized T cells are essential to control *H pylori* infection[27, 28].

Several lines of evidence suggest that the variation in *cagY* and T4SS function that we have observed in animal models is relevant to human *H pylori* infection. First, it is not simply an artifact of the mouse model, because *cagY* mediated loss of T4SS function also occurs in rhesus macaques[8], which most closely mimic human infection. Second, *cagY* recombination can both up- and down-modulate T4SS function[8], in a graded fashion (Figure 3), suggesting that this observation is a window into the biology of *H pylori*, which actually has the capacity to "tune" or optimize the host inflammatory response to achieve a homeostatic balance. Third, there is marked variability in the capacity of different PAI positive *H pylori* strains to induce IL8, varying by up to 20-fold[19]. Since *cagY* is second only to *cagA* in the percentage of codons under positive selection[19], CagY diversity may be important for adaptation to chronic human infection. Most importantly, here we demonstrate that *cagY* recombination within an individual patient can modulate T4SS function (Figure 7). The *cagY* variants in the sequential isolates might represent the dominant population present at each time point, which underwent recombination and functional change under pressure from changes in, for example, host physiology. Alternatively, we cannot exclude the possibility that the *cagY* variants we observed represent diversity that was present at each time point, because only a single A and B isolate were examined. Regardless of which scenario is correct, these results provide proof-of-principle that *cagY* recombination can modulate T4SS function during *H pylori* infection in humans. *H pylori* adaptation during acute and chronic human infection has also been demonstrated at other virulence loci, such as the *babA* adhesion[29, 30], and more broadly using whole genome sequencing[31].

When might *cagY* recombination occur during human infection, and why? Clearly, a functional T4SS enhances bacterial fitness, probably by increasing its capacity to acquire iron and other nutrients[32–34]. But rearrangement in *cagY* that confers loss of T4SS function may be more advantageous under conditions that are unfavorable for *H pylori* growth, because loss of T4SS function decreases the host inflammatory response, increases bacterial load[16, 35], and thus increases the likelihood of transmission to a new host. It may occur soon after acquisition, as in mice and monkeys, or perhaps during some environmental event, which from the bacterial perspective tips the balance for or against inflammation. If so, it may be difficult to "catch it in the act" because acute *H pylori* infection is rarely detected in humans and the hypothetical environmental events are unknown. One possibility is that *cagY*-mediated down regulation of T4SS function is a bacterial strategy to persist in the setting of an intercurrent infectious disease such as malaria, tuberculosis or typhoid—which, along with *H pylori*, have evolved with humans since antiquity and might cause sufficient systemic inflammation that would otherwise reduce *H pylori* colonization and perhaps even clear the infection. A similar phenomenon has been observed experimentally in the mouse model of herpes viruses, where viral infection can non-specifically protect against bacterial challenge with *Listeria* or *Yersinia pestis* by inducing IFNγ and systemic activation of macrophages[36]. This hypothesis is also supported by the results in *Il10*−/− mice, which have an exaggerated inflammatory response to *H pylori* and demonstrate a strong inverse correlation between bacterial load and *cagY*-mediated loss of T4SS function (Figure 6). Alternatively, perhaps *cagY*-mediated loss of T4SS enhances bacterial persistence in the setting of atrophic gastritis, in which elevated gastric pH reduces *H pylori* burden and sometimes leads to bacterial clearance[37]. Both are testable hypotheses that we are currently examining.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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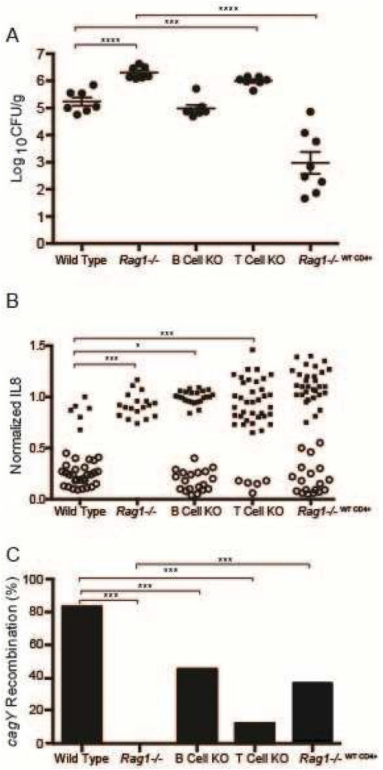

Figure 1. CD4+ T cells are required to control *H pylori* colonization density and select strains with loss of T4SS function and recombination in *cagY*

(A) *H pylori* colonization density was significantly greater in *Rag1*−/− and T cell KO mice than in wild type mice. Adoptive transfer of WT CD4+ T cells into *Rag1*−/− mice markedly reduced *H pylori* colonization compared to *Rag1*−/−. Each data point represents CFU/g for an individual mouse 8 weeks PI (N=7–8 mice/group). Horizontal lines indicate mean ± standard error of the mean (SEM). (B) Single colonies recovered from WT and B cell KO mice, and mice adoptively transferred with WT CD4+ T cells, showed marked loss in the capacity to induce IL8 that was accompanied by recombination in *cagY* (open circles). In contrast, all colonies from *Rag1*−/− mice and most from T cell KO mice induced IL8 and had the same *cagY* RFLP (closed circles) as WT *H pylori* PMSS1. Each data point represents the result from a single colony (N=3-6 colonies/mouse). (C) Percent of colonies that underwent *cagY* recombination (open circles divided by total colonies for each group in panel B). *P≤0.05, **P≤0.01, ***P≤0.001.

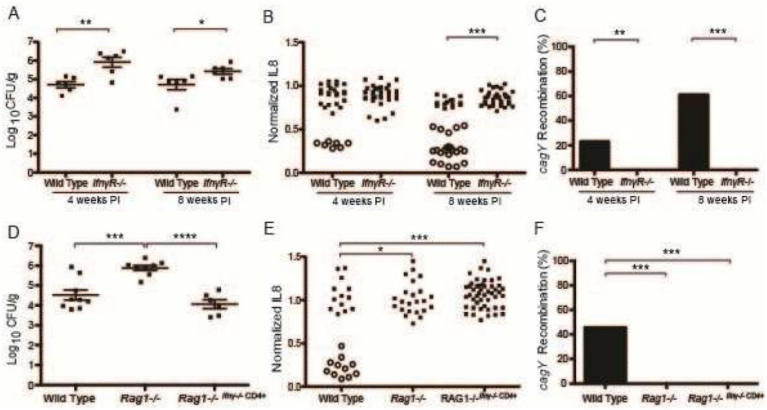

Figure 2. Selection of CagY variants is mediated downstream of IFN-γ signaling

(A) *H pylori* colonization density was significantly higher in *Ifnγ R*−/− mice compared to WT at both 4 and 8 weeks PI. Each data point represents CFU/g from an individual mouse (N=6/group). (B) Single colonies (N=3-6/mouse) recovered from WT mice showed loss in the capacity to induce IL8 that was associated with recombination in *cag Y* (open circles), but colonies from *Ifnγ R*−/− mice induced IL8 similarly to WT PMSS1 and had no changes in *cag Y* (closed circles). (C) Percent of colonies that underwent *cag Y* recombination (open circles divided by total colonies for each group in panel B). Adoptive transfer of *Ifnγ*−/− CD4+ T cells into *Rag1*−/− mice was sufficient to control bacterial load 8 weeks PI (D), but did not select *H pylori* variants with loss of IL8 induction or change in *cag Y* PCR-RFLP (E,F). Horizontal lines indicate mean ± SEM. *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001

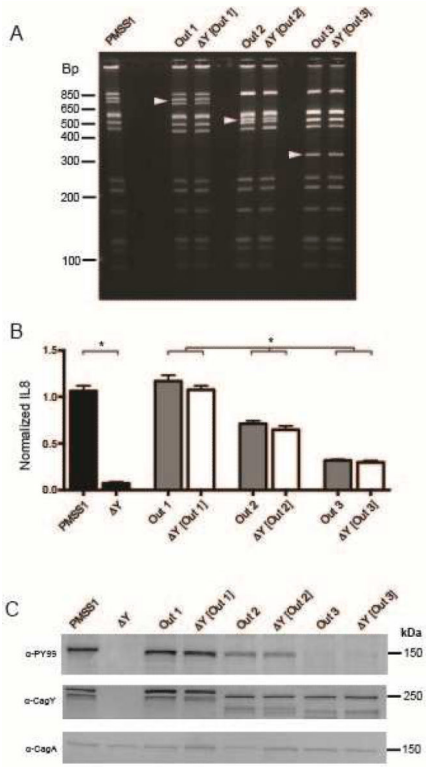

Figure 3. CagY is a molecular rheostat that alters the inflammatory capacity of *H pylori*

Three single colonies recovered from WT mice infected with PMSS1 (Out1, with *cagY* PCR-RFLP equivalent to wild type *cagY* from PMSS1; Out2; Out3) had unique *cagY* PCR-RFPL patterns (A), and induced high, intermediate, or low IL8, respectively (B) (gray bars) compared to PMSS1 and its *cagY* deletion mutant (black bars). Complementation of Δ*cagY* with Out1 (ΔY [Out1]), Out2 (ΔY [Out2]), or Out3 (ΔY [Out3]) phenocopied the IL8 induction of the respective output strain (white bars). Data represent mean ± SEM of four replicates. *P≤0.05. (C) Out1, Out2 and Out3 also demonstrated decreasing translocation of phosphorylated CagA (α-PY99), which was phenocopied when PMSS1Δ*cagY* was complemented with the respective *cagY* gene. Differences in CagY (α-CagY) were also apparent by immunoblot. Arrowheads in panel A indicate unique bands.

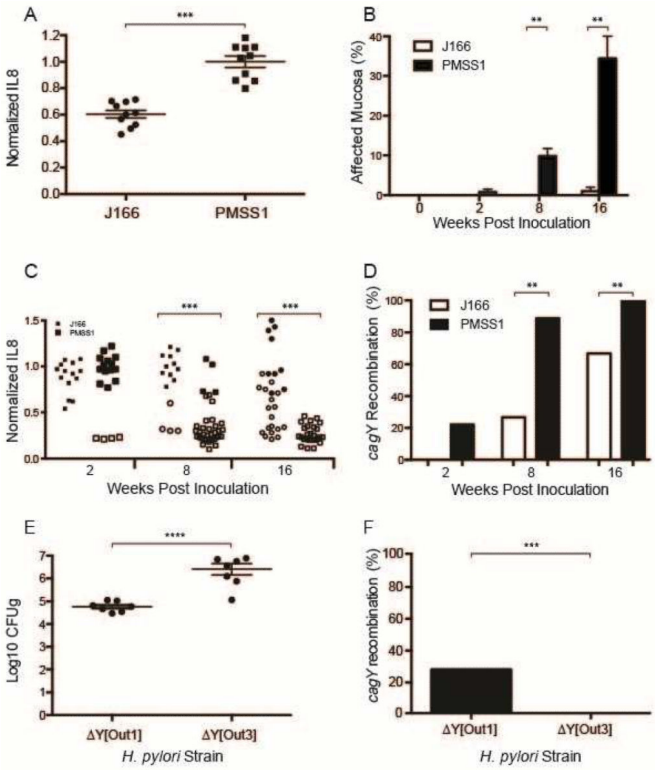

Figure 4. Kinetics of *cagY* recombination and loss of T4SS function are associated with the capacity of *H. pylori* to induce inflammation

(A) Replicate IL8 assays (N=10) for WT *H pylori* strains PMSS1 and J166. Data for both strains are normalized to PMSS1. (B) Mice infected with *H pylori* PMSS1 showed increased inflammation in gastric tissue compared to J166, which was statistically significant at 8 and 16 weeks PI (B). (C) Colonies recovered from PMSS1-infected WT mice lost the capacity to induce IL8 and changed *cagY* (open symbols) more rapidly and more completely than colonies recovered from J166-infected mice. Data for each strain are normalized to their respective WT. (D) Percent of colonies that underwent *cagY* recombination (open circles divided by total colonies for each group in panel C. *H pylori* recovered 8 weeks after challenge of WT mice with ΔY[Out1], which induces high IL8, were at a lower bacterial density (E) and underwent *cagY* recombination more frequently (F) than *H pylori* from mice colonized with ΔY[Out3]. Bars represent mean ± SEM. ***P*≤0.01, ****P*≤0.001, *****P*≤0.0001.

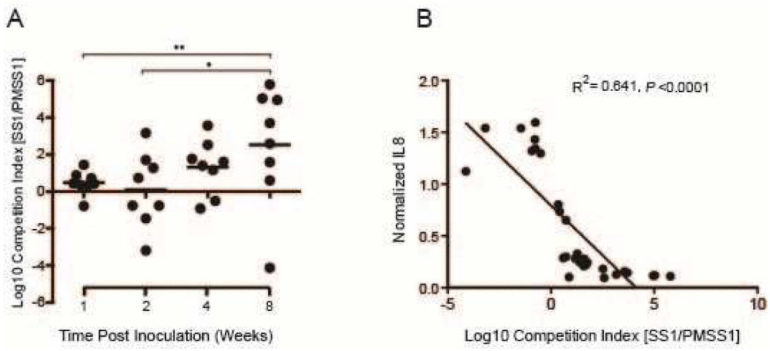

Figure 5. Competitive advantage of CagY-mediated loss of T4SS function increases progressively over time

(A) Output colonies from mice infected with an equal mixture of isogenic *H pylori* PMSS1 strains bearing either the functional (PMSS1) or non-functional (SS1) *cagY* allele were enumerated by selective plating, and used to calculate the log10 competition index. Each data point represents a single mouse; horizontal lines=geometric mean. At early time points there was no selective advantage, but by 8 weeks PI the PMSS1 strain bearing the SS1 *cagY* was present at > 300-fold greater abundance. *P≤0.05, **P≤0.01. (B) Normalized IL8 induction of a sweep culture from each mouse showed a strong inverse correlation with log10 competition index.

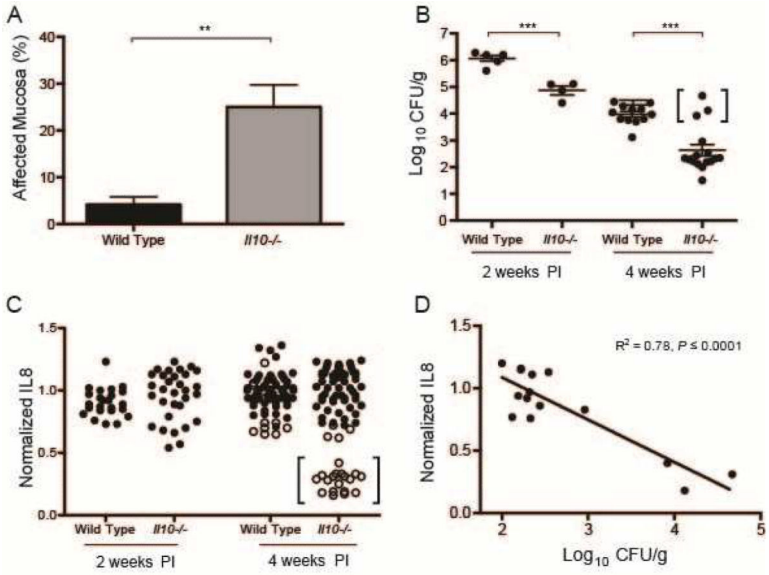

Figure 6. CagY-mediated loss of T4SS function promotes bacterial persistence during an intense inflammatory response

(A) *Il10*−/− mice inoculated with *H pylori* PMSS1 showed significantly increased gastritis compared to WT mice 4 weeks PI. (B) *H pylori* bacterial burden was significantly higher in WT compared to *Il10*−/− mice. By 4 weeks PI, bacterial burden in *Il10*−/− mice was frequently near the level of detection and 3 mice were uninfected (not shown). Mice whose CFU are shown in brackets yielded the colonies whose IL8 induction is shown in brackets in panel C. (C) Loss of the capacity to induce IL8 associated with changes in *cagY* PCR RFLP (open circles) was more apparent in *H pylori* colonies recovered from *Il10*−/− compared to WT mice, particularly in colonies from mice that showed colonization density that resembled that in WT mice. All colonies whose IL8 induction is shown in brackets in panel C were recovered from the mice whose CFU are bracketed in panel B. (D) Average normalized IL8 induction of all colonies from each mouse showed a strong inverse correlation with bacterial burden (R^2=0.78, P≤0.0001). **P≤0.01,***P≤0.001.

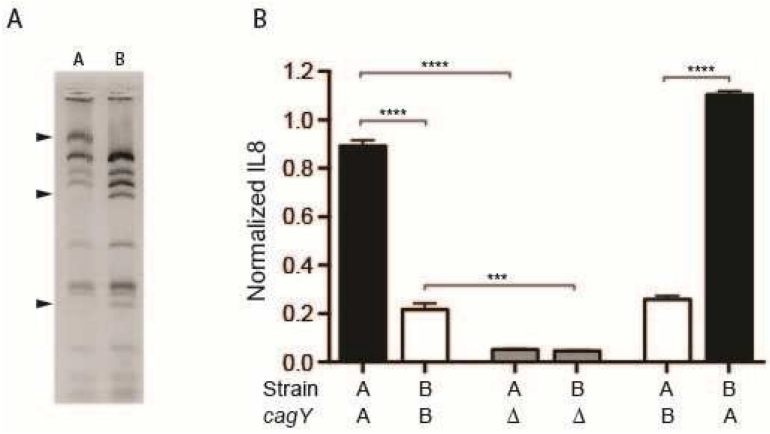

Figure 7. Recombination in *cagY* modulates T4SS function during chronic infection in humans

(A) *cagY* PCR-RFLP analysis of sequential A and B *H pylori* isolates. Arrowheads denote bands that changed in isolate A and B, which were collected from the same patient 7.4 years apart. (B) IL8 induction normalized to strain PMSS1 for sequential *H pylori* isolates A and B, their *cagY* knockouts (Δ), and strains in which their *cagY* genes have been exchanged. ***P≤0.001,****P≤0.0001.