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The C-Terminally Encoded, MHC Class II-Restricted T Cell Antigenicity of the *Helicobacter pylori* Virulence Factor CagA Promotes Gastric Preneoplasia

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Chronic infection with the human bacterial pathogen *Helicobacter pylori* causes gastritis and predisposes carriers to an increased gastric cancer risk. Consequently, *H. pylori*-specific vaccination is widely viewed as a promising strategy of gastric cancer prevention. *H. pylori* strains harboring the Cag pathogenicity island (PAI) are associated with particularly unfavorable disease outcomes in humans and experimental rodent models. We show in this study using a C57BL/6 mouse model of *Cag*-PAI* H. pylori* infection that the only known protein substrate of the Cag-PAI-encoded type IV secretion system, the cytotoxin-associated gene A (CagA) protein, harbors MHC class II-restricted T cell epitopes. Several distinct nonoverlapping epitopes in CagA's central and C-terminal regions were predicted in silico and could be confirmed experimentally. CagA* infection elicits CD4+ T cell responses in mice, which are strongly enhanced by prior mucosal or parenteral vaccination with recombinant CagA. The adoptive transfer of CagA-specific T cells to T cell-deficient, *H. pylori*-infected recipients is sufficient to induce the full range of preneoplastic immunopathology. Similarly, immunization with a cholera toxin-adjuvanted, CagA* whole-cell sonicate vaccine sensitizes mice to, rather than protects them from, *H. pylori*-associated gastric cancer precursor lesions. In contrast, *H. pylori*-specific tolerization by neonatal administration of *H. pylori* sonicate in conjunction with a CD40L-neutralizing Ab prevents *H. pylori*-specific, pathogenic T cell responses and gastric immunopathology. We conclude that active tolerization may be superior to vaccination strategies in gastric cancer prevention. 

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P ersistent gastric infection with the bacterial pathogen *Helicobacter pylori* results in chronic gastritis (1) and predisposes carriers to a high risk for development of gastric and duodenal ulcers, gastric cancer, and gastric MALT lymphoma (2–4). Despite a recent decline in infection rates in industrialized countries, the prevalence rate of *H. pylori* remains at nearly 100% in the developing world (5). The infection can be eradicated in a majority of individuals by antibiotic therapy; however, resistance rates are rising (6), and vaccination against *H. pylori* is viewed as a cost-effective alternative to eradication therapy (7). Several vaccination regimens induce protective immunity in animal models; these include subunit vaccines containing *H. pylori* urease (8), neutrophil-activating protein (9), or adhesin A (10), but also recombinant live *Salmonella* vaccines expressing *Helicobacter* Ags (11). Clinical trials have demonstrated the immunogenicity of experimental vaccines in human volunteers (12–14). However, all current vaccine development efforts are hampered by their failure to achieve sterilizing immunity in rodent models, let alone in humans. 

*Helicobacter* strains harboring the cytotoxin-associated gene A-encoded virulence factor CagA have been associated with high levels of gastric inflammation (15) and an increased gastric cancer risk compared with CagA* strains (16, 17). CagA is the only known protein substrate of a pathogenicity island-encoded type IV secretion system (T4SS), which allows the bacteria to deliver the virulence factor directly into their host cell’s cytosol (18). Upon injection, CagA is tyrosine phosphorylated on C-terminal motifs, leading to the loss of cell-to-cell contacts, cell scattering, and increased motility (19). CagA delivery to the host cell further disrupts cell polarity (20) and allows the bacteria to colonize the apical surface of cultured cells (21). In vivo, transgenic expression of CagA under a stomach-specific promoter is by itself sufficient to induce epithelial hyperplasia and, in a subset of mice, gastric polyps and adenocarcinoma (22), implying that CagA can function as a bacterial oncoprotein. In a Mongolian gerbil model of CagA* *H. pylori* infection, the bacteria induce gastric cancer precursor lesions that resemble *H. pylori*-associated lesions in humans (23). We have recently introduced a C57BL/6 mouse model of infection with a CagA* *H. pylori* patient isolate that induces atrophic gastritis, epithelial hyperplasia, and intestinal metaplasia in its host in a T4SS-dependent manner (24). In this model, the age of the host at the time of infection determines disease outcome. Mice that are experimentally infected during the neonatal period develop immunological tolerance rather than immunity to *H. pylori* and are protected from the immunopathological T cell responses that are a hallmark of CagA* infection in adults (24).
In this study, we pursued the hypothesis that the CagA protein itself functions as a “pathogenic,” procarcinogenic T cell Ag. By analyzing the antigenicity of recombinantly expressed full-length CagA, as well as truncated epitopes and in silico-predicted CagA peptides, we have identified several distinct MHC class II-restricted T cell epitopes in the central and C-terminal regions of the protein. We further show in this article that immunization with CagA, either systemically or mucosally, greatly enhances the host’s T cell response to challenge infection but fails to afford protective immunity. Immunization with a CagA whole-cell sonicate vaccine sensitizes mice to, rather than protects them from, gastric cancer precursor lesions. In contrast, active tolerization of neonatal mice by administration of *H. pylori* sonicate in conjunction with a CD40L blocking Ab before experimental infection prevents pathogenic T cell responses and protects mice from preneoplastic gastric changes. We conclude that tolerization strategies may hold more promise than vaccination for the prevention and management of *H. pylori*-associated gastric disease manifestations.

Materials and Methods

**Animal experimentation and H. pylori cultures**

C57BL/6 and TCR-β−/− BL6 mice were purchased from Charles River Laboratories (Sulzfeld, Germany) and bred at a University of Zurich specific pathogen-free facility. Mice were maintained in individually ventilated cages and included in studies at 6 wk of age. All animal experimentation was conducted in accordance with cantonal and federal guidelines for the care and use of laboratory animals, and was reviewed and approved by the Zurich cantonal veterinary office (permit number 63/ 2008 to A.M.). Mice were immunized four times at weekly intervals with 100 μg recombinant CagA (rCagA) or 1 mg *H. pylori* (premouse Sydney strain 1 [PMSS1]) sonicate, either adjuvanted with 10 μg cholera toxin (CT, List Biologicals, Campbell, CA) and given orally, or administered s.c. with CAP01. A stable CAP01 formulation consisting of dimethyldioctylammonium bromide and a,a'-dihexyl-6,6'-dibehenate (Avanti Polar Lipids) was prepared by the lipid film hydration method as previously described (25). All mice were challenged 1 wk after the last immunization with *H. pylori* PMSS1 by oral gavage of 10^8 bacteria. Anti-CD40L mAb (clone MR1) and anti-CD40 mAb (clone FGK 4.5; both Bio X Cell, West Lebanon, PA) were i.p. injected at 50 or 75 μg/dose into neonatal mice on days 7, 10, 12, and 14 after birth. Neonatal mice were infected at 7 d of age with 10^5 CFU *H. pylori* PMSS1 in 50 μl. For adoptive transfer experiments, 300,000 immunomagnetically sorted splenic CD4+CD25+ T cells (CD4+CD25+ T cell purification kit; R&D Systems, Minneapolis, MN) were injected into the tail veins of TCR-β−/− mice. The *H. pylori* PMSS1 used in this study, as well as agar and liquid culture conditions, was described in detail previously (24).

**Preparation of gastric tissue and gastric/mesenteric lymph node single-cell suspensions; assessment of H. pylori colonization, histopathology, and IFN-γ production**

Stomachs were retrieved and dissected longitudinally into equally sized pieces. For quantitative assessment of *H. pylori* colonization, one section of each stomach was homogenized in *Escherichia coli* BL21 by isopropyl β-D-thiogalactopyranoside for 4 h at 27°C, followed by glutathione Sepharose affinity chromatography. MHC class II-binding 15-aa peptides were predicted with Rankpep (http://immunax.dfci.harvard.edu/Tools/rankpep.html) and synthesized by Thermo Fisher Scientific (peptide 11: NFNKAVAAEKTNGTY; peptide 14: EEPYIPYAVKKNVK; peptide 15: AESAKKVPASLSAKL; peptide 16: TGYCCLAEENAEGHI). For the analysis of CagA-specific T cell responses, dendritic cells (DC) were enriched from MLN suspensions of C57BL/6 and TCR-β−/− mice by CD11c-specific immunomagnetic isolation (BD Bio-Sciences). A total of 80,000 DC were pulsed overnight with 50 μg/ml rCagA or its fragments and cocultured with 150,000 immunomagnetically isolated MLN-derived CD4+CD25− T cells. CagA peptides were added directly to the cocultures at 10 μg/ml. IFN-γ production was assessed by ELISA (BD Biosciences) or by intracellular cytokine staining (see later).

**Flow cytometry**

The following Abs were used for FACS analysis: IFN-γ–PE–CY7, CD4–FITC (BD Biosciences, San Jose, CA), CD45-PB, CD62L-allopolycoyycin, CD44-PB, GR-1 Ly6-G–allophycocyanin, and rat anti-mouse c-Kit/CD117 (all from BioLegend, San Diego, CA). For intracellular IFN-γ staining, cells were restimulated and blocked for 5 h in medium containing 2.5 μg/ml brefeldin A (AppliChem, Darmstadt, Germany), 0.2 μM ionomycin (Santa Cruz Biotechnology, Santa Cruz, CA), and 50 ng/ml phorbol 12-myristate 13-acetate (Sigma-Aldrich). Cells were first stained for CD4 and then fixed in 4% paraformaldehyde and stained for IFN-γ in 10% saponin permeabilization buffer. Flow cytometric analysis was performed on a CyanAPD instrument and analyzed with Summit software (Beckman Coulter, Brea, CA).

**Generation of rCagA fragments and synthetic peptides, and assessment of CagA-specific T cell responses**

For the generation of full-length CagA and its fragments, PCR products were obtained with Hotstart High Fidelity DNA Polymerase (Qiagen) and cloned into the BamH1 and Sall restriction sites of the pGEX 4T3 expression vector with an N-terminal GST-tag. All primer sequences and PCR conditions are listed in Supplemental Table I. Protein expression was induced in *Escherichia coli* BL21 by isopropyl β-D-thiogalactopyranoside for 4 h at 27°C, followed by glutathione Sepharose affinity chromatography. MHC class II-binding 15-aa peptides were predicted with Rankpep and synthesized by Thermo Fisher Scientific (peptide 11: NFNKAVAAEKTNGTY; peptide 14: EEPYIPYAVKKNVK; peptide 15: AESAKKVPASLSAKL; peptide 16: TGYCCLAEENAEGHI). For the analysis of CagA-specific T cell responses, dendritic cells (DC) were enriched from MLN suspensions of C57BL/6 mice by CD11c-specific immunomagnetic isolation (BD Biosciences). A total of 80,000 DC were pulsed overnight with 50 μg/ml rCagA or its fragments and cocultured with 150,000 immunomagnetically isolated MLN-derived CD4+CD25− T cells. CagA peptides were added directly to the cocultures at 10 μg/ml. IFN-γ production was assessed by ELISA (BD Biosciences) or by intracellular cytokine staining (see later).

**FIGURE 1.** CagA harbors central and C-terminally encoded MHC class II-restricted T cell epitopes. A, Schematic representation of full-length CagA and the fragments used in B, B, and C. A total of 80,000 MLN DC were pulsed with 50 μg/ml of either the GST-tag alone or the indicated CagA fragments (B), or with increasing concentrations of full-length rCagA (C), and cocultured for 4 d with 150,000 pooled CD4+CD25− MLN T cells from 5 *H. pylori*-infected mice. IFN-γ secretion of cocultures was quantified by ELISA (B) or by intracellular cytokine staining (C). *p > 0.05, **p < 0.01 in relation to the GST-loaded DC. The correlation in C between the CagA concentration used for DC loading and the fraction of IFN-γ− cells of the total CD4+ population was calculated by regression analysis; the 1/p and r values are indicated. Data are representative of three to five independent experiments.
CagA ELISA.s and IFN-γ real-time RT-PCR

For the evaluation of CagA-specific Ab titers, serum was diluted 1/10,000 and assessed in triplicate by ELISA on 96-well plates (Nunc, Roskilde, Denmark) precoated with 5 μg rCagA in carbonate buffer. Bound Abs were detected by HRP-coupled goat anti-mouse IgG, IgG1, or IgG2c (all from AbD Serotec, Kidlington, U.K.) Abs according to the manufacturer’s recommendations. After addition of tetramethylbenzidine substrate (Sigma-Aldrich), the OD was measured at 655 nm. For real-time RT-PCR of IFN-γ, RNA was isolated from scraped gastric mucosa (antrum and corpus) using a Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany). A total of 1.5 μg total RNA was used for cDNA synthesis with Superscript Reverse Transcriptase III (Life Technologies). The resulting cDNA served as a template for real-time PCR performed with a LightCycler 480 using the SYBR green I master kit (Roche, Basel, Switzerland). Absolute values of IFN-γ expression were normalized to GAPDH expression. Primers and conditions are listed in Supplemental Table I.

Statistics

GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA) was used for statistical analyses. The significance of differences in histopathology categories was calculated by Mann–Whitney U test; all other indicated p values were calculated by Student t test. Linear regression analysis was performed when increasing concentrations of Ag were used in T cell priming experiments. In all scatter plot graphs, the medians are indicated by horizontal bars. In column bar graphs, standard deviations are indicated by vertical bars.

Results

H. pylori CagA harbors MHC class II-restricted T cell epitopes in its central and C-terminal domains

H. pylori harboring a functional T4SS triggers Th1 cell infiltration into the chronically infected gastric mucosa and the subsequent formation of atrophic, hyperplastic, and metaplastic lesions (24). We hypothesized that the only known T4SS substrate and strong humoral Ag, the CagA protein, might itself function as an MHC class II-restricted T cell Ag and trigger T cellular IFN-γ production. To test this possibility, we expressed and purified full-length CagA derived from H. pylori PMSS1, as well as fragments corresponding to the N-terminal, C-terminal, and central domains of the protein as N-terminally tagged GST fusions (Fig. 1A). Immunomagnetically isolated DC were pulsed with purified protein and cocultured with CD4+CD25− T cells isolated from the gut-draining MLN of H. pylori-infected mice before the quantification of IFN-γ secretion. Full-length CagA, as well as the fragments corresponding to aa 400–838 and aa 838–1180, efficiently stimulated IFN-γ production of the cocultures; a fragment containing aa 1–400 was inactive in this regard, as was the GST tag itself (Fig. 1B, Supplemental Fig. 1). Further subcloning and testing of 150-aa-long subfragments revealed the existence of at least three to four distinct central and C-terminal T cell epitopes (Fig. 1A, 1B). T cells from infected mice generally responded more strongly than T cells from uninfected mice in this experimental setup (Supplemental Fig. 1). CagA did not trigger T cellular IFN-γ production in the absence of APCs (data not shown). IFN-γ production in the cultures was restricted to CD4+ T cells as determined by intracellular staining, and was positively correlated with the CagA concentration used for DC loading, as determined by linear regression analysis (Fig. 1C). The combined results imply that the central and C-terminal domains of the CagA protein contain MHC class II-restricted T cell epitopes that stimulate CD4+ T cells to produce and secrete IFN-γ.

FIGURE 2. Immunization with rCagA induces local and systemic immune responses. Mice were immunized four times in weekly intervals with rCagA adjuvanted with either CT (rCagA+CT Vac) or CAF01 (rCagA+CAF Vac), challenged with live H. pylori together with a nonimmunized group (Inf), and sacrificed 2 and 6 wk p.i. Uninfected and immunized-only mice (rCagA+CT Vac Uninf) served as controls. A. Gastric infiltration of leukocytes (CD45+), T cells (CD4+), memory T cells (CD4+CD62L−), mast cells (c-Kit+), and neutrophils (Ly6-G) as quantified by FACS of gastric single-cell preparations. B, IFN-γ secretion of MLN cultures as determined by ELISA. Averages ± SDs are shown for MLN cultures isolated from all four to seven individual mice per group. C, CagA-specific IgG1 and IgG2c serum titers as determined by ELISA. D, H. pylori colonization densities as determined by plating and colony counting. Data are representative of three independent immunization experiments. n.s., not significant.
Mucosal or systemic immunization with rCagA induces strong Th1-polarized responses to challenge infection but fails to confer protective immunity

To assess the immunogenicity of full-length rCagA in vivo in mucosal and systemic immunization models, we either administered CagA orally with CT or s.c. with a cationic adjuvant formulation derived from Mycobacterium tuberculosis (CAF01). CAF01 consists of the synthetic analog of a mycobacterial cell wall glycolipid (trehalose 6,6'-dibehenate) delivered in dimethyldioctadecylammonium liposomes; it triggers mixed Th1/Th17 responses and is successfully used in mycobacterial vaccine formulations (26). Both vaccines were administered four times at weekly intervals before challenge infection with CagA+ H. pylori. Challenged mice were compared at 2 and 6 wk postinfection (p.i.) with nonimmunized, infected, and uninfected controls with respect to gastric infiltration of various immune cell types, T cell responses in the MLN, serum titers to CagA, and colony counts.

All immunized mice, independent of the route and adjuvant used for their vaccination, differed strongly from nonimmunized mice with respect to all parameters analyzed at both time points p.i. (Fig. 2A–C). CagA-vaccinated mice showed higher gastric infiltration of CD45+ leukocytes, CD4+ T cells, CD44+CD62L memory T cells, c-Kit+ mast cells, and Ly6-G+ neutrophils than infected-only controls and uninfected controls (Fig. 2A). Helicobacter-specific T cell priming and Th1 differentiation in the MLN in response to challenge infection was stronger in immunized mice as determined by IFN-γ ELISA of MLN single-cell suspension cultures of individual mice (Fig. 2B). A group of immunized but unchallenged mice was indistinguishable from unimmunized, uninfected controls with respect to all parameters analyzed at the 6 wk p.i. time point (Fig. 2A, 2B). High CagA-specific IgG titers were measured in the systemically immunized, but not the mucosally immunized or control infected mice (Fig. 2C). The IgG subclass profile further suggests a bias toward IgG1 over IgG2c production (Fig. 2C). The results indicate that parenteral or mucosal vaccination with rCagA enhances the cellular and humoral immune responses to challenge infection, both locally at the site of infection and in the draining lymph nodes, thereby providing evidence for the in vivo immunogenicity of CagA.

Surprisingly, however, no significant differences in colonization were observed between immunized and naïve mice on challenge infection (Fig. 2D), indicating that immunization with adjuvanted rCagA alone does not confer protective immunity. In contrast, roughly half of all immunized mice showed widespread atrophy and hyperplasia at both 2 and 6 wk p.i., a phenomenon that is only rarely seen in infected-only controls at such early time points (Supplemental Fig. 2A, 2B). In conclusion, immunization with rCagA efficiently stimulates memory T cell responses that are reactivated on challenge infection and result in increased gastric immune cell infiltration; however, these responses fail to clear or reduce the bacteria in the vaccinated hosts.

Several distinct MHC class II-restricted T cell epitopes are encoded in the central and C-terminal domains of CagA

We next aimed to identify specific MHC class II-restricted T cell epitopes in the central and C-terminal regions of CagA. Postulating that CagA-specific T cells should be particularly abundant in CagA-vaccinated mice, and should further be enriched in the draining MLN on challenge infection, we cultured single-cell MLN preparations from individual vaccinated and control mice with rCagA. rCagA, but not the GST-tag alone, stimulated IFN-γ production by MLN cells that was stronger in the vaccinated/challenged than in the infected-only mice (Fig. 3A), reflecting their overall stronger reactivity to H. pylori (Fig. 2). MLN cultures from the same groups of mice were further restimulated in a parallel experiment with various peptides corresponding to silico-predicted MHC class II-restricted T cell epitopes located in the central and C-terminal domains of CagA (Fig. 3B, 3C). Four of the

**FIGURE 3.** The central and C-terminal domains of CagA encode MHC class II-restricted T cell epitopes. A–C, MLN cultures from mice immunized and challenged as described in Fig. 2 were restimulated ex vivo for 4 d with 50 μg/ml full-length rCagA (A) or 10 μg/ml peptides (C) and assessed for IFN-γ secretion by ELISA. B, Schematic representation of the position of the peptides used in C. Data are representative of two independent experiments. n.s., not significant.
15 predicted peptides stimulated IFN-γ production in the MLN culture of at least one mouse (Fig. 3B, 3C). Most mice responded to more than one peptide; no single peptide elicited recall responses in all mice (Fig. 3C). Overall, the vaccinated mice of both treatment arms responded more strongly to the CagA peptides than infected-only or control mice; this reflects their overall stronger reactivity to rCagA (Fig. 3A). In conclusion, we show in this article that at least four distinct, nonoverlapping sequences in the C terminus of the CagA protein represent MHC class II-restricted T cell epitopes and trigger ex vivo recall responses in the draining lymph nodes.

**CagA-specific T cells are sufficient to trigger gastric preneoplastic immunopathology in an adoptive transfer model**

Based on our results showing that CagA-vaccinated mice respond more strongly to challenge infection than infected-only mice, and exhibit more severe gastric immunopathology, we postulated that CagA-specific T cells should be sufficient to induce gastric pathology in a host that is otherwise devoid of T cells. We used TCR-β−/− recipients, which cannot generate α/β+ T cells and are, on the one hand, incapable of controlling a *Helicobacter* infection, and on the other hand, completely protected from the T cell-driven *Helicobacter*-associated gastric immunopathology typical of immunocompetent mice (24, 27). We adaptively transferred immunomagnetically isolated, >85% pure, splenic CD4+CD25− T cells from CagA/CT-immunized, unchallenged mice to TCR-β−/− recipients, which were either experimentally infected with *H. pylori* PMSS1 on the same day or remained uninfected (Fig. 4). Additional groups of TCR-β−/− recipients received T cells from naive donors or from CagA/CT-immunized, challenged donors. In line with the presumed “pathogenicity” of CagA-specific T cells, the recipients of T cells isolated from CagA/CT-immunized, unchallenged mice developed extremely severe gastric pathology. This response was seen only in *H. pylori*-infected recipients; the same cells were harmless in uninfected recipients (Fig. 4A, 4B). T cells from immunized donors produced more severe pathology in infected recipients than T cells from naive donors, indicating that CagA-specific T cells must account for the infection-dependent immunopathology in the new hosts. Quite unexpectedly, the recipients of T cells from CagA-immunized mice that had been challenged with live bacteria were less pathogenic in their new hosts than cells from immunized-only mice (Fig. 4A, 4B), suggesting that an active infection in the donor suppresses T cell activity in a robust and very sustained manner. The strong pathology observed in the recipients of T cells from CagA/CT-immunized, unchallenged mice correlated with low colonization levels (Fig. 4C), high levels of infiltration of CD4+ T cells into the gastric mucosa (Fig. 4D), and high levels of gastric IFN-γ production (Fig. 4E). In contrast, the recipients of naive T cells, immunized/challenged T cells, and the uninfected recipients of immunized T cells all had relatively lower levels of gastric CD4+ T cell infiltration and IFN-γ production (Fig. 4D, 4E), and were colonized more heavily (Fig. 4C). In conclusion, the adoptive T cell transfer model revealed that CagA-specific T cells targeting the infection site in the otherwise T cell-deficient host are by themselves sufficient to produce the gastric pathology typically associated with *H. pylori* infection in immunocompetent animals, lending further support to our model that a CagA+ infection triggers gastric pathology through CagA’s T cell antigenic properties.

![FIGURE 4](http://www.jimmunol.org/)

CagA-specific T cells trigger gastric preneoplastic immunopathology. Pooled splenic CD4+CD25− T cells were isolated from groups of mice that had been vaccinated with CagA/CT and challenged (Vac inf) or not (Vac uninf) or had remained untreated (Uninf); cells were adoptively transferred to TCR-β−/− recipients, which were either infected with *H. pylori* (TCR-β−/− inf) or remained uninfected (TCR-β−/− ctrl). A–C, Histopathology scores (A), representative micrographs of Giemsa-stained gastric sections (original magnification ×200) (B), and bacterial colonization densities (C) are shown for all mice 4 wk post cell transfer. D, Stomach-infiltrating CD4+ T cells as determined by FACS. E, Gastric IFN-γ expression as assessed by real time RT-PCR and normalized to GAPDH. A–E, Data are representative of two independent adoptive transfer experiments. n.s., not significant.
H. pylori-specific vaccination promotes preneoplasia even if it confers "protective" immunity

Vaccination with adjuvanted CagA induces local and systemic T cell responses to challenge infection that are not observed in naive mice infected for the same length of time, but nevertheless fails to confer protective immunity (Fig. 2). To assess whether a vaccination strategy conferring protective immunity sensitizes mice to enhanced gastric immunopathology, we immunized mice orally with an H. pylori PMSS1 whole-cell sonicate vaccine adjuvanted with CT, and challenged them with the autologous strain 2 wk after the last dose. The vaccinated mice reduced their bacterial burdens by almost two orders of magnitude compared with infected-only controls (Fig. 5A). However, the vaccinated mice further also developed more severe immunopathology than infected-only mice with respect to all parameters scored at 2 mo p.i. (Fig. 5B, 5C), implying that vaccination is not a suitable strategy for the prevention of gastric preneoplasia induced by CagA+ strains.

Active tolerization of neonatal mice to H. pylori prevents gastric preneoplasia

We have shown previously that the development of immunological tolerance to H. pylori during the neonatal period prevents local and systemic immune responses to the pathogen and confers long-lasting protection of the host from gastritis and premalignant changes of the gastric mucosa (24). To test whether tolerization to H. pylori can also be achieved in the absence of active infection as an alternative strategy of gastric cancer prevention, we treated neonatal mice repeatedly with a PMSS1 whole-cell sonicate combined with anti-CD40L–neutralizing Ab during the second week of life, that is, during a time when a newborn’s immune system is inherently prone to develop tolerance to self-Ags and foreign Ags (28). CD40L antagonization is used for allograft tolerization and functions by preventing costimulatory signaling during alloreactive T cell priming (29). The tolerized mice were subsequently infected as adults with live bacteria and compared with untreated mice with respect to H. pylori colonization, gastric T cell infiltration, and gastric histopathology (Fig. 6A–C). Neonatal treatment with H. pylori sonicate and anti-CD40L mAb was indeed sufficient to tolerize neonatal mice efficiently to the bacterium; anti-CD40L mAb-treated mice were colonized at significantly higher levels (Fig. 6A), exhibited lower levels of gastric T cell infiltration (Fig. 6B), and had been protected from the gastric inflammation and associated immunopathology that are a hallmark of CagA+ infection after 2 mo (Fig. 6C). Similar, but somewhat weaker results were obtained when rCagA was used for neonatal tolerization in conjunction with anti-CD40L mAb (Supplemental Fig. 3). Conversely, the infection-induced development of H. pylori-specific tolerance in neonates could be prevented by four doses of an agonistic anti-CD40 Ab administered during the first week of neonatal H. pylori infection; neonatally infected mice treated in this manner had significantly reduced their bacterial burdens compared with untreated, infected mice at 2 mo p.i. (Fig. 6D), exhibited higher levels of gastric T cell infiltration (Fig. 6E), and had developed significant gastritis, atrophy, and hyperplasia at this time point (Fig. 6F). In summary, our data suggest that the modulation of the CD40–CD40L interaction by neutralizing and/or activating Abs is a suitable method to shift the balance to either immunological tolerance or immunity, and to thereby influence disease outcome.

Discussion

Substantial epidemiological and experimental evidence is now available to support the notion that CagA functions as a bacterial oncoprotein. A large meta-analysis of all epidemiological data available at the time showed that infection with CagA+ H. pylori strains increases the gastric cancer risk above and beyond the risk conferred by infection alone (17). Experimental infection of Mongolian gerbils (23) and C57BL/6 mice (24) with wild type, but not CagA translocation-deficient H. pylori results in the rapid development of gastric cancer precursor lesions. Transgenic expression of CagA in the gastric mucosa further revealed that CagA is in itself sufficient to cause gastric hyperplasia, gastric polyps, and adenocarcinomas (22). In addition to the direct effects that the ectopic expression or natural delivery of CagA have on the cell biology of host cells, we demonstrate in this article that CagA has strong T cell antigenic properties. The central and C-terminal parts of the protein harbor in silico predicted MHC class II-restricted T cell epitopes, some of which were confirmed experimentally by ex vivo restimulation of T cells from CagA-vaccinated mice. Vaccination with CagA through two complementary routes, parenteral or mucosal, induced CagA-specific pathogenic T cells, which cause excessive gastric immunopathology in T cell-deficient recipients but fail to confer protective immunity in immunocompetent mice. Several previous findings make it seem likely that the T cell immunogenicity of CagA is at least partially responsible for CagA’s oncogenic properties. First, we have shown earlier that T cells are indispensable for the induction of gastric pathology, not just in H. pylori, but also in Helicobacter felis infection models (27, 30). TCR-β−/− mice that lack functional αβ+ T cells are protected from gastric cancer precursor lesions, and the adoptive transfer of CD4+CD25+ T cells is sufficient to trigger these lesions in resistant mice (24,
The pharmacological inhibition of T cell activation prevents and even reverses pre-existing lesions (31, 32), and neonatally infected (24) and actively tolerized mice are protected from gastric preneoplasia. Our finding that tolerant mice, despite being colonized very densely by CagA translocation-proficient bacteria, do not develop preneoplasia is perhaps the most important piece of evidence for an indirect pathogenic role of CagA (24).

Significant progress has been made in recent years with respect to the identification of new protective Helicobacter Ags, understanding the mechanisms of protective immunity and the development of immunization/challenge protocols in human volunteers (33). We and others have reported previously that the challenge of whole-cell-immunized mice with avirulent mouse-adapted \( \text{H. pylori} \) strains such as SS1 induces more gastritis and preneoplastic lesions than infection of nonimmunized animals (27, 34, 35). In this study, we expand these findings by challenging immunized mice with a patient isolate that harbors a functional CagA translocation-proficient \( \text{H. pylori} \) and an isogenic CagE-deficient mutant differ significantly with respect to the gastric mucosal Th1 infiltration and the gastric production of IFN-\( \gamma \) they induce (24).

We propose in this article that tolerization of the host to \( \text{H. pylori} \) may hold more promise than vaccination strategies in gastric cancer prevention. We base this proposition on our observation that neonatal mice develop natural tolerance to the bacteria if they are infected during the first 2 wk of life, at a time when the immune system is immature and inherently biased toward tolerogenic over immunogenic responses (28). Neonatally acquired, \( \text{Helicobacter} \)-specific tolerance is mediated and maintained by long-lived, inducible regulatory T cells, and protects the host from gastric preneoplasia not only during the neonatal period, but long into adulthood. We have expanded these findings in this study by showing that newborn mice can be tolerized actively by administration of \( \text{H. pylori} \) whole-cell sonicate in conjunction with anti-CD40L blocking Ab, which has been used extensively to prevent allograft rejection in preclinical models (29), and effectively prevented the gastric T cell responses and immunopathology typically associated with CagA\(^+\) infection in adult-infected mice. Similar results were obtained by tolerization with CagA in conjunction with anti-CD40L Ab. The antigraft tolerization by CD40L inhibition was shown to be due to the induction of anergy in the alloreactive effector T cell pool as measured by their failure to expand and produce cytokines, but also required regulatory T cells (29). Antigraft tolerance can be broken by administration of an agonistic anti-CD40 Ab (29). Similarly, the anti-\( \text{Helicobacter} \) tolerance of neonatally infected mice could be broken in our hands by systemic CD40 activation.

In conclusion, we propose in this article that the increased gastric cancer risk associated with CagA\(^+\) strains is due to the protein’s central and C-terminally encoded T cell antigenicity. Not only do wild type \( \text{H. pylori} \) induce more gastric pathology than a CagA translocation-deficient isogenic mutant, but CagA\(^+\) infection-associated pathology can be further exacerbated by prior immunization with rCagA. Overall, our data imply that gastric cancer management and prevention strategies will only be successful if they take into account that gastric cancer precursor lesions may be
inflammation- and/or immunity-driven rather than the direct result of bacterially induced tissue damage.

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Disclosures

The authors have no financial conflicts of interest.

References


Suppl. Figure 1. Naive T-cells are less responsive to rCagA than T-cells from infected mice

100k MLN DC were pulsed with 50μg/ml rCagA, the GST-tag alone or the indicated fragments and co-cultured for four days with 200k CD4⁺CD25⁻ MLN T-cells isolated from *H. pylori*-infected or uninfected mice. IFN-γ secretion of co-cultures was quantified by ELISA.
Suppl. Figure 2

Suppl. Figure 2. Immunization with rCagA induces “pathogenic” rather than protective immune responses. Mice were immunized and challenged as described in main Figure 2 and assessed with respect to gastric histopathology. Scores assigned for the parameters gastric inflammation, atrophy, hyperplasia and metaplasia are shown in A; representative micrographs of the gastric corpus are shown in B.
Suppl. Figure 3. Tolerization with rCagA and anti-CD40L antibody increases H. pylori colonization and prevents gastric preneoplastic changes. For CagA-specific tolerization, neonatal mice received 4 oral doses of 25, 25, 50 and 50μg rCagA along with 50, 50, 75 and 75μg antagonistic anti-CD40L mAb (i.p.) on days 7, 10, 12 and 14 after birth. Sonicate-treated mice and untreated control mice were infected with the autologous strain at 6 weeks of age (i.e. 4 weeks after the last dose) and sacrificed 8 weeks later. A, H. pylori colonization. B, Histopathology scores.
Supplemental Table 1
Primer sequences, annealing temperatures and cycle number of PCR reactions performed for the generation of rCagA fragments and quantitative real-time assessment of cytokine genes.

<table>
<thead>
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<th>Gene</th>
<th>Nucleotide Sequence (5’ 3’)</th>
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<th>PCR cycles</th>
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<td></td>
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