**Helicobacter pylori-Pulsed Dendritic Cells Induce H. pylori-Specific Immunity in Mice**

Min Zhang,† Bradford E. Berndt,† Kathryn A. Eaton,† Sivaprakash Rathinavelu,† Anna Pierzchala† and John Y. Kao†

Departments of †Internal Medicine (Gastroenterology Division) and †Microbiology and Immunology, University of Michigan, Ann Arbor, Michigan 48109, USA

**Abstract**

**Background:** The growing concern over the emergence of antibiotic-resistant *Helicobacter pylori* infection is propelling the development of an efficacious vaccine to control this highly adaptive organism.

**Aim:** We studied the use of a dendritic cell (DC)-based vaccine against *H. pylori* infection in mice.

**Methods:** The cellular immune responses to murine bone marrow-derived DCs pulsed with phosphate-buffered saline (PBS-DC) or live *H. pylori* SS1 (HP-DC) were assessed in vitro and in vivo. The protective immunity against *H. pylori* SS1 oral challenge was compared between HP-DC or PBS-DC immunized mice. The effect of regulatory T-cell (Treg) depletion by anti-CD25 antibody on HP-DC vaccine efficacy was also evaluated.

**Results:** HP-DC induced a Th1-dominant response in vitro. In vivo, HP-DC immunized mice were characterized by a mixed Th1/Th2 peripheral immune response. However, in the stomach, HP-DC immunized mice expressed a higher level of IFN-γ compared to PBS-DC immunized mice; no difference was found for interleukin-5 expressions in the stomach. A lower bacterial colonization post-*H. pylori* challenge was observed in HP-DC immunized mice compared to PBS-DC immunized mice with no significant difference in gastritis severity. *H. pylori*-specific Th1 response and protective immunity were further enhanced in vivo by depletion of Treg with anti-CD25 antibody.

**Conclusion:** DC-based anti-*H. pylori* vaccine induced *H. pylori*-specific helper T-cell responses capable of limiting bacterial colonization. Our data support the critical role of effector cellular immune response in the development of *H. pylori* vaccine.

**Keywords**
Vaccine, dendritic cells, Th1/Th2, regulatory T cells, CD25 depletion.

Reprint requests to: John Y. Kao, Division of Gastroenterology, Department of Internal Medicine, University of Michigan Health System, 6520A Medical Science Research Building 1, Special Postal Code 5682, 1150 West Medical Center Drive, Ann Arbor, Michigan 48109, USA. Tel.: (734) 647-2964; Fax: (734) 763-2535; E-mail: jykao@umich.edu

*Helicobacter pylori* causes chronic gastritis in most infected individuals and it is associated with gastroduodenal ulcers and malignancies. Although *H. pylori* can be eradicated by a combination of antibiotics and proton-pump inhibitors, the emergence of antibiotic-resistant *H. pylori* strains [1–3] remains a significant threat to the half of the world’s population infected with this pathogen. An improved understanding of the interplay between *H. pylori* and the host immune response will contribute to the development of an effective immunotherapy targeting *H. pylori*.

Studies in mice have revealed that interleukin (IL)-12, a type 1 helper T cell (Th1)-promoting cytokine, plays a critical role in vaccine-induced protective immunity [4,5]. We have previously shown that dendritic cells (DCs) pulsed with *H. pylori* (HP-DC) secrete lower levels of IL-12 than *Escherichia coli* or *Acinetobacter lwoffi*, possibly leading to an ineffective host response and failure to eradicate *H. pylori* [6]. Since immunization with *ex vivo* antigen-pulsed DCs leads to the induction of in vivo antigen-specific protective immunity against tumors [7], we speculate that an HP-DC vaccine may prime effective protective immunity against *H. pylori*. Although several prototype vaccines for *H. pylori* have been assessed with some degree of success [8,9], the advantage of DC vaccine is the priming of potent adaptive immunity which, in tumor models, were shown to be more efficacious than non-DC-based vaccines [10]. The goal of the current study was to examine the efficacy of an HP-DC vaccine against *H. pylori* infection in vivo.

Our results showed that HP-DC stimulate the in vitro proliferation of Th1 cells. In vivo, HP-DC stimulated
**Materials and Methods**

**Mice**

Specific pathogen-free female C57BL/6 mice aged 8–10 weeks were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and housed in the Animal Maintenance Facility at the University of Michigan Health System. Experiments were conducted on mice aged 10–14 weeks. All animal experiments were approved by the University of Michigan Animal Care and Use Committee.

**Media, Cytokines, and Neutralizing Antibodies**

Complete medium (CM) consisted of RPMI-1640 with 10% heat-inactivated fetal calf serum, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. Two recombinant cytokines (R&D Systems, Minneapolis, MN, USA) were diluted in CM: mouse granulocyte/macrophage colony-stimulating factor (GM-CSF, 10 ng/mL) and mouse IL-4 (10 ng/mL). The antibodies used to deplete CD25+ cells were purified using protein G columns (Amersham Biosciences, Piscataway, NJ, USA) from the supernatant of the PC61 hybridoma cell line (American Type Culture Collection, Manassas, VA, USA).

**Bacterial Strains and Culture Conditions**

*H. pylori* (SS1 strain) organisms were grown on *Campylobacter*-selective agar (BD Diagnostics, Bedford, MA, USA) supplemented with 5% sterile horse blood, trimethoprim (5 µg/mL), vancomycin (10 µg/mL), and nystatin (10 µg/mL) [11] for 2 days at 37 °C in a humidified microaerophilic chamber (BBL Gas System, with CampyPak Plus packs, BD Microbiology, Sparks, MD, USA).

**Generation of Bone Marrow-Derived DCs**

Erythrocyte-depletedmurine bone marrow cells were cultured in CM with GM-CSF (10 ng/mL) and IL-4 (10 ng/mL) at 1 × 10^6 cells/mL [12]. On day 6, nonadherent bone marrow-derived DCs were harvested by vigorous pipetting and enriched by gradient centrifugation using the Optiprep density solution (Sigma, St. Louis, MO, USA). The low-density interface containing the DCs was collected by gentle aspiration. The recovered bone marrow-derived DCs were washed twice with RPMI-1640 and cultured in CM with GM-CSF (10 ng/mL).

**Induction of Proliferative T-cell Responses In Vitro**

After overnight stimulation with *H. pylori* SS1 (1 × 10^8 CFU/mL) followed by washing to remove bacteria, irradiated (5000 rads) bone marrow-derived DCs (1 × 10^6 cells/mL) were cocultured with naïve syngeneic C57BL/6 splenocytes (1 × 10^5 cells/mL) for 48 hours. Tritiated deoxythymidine (Amersham Biosciences) was added to each microtiter well (1 µCi/well), and the plates were incubated for 24 hours [7]. The plates were harvested at completion and the radioactivity was measured using a scintillation counter. Responses were reported as the mean count per minute ± standard error of the mean (SEM) from duplicate samples in three separate experiments.

**Animal Studies**

Naïve C57BL/6 mice (n = 15 per group) were given an intraperitoneal (IP) injection of *H. pylori* SS1 (1 × 10^6 CFU/mL)-stimulated DCs (10^6 cells per injection) on day 0 and day 14. Phosphate-buffered saline-treated unstimulated DCs served as controls. Beginning on day 21, all mice were infected with *H. pylori* SS1 – an oral gavage of 10^6 colony-forming units (CFU) per milliliter of live organisms was given to each mouse, three times over 1 week. Mice were sacrificed 120 days after the first infection on day 0, and spleens and stomachs were removed for analyses. The stomachs were cut along the greater curvature into 2-mm strips that included fundic and antral tissue. Paraflin sections were prepared for hematoxylin and eosin (H&E). The spleens were flushed with RPMI-1640 and CD4+ T cells were isolated using Magnetic-Activated Cell Sorting (MACS) microbeads (Miltenyi Biotech, Auburn, CA, USA). Histologic scores were determined as mean ± SEM according to the Eaton scoring method [13]. The pathologist was blinded to the experimental manipulation of each mouse to eliminate observer bias.

**Delayed-Type Hypersensitivity Measurement**

To measure delayed-type hypersensitivity, mice were given 10 µg of *H. pylori* sonicate by injection into the right hind footpad 1 day before sacrifice. The left hind footpad was given an equal volume of sterile saline. Footpad thickness was measured with a dial thickness gauge 24 hours later, immediately before sacrifice, and the difference in thickness between the control and the sonicate-treated footpads was recorded.
Cytometric Bead Analysis
Mouse Th1/Th2 Cytokine Cytometric Bead Array Kit (BD Biosciences PharMingen, San Diego, CA, USA) was used according to the manufacturer’s instructions. Briefly, cell culture supernatants and standards were incubated with capture beads and PE Detection Reagent and analyzed with the BD FACSCalibur™ system (BD Biosciences, San Jose, CA, USA) using software supplied by the manufacturer.

Preparation of Conditioned Medium and Whole Cell Sonicate
Bacteria were grown overnight at a concentration of 1 × 10⁹ CFU/mL. H. pylori was grown in brain–heart infusion medium. The cultures were spun down for 15 minutes at 14,500 g. The culture supernatants were designated as conditioned media. The pellets were sonicated on ice as previously described [14] and then spun down for 10 minutes at 280 g. The supernatants were stored and designated as bacterial sonicate.

Quantification of Helicobacter Colonization and Gastric Tissue mRNA for IFN-γ and IL-5 by Real-Time Polymerase Chain Reaction
A standard curve was generated by extracting total RNA, using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), from H. pylori SS1 bacterial cultures with densities ranging from 10³ to 10⁹ total bacteria. Total RNA also was isolated from stomach tissue using TRIzol Reagent. Primer pairs C97 and C98 [15] were used to amplify the 16S rRNA species that is specific for Helicobacter, generating an amplicon of about 400 base pairs [16]. Additional primer sequences used were: IFN-γ (sense: GGCTGTTTCTGGCTGTTACTGCCACG, antisense: GACAATCTCTTCCCCACCCAGAAC) and IL-5 (sense: GCAATGGAAGGCTGAGGCTG, antisense: GGGTATGTGATCCTCCTGCGT). Polymerase chain reaction (PCR) amplifications were performed in a total volume of 25 µL, containing 10 × PCR buffer with MgCl₂, 10 nmol/L dNTPs, 200 nmol/L primers, 5 µL cDNA, 100 nmol/L Taq polymerase GOLD, and 2.5 µL Sybr Green (Molecular Probes, Carlsbad, CA, USA). Each PCR amplification was performed in duplicate wells in a Bio-Rad I-Cycler (I-Cycler IQ Real-Time PCR Detection System, Bio-Rad Laboratories, Hercules, CA, USA) at 94 °C for 10 minutes, followed by 35 two-temperature cycles at 94 °C for 1 minute and 55 °C for 1 minute.

Fluorescence-Activated Cell Sorting (FACS)
DCs were washed twice with ice-cold PBS containing 0.5% bovine serum albumin and sodium azide. After a 30-minute incubation with Fc Block (1 µg/100 µL, BD Biosciences PharMingen), the cells were incubated with either FITC and/or PE-conjugated antibodies or with isotype control antibodies (1 : 100 dilution). The cells were washed, resuspended in ice-cold 2% paraformaldehyde, and analyzed using a Coulter XL Flow Cytometer (Beckman Coulter, Miami, FL, USA). For intracellular cytokine staining, cells were permeabilized with Perm/Fix Solution (BD Biosciences PharMingen) before staining. Both dot plots and histograms were obtained using WinMDI version 2.8. The percentage of CD4+CD25+ Treg determination was measured using Mouse Regulatory T Cell Staining Kit (eBioscience, San Diego, CA, USA).

H. pylori-Specific In Vitro Stimulation
Erythrocyte-depleted splenic CD4+ T cells (1 × 10⁵ cells/mL) were isolated using MACS (Miltenyi Biotech) and then stimulated for 7 days with syngeneic bone marrow-derived DCs (1 × 10⁶ cells/mL) and H. pylori SS1 sonicate (5 µg/mL). CD4+ T cells stimulated with DCs alone or H. pylori sonicate alone served as controls.

Enzyme-Linked Immunosorbent Spot (ELISpot)
In vitro stimulated H. pylori-specific CD4+ T cells were cultured with bone marrow-derived DCs (1 : 10) and H. pylori SS1 sonicate (10 µg/mL) for 24 hours (to measure IFN-γ secretion) or for 48 hours (to measure IL-5 secretion). ELISpot (BD Biosciences Pharmingen) assays were performed.

In Vivo Depletion of Regulatory T Cells (Tregs)
C57BL/6 mice (n = 9 or 10 per group) were treated IP with either PBS or a single 1-mg dose of anti-CD25 mAb (PC61). The success of Treg depletion was determined after 30 days by FACS analysis of isolated splenocytes.

Statistical Analysis
Statistical significance was determined by nonparametric Student t-test using commercially available software (PRISM, GraphPad, San Diego, CA, USA). p < .05 was considered significant.

Results
H. pylori-Pulsed DCs Stimulated Th1 Response In Vitro
The activation of antigen-presenting cells by bacterial antigens can be measured by the surface expression of
Responses In Vivo

HP-DC Induced a Delayed-Type Hypersensitivity Reaction Against H. pylori Sonicate In Vivo

Research suggests that the induction of an H. pylori-specific Th1 response is a crucial component of protective immunity against H. pylori [4,5]. Since HP-DC prime a Th1 response in vitro, we examined this response in vivo. Using a general protocol for inducing protective antitumor immunity, mice were infected with HP-DC. A booster injection was given 2 weeks later. The mice were then challenged with live H. pylori to assess the induction of anti-H. pylori immunity. After footpad injection with H. pylori SS1 sonicate to measure H. pylori-specific delayed-type hypersensitivity reaction, a significant increase in footpad thickness was measured in the HP-DC injected mice compared to the PBS-DC injected mice (Fig. 2). These data indicate that HP-DC induce H. pylori-specific cellular immune responses.

Induction of H. pylori-Specific Th1 and Th2 Responses in Vivo

To determine the ability of HP-DC to induce H. pylori-specific adaptive immune responses, CD4+ T cells isolated from splenocytes of PBS-DC and HP-DC mice were stimulated ex vivo with H. pylori sonicate. Naïve BM-DCs were included as stimulators. ELISpot assays showed significantly higher numbers of H. pylori-specific Th1 (i.e. IFN-γ-producing CD4+) and Th2 (i.e. IL-5-producing CD4+) cells in HP-DC mice (Fig. 3A,B). Thus, HP-DC are capable of inducing Th1 and Th2 responses in vivo.

Induction of a Th1-dominant Gastric Response in HP-DC Mice

To characterize further the effective of HP-DC on host response to H. pylori infection, total gastric RNA of mice was assessed for IFN-γ and IL-5 expressions in order to better define the Th1/Th2 responses at the gastric tissue level. We found increased gastric IFN-γ mRNA in HP-DC mice compared to PBS-DC mice and no measurable difference for the IL-5 mRNA expression (Fig. 4A). Despite detecting a mixed Th1/Th2 response in the peripheral (spleen) immune compartment, HP-DC induced a Th1-dominant response in the gastric tissue. This finding indicates HP-DC primes a Th1-dominant response in the gastric tissue despite a mixed Th1/Th2 in the periphery. Therefore, it is critical to evaluate the gastric Th response separately from peripheral Th response when determining in vivo H. pylori-specific immunity.

A Reduced H. pylori Colonization was Observed in HP-DC Mice Compared to PBS-DC Mice

To determine whether the H. pylori-specific Th1-dominant response induced by HP-DC could limit H. pylori colonization, we performed quantitative reverse transcriptase (RT)-PCR, which is a sensitive method of determining the number of bacterial CFU in the stomach. There was a trend toward lower bacterial colonization in HP-DC mice compared to PBS-DC mice (p = .1) (Fig. 4B). To evaluate whether this trend was due to more severe gastritis in HP-DC mice, the degree of gastritis also was assessed using the Eaton gastritis score for H. pylori-induced gastritis [14]. Quantification of H. pylori by the culture method showed a similar trend (data not shown). No significant differences in gastritis or polymorphonuclear neutrophil infiltration scores were measured (Fig. 4C,D). Thus, HP-DC induced a H. pylori-specific Th response and reduced H. pylori colonization.

Depletion of Tregs by CD25 Antibody Further Enhanced the Ability of HP-DC Mice to Prime H. pylori-Specific Immunity

H. pylori-infected individuals have been shown to express higher levels of Foxp3, a regulatory T-cell (Treg) marker. We speculate that a depletion of Tregs by the anti-CD25 antibody PC61 may further enhance the ability of HP-DC to limit H. pylori colonization. To examine this, PC61-treated or nontreated mice vaccinated with HP-DC were challenged with H. pylori and then analyzed 7 days later.
Figure 1  H. pylori-pulsed dendritic cells (HP-DC) induce Th1 helper response in vitro. (A) H. pylori induces increased expression of DC costimulatory molecules and intracellular cytokines. DCs were cocultured with PBS or H. pylori SS1 (MOI 1 : 100) for 18 hours. The expressions of surface costimulatory molecules CD40, CD80, and CD86 and intracellular cytokines interleukin (IL)-4, IL-10, IL-12, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ were assessed by fluorescence-activated cell sorting (FACS). Representative histograms from at least three independent experiments are shown (isotype control: shaded area, phosphate-buffered saline (PBS) control: dotted line, and H. pylori-pulsed DCs: solid line) (B, C). HP-DC stimulate splenocyte proliferation. Irradiated (5000 rads) HP-DC were cocultured with naïve syngeneic splenocytes for 72 hours. Micrographs show intense splenocyte clustering in the HP-DC culture (×100). Splenocyte proliferation was quantified in a dose–response manner using a tritiated thymidine (3H) incorporation assay. (D) HP-DC stimulate a Th1 response. The production of Th1/Th2 cytokines was measured using cytometric bead assays. HP-DC induced a type 1 helper T cell (Th1)-dominant response with increased levels of TNF-α and IFN-γ. Data shown represent results from three independent experiments.
postinfection. We found that PC61-treated mice had a lower percentage of splenic CD4+CD25+ T cells (Fig. 5A,B) and an increased H. pylori-specific Th1 response (Fig. 5C) with a significant reduction of H. pylori colonization in the stomach (p = .0028) compared to non-PC61-treated HP-DC immunized mice (Fig. 5D). No significant gastritis was noted at this early time point in either group (data not shown). These data indicate further enhancement of H. pylori-specific Th1 response by removal of Tregs.

Discussion

Research has shown that human DCs pulsed with H. pylori stimulate a Th1-dominant response [17, 18]. Although the use of DC vaccine as a routine immunization strategy for H. pylori infection may appear impractical and too technically complex, the role of DC-based vaccine may benefit patients infected with antibiotic-resistant H. pylori, especially those with mucosa-associated lymphoid tissue (MALT) lymphoma in which eradication of H. pylori may lead to disease regression [19]. We explored the possibility of inducing bacteria-specific immunity with a DC-based vaccine in a mouse model of H. pylori infection. Similar to human DCs, mouse bone marrow-derived DCs pulsed with live H. pylori stimulated a Th1-dominant response. HP-DC mice challenged with H. pylori exhibited an H. pylori-specific delayed-type hypersensitivity reaction, whereas PBS-DC mice challenged with H. pylori did not. Analysis of HP-DC-stimulated helper T-cell responses showed that HP-DC induce a mixed peripheral Th1/Th2 response but a Th1-dominant gastric response that lowered H. pylori colonization. We also showed that enhancing the H. pylori-specific Th1 response by Treg depletion further augmented H. pylori immunity.
Otsu et al. reported that the transfer of *H. pylori*-pulsed immortalized murine DC cell line (JAWS II) effectively induced therapeutic immunity against *H. pylori* infection (reported as a 2-log reduction in bacterial colonization) [20]. In our study, HP-DC induced a 1.25-log reduction in bacterial colonization compared to PBS-DC. The difference in these results may be explained by their use of immortalized DC cell lines versus bone marrow-derived primary DCs used in this study. Immortalized cells may have higher efficacy in vivo due to prolonged antigen priming. Another difference between the two studies is the methods used to quantify *H. pylori* colonization (i.e. culture by Otsu et al. vs. real-time PCR in this study). In our laboratory, we find that real-time PCR quantification of *H. pylori* colonization is more sensitive than culturing methods (data not shown).

The current study shed light on the complexity of vaccine development against *H. pylori*. As the debate over the requirement of a Th1 response for vaccine-induced protective immunity continues [4,5], our finding supports the current paradigm that an effective Th1 response protects against *H. pylori* infection. Ineffective Th1 effector response, suggested by our previous study of a defective Th1 priming by dendritic cells [6], may result in the persistent colonization and the development of chronic gastritis. Using a potent inducer of antigen-specific cellular immunity with DC vaccine, we show that *H. pylori*-specific Th1 response can be induced in vivo. A Th2 response also was augmented by DC vaccine but its role is less well-defined as protective immunity could be achieved in B-cell-deficient mice [21,22]. Additional support for the importance of effector Th1 response was reported by Rad...
et al., where depletion of CD4+CD25+ Tregs correlated with a reduction in H. pylori colonization [23]. In our study, a depletion of CD4+CD25+ Tregs further enhances DC vaccine primed H. pylori-specific Th1 response and reduced bacterial colonization. As an intact Th1 response is essential for vaccine-induced protection against H. pylori, a balanced, nonrestrictive Treg response may be the other determinant of whether H. pylori sterilization can be achieved. Understanding the interaction between H. pylori and host immune system with respect to induction of Th and Treg response will be critical for the success of a vaccination strategy. Furthermore, since Treg is also involved in regulation of gastric inflammation [23], manipulations to upregulate this response may prove to be beneficial in suppressing chronic gastritis.

In summary, our study shows that DC-based vaccine is capable of inducing protective H. pylori-specific immunity. Treg depletion with anti-CD25 antibodies enhanced the ability of DC vaccine to prime a H. pylori-specific immunity. Our study supports the critical role of a Th1-skewed response in anti-H. pylori immunity. These studies may shed light on novel strategies to modulate the host response to eradicate H. pylori infection.

The preliminary results of this study were presented orally at the 2004 Digestive Disease Week and published in abstract form in Gastroenterology (Vol. 126(4) Suppl. 2: A790). This study was supported by grants from the National Institutes of Health (1 KO8 DK0669907-01 and P30 DK034933), the Foundation of Digestive Health and Nutrition (Fellow-to-Faculty Transition Award), and the GlaxoSmithKline Institute of Digestive Health (Basic Research Award).

References

5. Garhart CA, Heinzel PP, Czinn SJ, Nedrud JG. Vaccine-induced


