Dendritic Cells Prevent Rather Than Promote Immunity Conferred by a *Helicobacter* Vaccine Using a Mycobacterial Adjuvant

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BACKGROUND & AIMS: Immunization against the gastric bacterium *Helicobacter pylori* could prevent many gastric cancers and other disorders. Most vaccination protocols used in preclinical models are not suitable for humans. New adjuvants and a better understanding of the correlates and requirements for vaccine-induced protection are needed to accelerate development of vaccines for *H pylori*. **METHODS:** Vaccine-induced protection against *H pylori* infection and its local and systemic immunological correlates were assessed in animal models, using cholera toxin or CAF01 as adjuvants. The contribution of B cells, T-helper (Th)–cell subsets, and dendritic cells to *H pylori*–specific protection were analyzed in mice. **RESULTS:** Parenteral administration of a whole-cell sonicate, combined with the mycobacterial cell-wall–derived adjuvant CAF01, protected against infection with *H pylori* and required cell-mediated, but not humoral, immunity. The vaccine-induced control of *H pylori* was accompanied by Th1 and Th17 responses in the gastric mucosa and in the gut-draining mesenteric lymph nodes; both Th subsets were required for protective immunity against *H pylori*. The numbers of memory $CD4^+$ T cells and neutrophils in gastric tissue were identified as the best correlates of protection. Systemic depletion of dendritic cells or regulatory T cells during challenge infection significantly increased protection by overriding immunological tolerance mechanisms activated by live *H pylori*. **CONCLUSIONS: Parenteral immunization with a *Helicobacter* vaccine using a novel mycobacterial adjuvant induces protective immunity against *H pylori* that is mediated by Th1 and Th17 cells. Tolerance mechanisms mediated by dendritic cells and regulatory T cells impair *H pylori* clearance and must be overcome to improve immunity.**

Keywords: Gastric Cancer; Bacteria; Ulcer; Gastric Adenocarcinoma.

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Helicobacter pylori colonizes half of the world's population and chronic gastric infection with this bacterium leads to the development of gastric ulcers, gastric adenocarcinoma, or mucosa-associated lymphoid tissue lymphoma in 1%–10% of infected individuals.[1] The standard *H pylori* eradication therapy consists of 2 to 3 antibiotics and an acid suppressant; however, antibiotic resistance rates are rising,[2] a subset of individuals relapse even after efficient eradication,[3] and the infection is often not diagnosed until gastric premalignant lesions have progressed to an irreversible stage.[4] Immunization against *H pylori,* therefore, represents an attractive alternative strategy for the prevention of gastric cancer and other *Helicobacter*-associated gastric disorders.

No vaccine regimens are currently available for human use. The gold standard vaccination protocol that has been used extensively in preclinical models combines the mucosal adjuvant cholera toxin (CT) with whole-cell preparations of *H pylori* or *Helicobacter felis* into orally administered formulations.[5–8] Because of the toxicity of CT, human trials have bypassed this effective adjuvant in favor of other less toxic compounds, such as the heat-labile enterotoxin of *Escherichia coli*, which was combined with recombinant urease B- or whole-cell preparations to generate well-tolerated, but largely ineffective formulations.[9–11] A live recombinant *Salmonella enterica* serovar Typhi Ty21a vaccine expressing *H pylori*'s urease A and B subunits also did not show sufficient immunogenicity in human volunteers,[12,13] but confirmed a direct correlation between *H pylori*–specific T-cell responses and bacterial clearance.[14]

Protective immunity against *H pylori* can be achieved by either Th1 and/or Th17-polarizing adjuvants[5,15–18] or by aluminum-based adjuvants that stimulate Th2-polarized responses.[19,20] Both complete Freund's adjuvant and alum have been used successfully in preventive[20,21] and even therapeutic[19] vaccination strategies. An alum-adjuvanted parenteral vaccine using the *H pylori* antigens VacA, CagA, and NAP was recently reported to be immunogenic and safe in humans.[22] Here we focus on a novel adjuvant for Th1/Th17 vaccination that incorporates a synthetic analog (trehalose-6,6-dibehenate) of the mycobacterial cell wall glycolipid trehalose-6,6-dimycolate into a cationic liposome-based adjuvant formulation (CAF01).[23] CAF01 facilitates antigen uptake and presentation by dendritic cells (DCs) and macrophages[24,25] and confers protective immunity to a variety of infectious agents, including *Mycobacterium tuberculosis, Chlamydia muridarum,* and the malarial parasite *Plasmodium yoelii.*[26,27] The mixed Th1/Th17

Abbreviations used in this paper: APC, allophycocyanin; CT, cholera toxin; DC, dendritic cell; DT, diphtheria toxin; IFN-γ, interferon-γ; IL, interleukin; IP, intraperitoneal; MHC, major histocompatibility complex; MLN, mesenteric lymph nodes; PB, pacific blue; SC, subcutaneous; Th1/2/17, T-helper type 1/2/17; Treg, regulatory T cell.

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T-cell responses elicited by CAF01-adjuvanted vaccination have been particularly well-characterized in the context of *M tuberculosis* infection.[23]

Here, we compared various routes of administration of a CAF01-adjuvanted *H pylori* whole-cell vaccine to a CT-adjuvanted immunization protocol with respect to protection from autologous challenge infection. We show that parenteral—but not mucosal—immunization with CAF01 elicits protective immunity to *H pylori* challenge infection that is antibody-independent, but requires both Th1 and Th17 subsets. Other correlates of protection included gastric memory T-cell, neutrophil, and mast cell infiltration. We further report here that immunoregulatory mechanisms involving DCs and regulatory T cells (Treg) impair protective immunity; consequently, the systemic depletion of DCs or Treg during challenge infection significantly improved the efficacy of *H pylori*–specific vaccination.

Materials and Methods

Animal Experimentation

C57BL/6 wild-type, major histocompatibility complex (MHC) $II^{-/-}$, $JHT^{-/-}$ (which lack J_H segments and the intron enhancer in the IgH locus and therefore cannot generate B cells),[28] interleukin (IL)-12 $p35^{-/-}$, IL-12/IL-23 $p40^{-/-}$, and *CD11c*-DTR tg mice (expressing the diphtheria toxin [DT] receptor under the control of the *CD11c* promoter) were originally purchased from Charles River Laboratories (Wilmington, MA). *FoxP3*-EGFP-DTR tg mice[29] and IL-23 $p19^{-/-}$ mice were kindly provided by Tim Sparwasser and Regeneron Pharmaceuticals, respectively. All mice were bred at a University of Zurich specific pathogen-free facility. Mixed-sex groups were included in studies at 6 weeks of age. All animal experimentation was reviewed and approved by the Zurich cantonal veterinary office. Mice were immunized 3 times at weekly intervals. A stable CAF01 formulation consisting of dimethyldioctadecylammonium bromide and a,a′-trehalose 6,6′-dibehenate (Avanti Polar Lipids, Alabaster, AL) was prepared by the lipid film hydration method, as described previously.[26] For CAF01 vaccinations, 250 μg *H pylori* SS1[30] whole-cell sonicate was given per dose. In the case of intraperitoneal (IP), oral, or subcutaneous (SC) immunization at the base of the neck, the total volume was 200 μL. For intranasal immunizations, a total volume of 50 μL was given in drops of 12.5 μL to alternating nostrils of mice anesthetized with isoflurane (Minrad, Buffalo, NY). For CT immunization, mice were administered 500 μg sonicate along with 10 μg CT (List Biologicals, Campbell, CA) by oral gavage. Two weeks after the last immunization, immunized and naïve mice were infected with 10^8 *H pylori* SS1 (colony-forming unit estimated based on optical density) grown as described previously.[7] Mice were sacrificed 2 weeks after challenge. Bacterial colonization was assessed by colony count assay as described earlier.[7] DT (Sigma-Aldrich, St Louis, MO) was injected IP every 2 days for the depletion of DCs (4 ng/g body weight) or of Treg (40 ng/g body weight).

Preparation of Gastric and Mesenteric Lymph Node Single-Cell Suspensions, Flow Cytometry, Enzyme-Linked Immunosorbent Assay, Quantitative Reverse Transcription Polymerase Chain Reaction, and Treg Conversion Assay

One-sixth of every stomach (antrum and corpus) and corresponding mesenteric lymph nodes were digested in 1 mg/mL collagenase (Sigma-Aldrich) for 45 minutes at 37°C with shaking before mechanical disruption between glass slides and filtering. Single-cell suspensions were either stained directly for flow cytometric analysis or seeded at 200-k cells/well into 96-well round-bottom plates. The following antibodies were used: CD4–fluorescein isothiocyanate, CD4-allophycocyanin (APC) (BD Biosciences, San Diego, CA), CD45-pacific blue (PB), Ly6G-APC, CD62L-APC, CD44-PB, rat anti-mouse CD117 (all BioLegend, San Diego, CA), followed by goat anti-rat fluorescein isothiocyanate (Sigma-Aldrich) and CD11c-biotin (BD Biosciences), followed by streptavidin-PB (Life Technologies, Carlsbad, CA). Interferon-γ (IFN-γ)-phycoerythrin-Cy7 (BD), IL-17–APC, and FoxP3-APC (all eBioscience, San Diego, CA) were used for intracellular staining. Before intracellular cytokine staining, cells were stimulated and blocked in 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) for 5 hours, stained for extracellular markers, and fixed in 4% paraformaldehyde. Flow cytometry was performed on a Cyan ADP 9 instrument (Beckman Coulter, Brea, CA) and analyzed using FlowJo software (TreeStar, Ashland, OR). Enzyme-linked immunosorbent assay and quantitative reverse transcription polymerase chain reaction techniques, as well as the protocols for Treg conversion are described in the Supplementary Methods.

Statistics

GraphPad Prism (GraphPad Software, La Jolla, CA) was used for statistical analyses. Colonization counts were compared by Mann–Whitney test. All other indicated *P* values were calculated by Student *t* test.

Results

Parenteral CAF01-Adjuvanted Vaccination Confers Protective Immunity to H pylori

To test the efficacy of the synthetic mycobacterial adjuvant CAF01 in *H pylori*–specific vaccination, we immunized mice with either the gold standard CT-adjuvanted, orally administered *H pylori* sonicate vaccine, or mixed the same sonicate with CAF01 for SC, IP, intranasal, and oral administration. Two weeks after the last of 3 weekly doses, all immunized mice as well as nonimmunized controls were challenged with 10^8 live *H pylori* SS1 and sacrificed 2 weeks later (see timetable in Figure 1*A*).