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Vaccination of mice against *H. pylori* induces a strong Th-17 response and immunity that is neutrophil-dependent

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Abstract

Background & Aims—Vaccine efficacy against gastric *Helicobacter pylori* infection has been demonstrated in mice, but little is known about the effector mechanisms of bacterial clearance. Our aim was to investigate a possible T cell-neutrophil pathway of vaccine-induced protection.

Methods—Nonimmune and immunized mice were compared for their response to *H. pylori* challenge. T cell responses were assessed by recall assays. IL-17-induced chemokine production by leukocyte and nonleukocyte cells was evaluated by cytokine ELISA. In a kinetic study, biopsies were collected at multiple time points post-challenge and assessed for bacterial load and inflammation. Relative levels of T cells, IL-17, IFN γ , MIP-2, KC, and LIX were determined by quantitative PCR. The role of neutrophils was evaluated by antibody-mediated depletion of neutrophils following challenge.

Results—Immunization induced strong IFN γ and IL-17-producing T cell responses, and IL-17 was capable of inducing significant amounts of KC and MIP-2 from dendritic cells, macrophages, fibroblasts, and gastric epithelial cells. Challenge of immunized mice induced significantly greater gastritis than infected mice, preceding significantly lower bacterial loads by day 7. In immune mice, T cell recruitment to the gastric mucosa correlated with a continuous rise in IL-17 and IFN γ , followed by KC, MIP-2, and LIX production and the recruitment of significant numbers of neutrophils by day 5. Antibody-mediated depletion of neutrophils abrogated vaccine efficacy.

Conclusions—Vaccination of mice against *H. pylori* results in a significant Th-17 cell recall response associated with increases in chemokines that attract neutrophils to the stomach which are important for eradication of *H. pylori*.

Helicobacter pylori is a gram-negative bacteria that inhabits the gastric mucosa. Over half the world's population is currently infected.¹ Although the majority of individuals remain asymptomatic, infection can lead to the development of peptic ulcer disease, gastric MALT

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lymphoma, and gastric cancer, which are associated with significant morbidity and mortality. No vaccine is available for use in humans and antimicrobial therapies are complex, expensive, and not practical for use in many parts of the world where infection is endemic. Without therapeutic intervention, infection with *H. pylori* is life-long.

Vaccination in mouse models of *H. pylori* can achieve protection, and this model provides an excellent tool for comparing the immune responses of unimmunized and immunized mice. Infected mice develop low-grade, active, chronic inflammation which fails to clear the bacteria from the gastric mucosa. The immune response following challenge of immunized mice is rapid and severe and leads to a significant reduction in bacterial load, and sometimes clearance, of the bacteria. It is well established that CD4⁺ T cells are necessary for facilitating this protection,^{2, 3} but it is not understood how these T cells exert their effects and which immune cells act downstream of T cell activation to eliminate the bacteria.

IL-17 exhibits proinflammatory effects and is known to mediate the recruitment of neutrophils by inducing chemokine secretion in various cell populations.^{4, 5} Little is currently known about the role of IL-17 in *H. pylori* infection and immunity, although this cytokine has been identified in biopsies of *H. pylori*-infected patients and IL-17 levels have been shown to increase over time in the stomachs of infected mice.^{6, 7} Neutrophils are abundant in post-immunization gastritis and are capable of translocating across the gastric epithelium into the lumen where they can phagocytose *H. pylori*.⁸ *H. felis*-infected IL-10^{-/-} mice, which are normally capable of eliminating the bacteria, have delayed clearance when they are treated with a neutrophil-depleting antibody.⁹ A role for neutrophils in vaccine-induced protection against *H. pylori* has not been demonstrated, although a model of decreased immunity in mice that received a sialoadenectomy was also accompanied by reduced numbers of neutrophils in the gastric mucosa.¹⁰ Therefore, we performed a kinetic study of cellular recruitment and cytokine production along with a neutrophil depletion model to assess the contribution of neutrophils in immunized/challenged mice. We now demonstrate that the protective immune response includes the early recruitment of T cells and significant IL-17 production, followed by the elaboration of neutrophil-promoting chemokines and subsequent reduction in bacterial load. Additionally, depletion of neutrophils in immunized mice during challenge compromises the host's ability to eradicate *H. pylori*.

Our findings suggest that IL-17 plays a major role in the early immune response of immunized mice, contributing to the rapid, severe inflammation in which neutrophils are an important effector cell for clearance of *H. pylori*.

Methods

Bacteria

Helicobacter pylori Sydney strain 1 (Hp SS1) was grown on Columbia blood agar or in Brucella broth with supplements as previously described.³ Actively growing liquid broth cultures were used for infection and *in vitro* assays. Live Hp SS1 cultures from Columbia blood agar plates were sonicated in PBS and passed through a 0.2 µm filter to produce bacterial lysate used for immunization and *in vitro* assays.

Mice

Wild-type C57BL/6 female and G-CSF knockout male and female C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were housed under pathogen-free conditions in microisolator cages at Case Western Reserve University's Animal Resource Center (CWRU ARC). Mouse protocols were approved by the Institutional Animal Care and Use Committee. The CWRU ARC is accredited by the American Association for the Accreditation of Laboratory Animal Care International.

For immunization, mice received 100 µg Hp SS1 lysate and 5 µg cholera toxin adjuvant (Sigma, St. Louis, MO) in 20 µl PBS intranasally once a week for 4 weeks. For infection, 0.5 ml live Hp SS1 (approximately 10^7 CFU) in Brucella broth was administered by oral gavage on two consecutive days, unless otherwise noted.

in vivo Kinetic Study

Thirty five immunized and 35 unimmunized female C57BL/6 mice were infected on day 0 (approximately 1 month following the last immunization). Groups of five mice were sacrificed at days 1, 2, 3, 5, 7, 9, and 13. Five naïve mice were sacrificed at day 8. Mock-immunized (BSA and adjuvant) mice were infected with Hp SS1 and harvested at 2 weeks post-challenge. Stomachs were harvested for bacterial load determination, inflammation grading, and RNA isolation. Bacterial load (CFU/g tissue) was determined by homogenizing pre-weighed biopsies in Brucella broth and culturing serial dilutions on Columbia blood agar plates. Biopsies encompassing the outer curvature of the stomach were fixed in 10% buffered formalin, paraffin-embedded, and sectioned for histology. Gastritis and neutrophil levels were scored from H&E-stained sections by a pathologist (RWR) blinded to sample identities. The most inflamed area of each section was given a score of 0-5, as previously described.¹¹ Gastric neutrophil counts were the number of neutrophils per high-power field of that most inflamed area. A third biopsy was stored in RNeasy lysis buffer (Qiagen, Crawfordsville, IN) at -20°C, and then later used as a source of RNA for reverse transcription quantitative PCR for CD3, IFN γ , IL-17, KC, MIP-2, and LIX.

RT-quantitative PCR

Total RNA samples were quantified, and archive cDNA was generated using an ABI high-Capacity cDNA archive kit using similar amounts of total RNA as starting material in a 100ul RT reaction in an ABI 9700 PCR unit. 384-well plates were set up to accommodate triplicate reactions. Mouse beta actin was used as an endogenous control. The ABI assays for murine CD3, IFN γ , IL-17, KC, MIP-2, and LIX were purchased from ABI and run according to manufacturer's instructions for a 40 cycle run. Results were generated using ABI SDS 2.0 software and presented as relative fold changes versus a designated calibrator sample, which was a naïve mouse sample except for IFN γ and IL-17. For these samples, nothing was detected in the naïve controls, so an infected mouse from day one was used as a calibrator sample. Results include 95% confidence limits.

Cell Culture assays

Bone marrow (BM) was isolated from mouse hind leg femurs and tibias by standard technique. Macrophages were grown from BM cells in DMEM supplemented with 10%

FBS, 1mM sodium pyruvate, 10mM HEPES, 20 µg/ml gentamicin, 50 µM β-mercaptoethanol, and 20% GM-CSF-conditioned media from Ladmec cell culture. Nonadherent cells were removed by washing. For DC cells, 10⁶ BM cells were plated in 1 ml of RPMI (supplemented with 5% FBS, 10mM HEPES, 20 µg/ml gentamicin, 50 µM β-mercaptoethanol, and 10 ng/ml GM-CSF)/well in 24-well plates. Wells were gently washed on days 2 and 4. Loosely adherent cells were transferred on day 6 to petri dishes. Loosely adherent DC were collected from these dishes and used on day 7. Neutrophils were purified by percoll gradient centrifugation¹² which yielded approximately 85% pure neutrophils by examination of H&E-stained cytospin slides. Fibroblasts were derived from mouse tail skin which was minced and incubated in DMEM containing 0.5 mg/ml dispase for 2 hours at 37°C. The cell-containing media was filtered and plated. Attached fibroblasts became confluent by day seven. The temperature sensitive GSM06 mouse gastric epithelial cell line originally cultured from SV40 T antigen-transgenic mice was purchased from the RIKEN Cell Bank (Tsukuba Science City, Japan).¹³ They were grown to confluency in DMEM/F-12 (supplemented with 2% FBS and 1X ITES) at 33°C but used at 37°C in assays. All other cultures were maintained at 37°C. For assays, triplicate wells of 10⁵ cells in 1.2 ml media were plated in 48-well plates and stimulated with 20 ng/ml recombinant mouse IL-17 or IFNγ (eBioscience, San Diego, CA).

in vitro Antigen Presenting Cell (APC)-T cell Co-cultures

Murine macrophages or DC were left untreated or stimulated with Hp SS1 lysate (10 µg/ml) for 4 hours, washed twice, and plated at 10⁵ cells per well in 96-well, round-bottom plates in RPMI (supplemented with 10% FBS and 20 µg/ml gentamicin). For T cells, CD4⁺ cells were purified from bulk splenocytes using positive selection on MACS magnetic columns (Miltenyi Biotec, Auburn, CA). 10⁶ CD4⁺ T cells from each mouse were co-cultured with APCs in triplicate. Supernatants were stored at -70°C until assessed by ELISA.

Enzyme-linked Immunosorbent Assays

Culture supernatants were analyzed for IL-17, IFNγ, KC, MIP-2, and LIX levels using DuoSet ELISA Development Systems according to the manufacture's protocols (R&D Systems, Minneapolis, MN). Concentrations were determined by comparison to a standard curve included on each plate.

Neutrophil Depletion study

The neutrophil-specific NIMP-R14 hybridoma was a generous gift from Dr. Eric Pearlman (CWRU SOM, Cleveland, OH).¹⁴ Monoclonal rat IgG antibody was purified from hybridoma supernatants by protein G affinity chromatography. Male and female G-CSF KO mice were used at approximately six weeks of age and distributed equally among groups. Hp SS1-immunized G-CSF KO mice were given 1 mg NIMP-R14 in PBS i.p. beginning one day prior to Hp SS1 challenge and continuing every other day until harvest (2 weeks post-challenge). Another group of immunized/challenged G-CSF KO mice were injected with control rat IgG monoclonal antibody (BioExpress, West Lebanon, NH). Immunized/challenged G-CSF KO and WT mice without antibody treatment were also included. Venous blood collected at harvest was analyzed for all white blood cell levels on a Hemavet 950 (Drew Scientific, Oxford, CT). Stomach biopsies were harvested and analyzed for bacterial

load and inflammation as described above. Gastric histology was also analyzed for neutrophil and mast cell levels by H&E and toluidine blue staining, respectively.

Statistics

Statistics were determined using the Student's t test. Differences between groups were considered significant at an interval level of $p < 0.05$.

Results

CD4⁺ T cells from immunized mice exhibit a robust IL-17 recall response in vitro

Recall assays were performed to investigate potential differences in the proinflammatory properties of splenic CD4⁺ T cells taken from immunized (unchallenged) mice a week after the final boost, compared to cells from chronically infected mice taken at least three weeks after infection. *H. pylori*-pulsed BM-derived macrophages or DC were used to activate CD4⁺ cells from naïve, infected, and immunized mice. IFN γ was produced by CD4⁺ cells from immunized mice stimulated with pulsed DC by 48 hours, but not by cells from non-immunized infected mice (Figure 1A). When macrophages were used, IFN γ was also produced by the CD4⁺ cells from immunized mice, but at approximately half the levels observed in DC-stimulated cultures (Figure 1B). IL-17 was also detected at higher concentrations than IFN γ (Figure 1C and D). Similar to IFN γ production, the use of DC resulted in approximately twice the amount of IL-17 compared to macrophages. Little, if any, IL-17 was produced by cells from unimmunized, infected mice. No cytokine was detected from pulsed APC monocultures (data not shown), cultures of CD4⁺ cells in the absence of APC, or from any cultures using cells from naïve mice.

IL-17 and *H. pylori* induce the production of chemokines for neutrophil recruitment in vitro

In vitro culture models of cell types likely to be present in the gastric mucosa, including macrophages, DC, fibroblasts, and epithelial cells, were stimulated with IFN γ or IL-17, and the supernatants were evaluated for KC, MIP-2, and LIX. IFN γ failed to induce the production of chemokines beyond the levels observed for unstimulated cells (Figure 2A and B). Recombinant IL-17, however, induced fibroblasts and epithelial cells to produce significant amounts of both KC (Figure 2A) and LIX (Figure 2B) by 24 hours. No MIP-2 could be detected in any cultures stimulated with IL-17 or IFN γ . Direct stimulation of cells with live *H. pylori* or *H. pylori* lysate antigen induced differing patterns of secretion. DC and fibroblasts produced KC, MIP-2, and LIX, macrophages produced KC and MIP-2, and epithelial cells produced some KC and LIX. Neutrophils were shown to produce MIP-2 only (data not shown).

T cells and IL-17 are detected at day 3 post-challenge in immunized mice, prior to increase in inflammation

Immunized mice were assessed at days 1, 2, 3, 5, 7, 9, and 13 post-challenge to characterize the early immune response. Significant gastritis developed by day five and inflammation continued to increase through day 13 (Figure 3A). In contrast, unimmunized mice did not develop statistically significant inflammation until day 13. The increase in inflammation in

immunized mice at day five preceded a subsequent decrease in bacterial load that was evident by day seven (Figure 3B). Unlike the immunized mice, unimmunized mice displayed levels of bacteria that continued to increase over time.

Recruitment of T cells in these mice correlated with the development of inflammation. Significant levels of CD3 mRNA were detectable in the stomachs of immunized mice by three days post-challenge, and similar to the overall gastritis scores in these mice, continued to increase throughout the entire 13 days (Figure 4A). This suggests T cell recruitment commences immediately prior to the development of significant gastritis on day five (Figure 4A). Unimmunized mice did not develop levels of CD3 that were significantly greater than naïve mice until day 13. Increased expression of CD3 was accompanied by the production of IL-17 that was significantly greater than unimmunized mice by day three (Figure 4B). IFN γ levels also increased by day five but were significantly lower than IL-17 expression on days five through 13. Unimmunized mice produced very low levels of both cytokines beginning at day 13 post-challenge. Because mock immunization with CT alone has occasionally been shown to elicit some degree of *Helicobacter* immunity, in a separate experiment we assessed the efficacy of mock immunization and the amount of IL-17 in the gastric mucosa. Although the CT immunized mice had less bacteria than unimmunized/challenged mice, they had a significantly elevated bacterial load ($P = 0.01$) compared to mice immunized by the standard vaccine. Gastric IL-17 and IFN γ mRNA expression in CT-immunized mice was comparable to unimmunized/challenged mice and significantly less than mice immunized by our standard protocol ($P < 0.004$; data not shown).

Immune mice recruit neutrophils to the gastric mucosa prior to reductions in bacterial load

To determine whether the IL-17 observed above promoted neutrophil recruitment, KC, MIP-2, and LIX mRNA was also measured. Although the relative quantity of LIX mRNA was significantly elevated by day three in immunized mice ($P < 0.02$; Figure 5A), notably elevated mRNA for all three chemokines were observed by day five ($P = 0.02$; Figure 5A, B, and C). Little or no expression of KC, MIP-2, or LIX was detected in the stomachs of nonimmunized mice. Histologic scoring of sections from these stomach tissues revealed significant increases in the number of neutrophils by day five post-challenge in immune mice. Neutrophil levels remained elevated throughout the time course, although levels did decline following significant decreases in bacterial load (Figure 5D – 5F).

Neutrophil depletion of immunized G-CSF^{-/-} mice results in reduced protective immunity against *H. pylori* challenge

Neutrophils were depleted to determine if they represent an important effector mechanism in the clearance of *H. pylori* from the gastric mucosa of immunized mice. G-CSF^{-/-} mice were used to decrease the baseline amount of neutrophils. NIMP-R14 antibody was administered to immunized mice throughout the challenge phase and peripheral blood cell counts were performed at harvest to assess the efficacy of antibody treatment. NIMP-R14-treated immunized mice had significantly lower peripheral blood neutrophils than either control untreated groups or mice treated with control rat monoclonal antibody (Figure 6A; $P < 0.01$). Immunized mice treated with control rat IgG or left untreated had comparable blood

neutrophil levels. The majority of NIMP-R14-treated immunized mice also had reduced neutrophil counts in the gastric mucosa compared to control antibody treated mice (Figure 6B). Mast cell levels in the gastric mucosa of these mice were low and did not vary among groups (data not shown).

Gastric inflammation scores in the G-CSF^{-/-} mice were equivalent in all three immunized groups 14 days post-challenge suggesting that reduction in neutrophil counts did not affect the overall intensity of inflammation (Figure 6C). However, although gastritis levels were comparable among these groups of mice, only the NIMP-R14-treated mice had a reduced capacity to eradicate the *H. pylori* from the gastric mucosa (Figure 6D). Untreated and control IgG-treated immunized mice achieved protective immunity, while the bacterial load in neutrophil-depleted mice was equivalent to unimmunized mice.

Discussion

The ability to achieve protective immunity against *H. pylori* in mice has been well documented. The immunologic events responsible for reduced bacterial loads in immunized mice, however, are still being elucidated. CD4⁺ T cells are necessary for the development of protective immunity^{2, 3} and the p40 subunit of both IL-12 and IL-23 is also important as immunized mice are not fully protected in its absence.¹⁵ More recently mast cells have also been implicated as an important mediator of protection in an *H. felis* immunization model.¹⁶ We now demonstrate that depletion of neutrophils in immunized mice compromises the host's ability to eradicate *H. pylori* following challenge.

The capacity of neutrophils to kill *H. pylori* *in vitro*¹⁷ and *in vivo*⁸ has been described, and the abundance of these phagocytes combined with their ability to cross the epithelium make them attractive candidates as effectors of bacterial clearance. Additional evidence for the importance of neutrophils in *Helicobacter* eradication comes from a study in IL-10^{-/-} mice.⁹ These mice develop severe inflammation capable of clearing *H. felis* from the gastric mucosa. However, when neutrophils were depleted, the mice failed to significantly reduce the bacterial load.

Data from our kinetic study suggest that challenge of immunized mice results in a temporally-ordered sequence of events involving proinflammatory T cell cytokines, resident nonleukocyte cells, and the production of chemokines that favor neutrophil recruitment. T cells were present by day three post-challenge and the production of IFN γ , and, to a greater extent, IL-17 occurred concomitantly with recruitment of the T cells. The production of these cytokines was rapidly followed by significant increases in KC, MIP-2, and LIX and a significant increase in general inflammation. The recruitment of significant numbers of neutrophils did not occur until the relevant chemokines were evident on day five. As the levels of neutrophils continued to increase on day seven, the bacterial load began to significantly decrease.

The rapid induction of inflammation post-challenge in immunized mice reported here are consistent with previous reports that included early timepoints.^{11, 18} Also similar to our analysis, Walduck *et al.* reported that gastric CD4⁺ and CD8⁺ cells were detectable in

immune mice by day three post-challenge followed by a significant decrease in bacterial load by day seven.¹⁸ Of particular interest is the link between the CD4⁺ T cell response and recruitment and activation of neutrophils. In the absence of CD4⁺ T cells, mice fail to develop significant gastritis when infected with *Helicobacter* strains.¹⁹ Thus, neutrophils do not inherently participate in the acute phase response to *H. pylori* infection and become prevalent only following appropriate T cell help signals. During natural and experimental infection T regulatory cells can suppress the activity of proinflammatory T cells.^{20, 21} In immunized mice, however, T helper cells are sufficiently activated to drive a proinflammatory response that ultimately reduces or eliminates *H. pylori*. The amount of IL-17 produced *in vitro* by isolated T cells from immune mice, and the prevalence of IL-17 in the gastric mucosa following challenge of immune mice compared to nonimmune mice indicate that IL-17-producing T cells promote this response.

Th-17 cells have recently been described as a distinct population of proinflammatory T cells that play an important role in the host response to infectious disease.^{4, 22} Cells expressing receptors for IL-17 include fibroblasts and epithelial cells.^{4, 23} Thus our experiments in which IL-17 induced the production of KC, MIP-2, and LIX primarily from murine fibroblasts and gastric epithelial cells are consistent with the known activity of IL-17 and supports our tissue-based observations that these three chemokines show a rapid and significant increase following the local production of IL-17.

IL-17 is elevated in biopsies from infected patients⁶ but since infection persists in humans, little can be concluded about the role of IL-17 in the host response. Our results in the mouse model show that immunized mice exhibit a robust IL-17 recall response not seen with cells or tissue from unimmunized/challenged mice. IL-17 expression occurred within a few days of bacterial challenge and remained elevated throughout the effector phase of bacterial eradication. A central role for Th-17 cells as mediators of protective *Helicobacter* immunity may explain why several IFN γ -deficient mice can still be protected by vaccination.^{15, 24} Such results suggest that it is IL-17 and not IFN γ that drives the protective inflammation, or that in the absence of IFN γ , IL-17 serves as a surrogate pathway for developing protective gastritis. A crucial role for IL-17 is also consistent with the inability to protectively immunize p40 knockout mice since IL-23 may be required for the proper development of Th-17 cells.¹⁵

Our results contrast with a report by Velin *et al.* in which antibody-mediated depletion of neutrophils failed to ameliorate vaccine-induced protective immunity against *H. felis*.¹⁶ Differences in results may be due to differences between the *H. pylori* and *H. felis* models of infection, or the different time points used for evaluation. However, as noted above, Ismail *et al.* demonstrated a substantial contribution of neutrophils to non-vaccination based bacterial clearance in IL-10^{-/-} mice using *H. felis*.⁹ It is more likely the different results are due to the effectiveness of neutrophil depletion. Although Velin *et al.* reported significant reductions of neutrophil levels in peripheral blood, such observations cannot necessarily be extrapolated to the tissue in which inflammation is induced. Ismail *et al.* observed a decrease in bacterial load as neutrophil levels began to rebound in the stomach, despite the continued significant reduction of peripheral neutrophils. *H. felis* typically induces robust inflammation, so it is possible that whatever neutrophil levels remained following antibody treatment were

concentrated in the gastric mucosa where they could effectively kill the bacteria. Since gastric neutrophil levels were not assessed by Velin *et al*, it is impossible to know whether in fact the neutrophil depletion obtained in the periphery was apparent in the gastric mucosa as well.

In our study, to better evaluate the contribution of neutrophils in the protective response, we used G-CSF^{-/-} mice combined with treatment with the anti-neutrophil antibody NIMP-R14. Our preliminary studies in C57BL/6 wild type mice showed that antibody treatment becomes less effective over time (data not shown), possibly due to the host's eventual immune response to the presence of foreign antibody. The inability to suppress neutrophil levels for prolonged periods is problematic when studying models of chronic infection like *H. pylori* where treatment regimens last for weeks. The use of G-CSF^{-/-} mice in addition to antibody-mediated depletion achieved sufficiently low neutrophil levels for most mice for the entire two week study. The importance of maintaining low levels of neutrophils for the duration is illustrated by our observation that two of the NIMP-R14-treated mice with higher gastric neutrophil counts than average were still able to clear the bacteria.

One concern with the use of G-CSF^{-/-} mice is the possibility that the lack of G-CSF might affect the development of other cell populations. While these mice do tend to have fewer circulating monocytes, the deficiency becomes significant only in older mice.²⁵ Additionally, peripheral blood analyses at harvest confirmed that no differences in erythrocyte, lymphocyte, or eosinophil levels were observed compared to wild type mice. Interestingly, there were no differences in gastric mast cell levels among any of the groups of G-CSF^{-/-} mice in our study (data not shown). Since Velin *et al*. demonstrated that mast cells may be a mediator of *H. felis* eradication in immunized mice, it was important to verify that the failure of the immunized NIMP-R14-treated G-CSF^{-/-} mice to reduce their bacterial load was not due to reduced mast cell numbers.

Our study does not negate a role for mast cells in vaccine-induced protective *Helicobacter* immunity, but suggests that neutrophils are also important and play an independent role from mast cells. It is possible that mast cells are required for the activation of neutrophils. Indeed mast cells have been shown to recruit neutrophils in other models, including allergic gastric inflammation.²⁶ Additionally, others have reported that mast cell-mediated neutrophil recruitment via IL-8 was dependent on the heterotypic adhesion of mast cells and activated T cells *in vitro*.²⁷ Such findings may explain the necessity of CD4⁺ T cells, mast cells, and neutrophils for the clearance of *H. pylori* in immunized mice.

In conclusion, our study demonstrates that neutrophils are required for the clearance of *H. pylori* in a model of vaccine-induced immunity. We also suggest that unlike unimmunized mice, immune mice are able to eliminate the bacteria due to the rapid activation of Th-17 cells which act on resident nonleukocyte cell populations to produce chemokines that subsequently recruit and activate the neutrophils. The means by which immunization predominantly promotes Th-17 cells rather than IFN γ -producing T cells and the relationship between the Th-17 cells and nonlymphocyte effector cells will be the subject of future investigation.

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Figure 1.

Cytokine recall response of T cells from *H. pylori*-infected and immune mice. Purified CD4⁺ cells were isolated from the spleens of naïve mice, immunized unchallenged mice, or mice experimentally infected with *H. pylori* and co-cultured for 48 hours with antigen-pulsed APCs. The culture supernatants were assessed by quantitative ELISA for the presence of IFN γ or IL-17. (A) IFN γ production in the presence of pulsed dendritic cells (* P = .004 at 48 hr). (B) IFN γ production in the presence of pulsed macrophages (* P < .03 at 48 hr). (C) IL-17 production in the presence of pulsed DC (* P = .005 at 24 and 48 hr). (D) IL-17 production in the presence of pulsed macrophages (* P = .02 at 24 and 48 hr). U/C = Unimmunized/challenged. I = Immunized. DC = dendritic cells. Macs = macrophages.

Figure 2. Chemokine secretion induced by IL-17 and IFN γ *in vitro*. Bone marrow-derived macrophages and DC, primary cultures of fibroblasts, and the gastric epithelial line GSM06 were cultured in the presence of 20 ng/ml of either IFN γ or IL-17 for 24 hours, and the culture supernatants were assessed for (A) KC or (B) LIX by quantitative ELISA. * $P < .0005$ when compared to unstimulated cells. No MIP-2 was produced.

Figure 3.

Kinetic evaluation of the gastric mucosa following challenge of immunized and unimmunized mice with *H. pylori*. Mice were immunized with *H. pylori* lysate antigen plus cholera toxin adjuvant and then challenged with *H. pylori*. (A) Subgroups of mice were harvested and evaluated at multiple timepoints through day 13 post-challenge for the degree histologic gastritis. (B) Longitudinal biopsies encompassing the entire length of the gastric mucosa were homogenized and plated on nutrient agar for CFU determination. * $P < 0.05$ for U/C versus I/C groups. U/C = Unimmunized/challenged. I/C = Immunized/challenged.

Figure 4.

T cell response in the gastric mucosa following challenge of immunized mice with *H. pylori*. Longitudinal gastric biopsies from both nonimmune and immune mice were collected at multiple time points following challenge with 1×10^7 *H. pylori*, and RNA was isolated for quantitative PCR. A single group of naïve mice were evaluated for use as T = 0. Tissues were assessed for relative expression of (A) CD3 mRNA as a marker for total T cells, (B) IL-17 mRNA, and (C) IFN γ mRNA. * $P < .03$ for immunized/challenged mice compared to

nonimmune/challenged mice. U/C = Unimmunized/challenged. I/C = Immunized/challenged. RU = Relative Units.

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Figure 5.

Local chemokine production in the gastric mucosa following challenge of immunized mice with *H. pylori*. Longitudinal gastric biopsies from both nonimmune and immune mice were collected at multiple time points following challenge with *H. pylori*, and RNA was isolated for quantitative PCR. A single group of naïve mice were evaluated for use as T = 0. Tissues were assessed for relative expression of (A) LIX mRNA, (B) KC mRNA, and (C) MIP-2 mRNA (**P* .02 for immunized/challenged mice compared to nonimmune/challenged mice). (D) Neutrophils were quantified by histologic evaluation for days 1 through 13 (**P* .04 for immunized/challenged mice compared to nonimmune/challenged mice). (E) H & E stained section of mouse gastric mucosa of immunized mouse three days post-challenge (magnification 20X). (F) H&E stained section of mouse gastric mucosa five days post-challenge illustrating significant neutrophil recruitment (Magnification 20X). U/C = Unimmunized/challenged. I/C = Immunized/challenged. RU = Relative Units.