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# Depletion of Neutrophils in IL-10<sup>-/-</sup> Mice Delays Clearance of Gastric *Helicobacter* Infection and Decreases the Th1 Immune Response to *Helicobacter*<sup>1</sup>

Hanan F. Ismail,\* Pamela Fick,\* Juan Zhang,\* Richard G. Lynch,<sup>†</sup> and Daniel J. Berg<sup>2\*</sup>

Gastric infection with *Helicobacter* induces a lymphocyte-rich mucosal inflammation that contains a minor population of neutrophilic granulocytes. The function of neutrophils in the local immune response to gastric *Helicobacter* infection remains unknown. To investigate this issue, we conducted experiments in neutrophil-depleted control wild-type (wt) and IL-10<sup>-/-</sup> mice infected with *Helicobacter felis* by gastric lavage. Infection of wt mice elicited a mild, focal gastritis and a *Helicobacter*-specific Th1 immune response. In wt mice *Helicobacter* colonization of the stomach was persistent and progressively increased during the 29 days of observation. Infection of IL-10<sup>-/-</sup> mice with *H. felis* elicited a severe chronic gastritis and a greatly enhanced *Helicobacter*-specific Th1 immune response, as compared with wt mice. After initial colonization, the IL-10<sup>-/-</sup> mice completely cleared *Helicobacter* from the stomach by day 8. The gastric inflammation in wt and IL-10<sup>-/-</sup> mice contained modest numbers of neutrophils. The intensity of gastric inflammation and the extent of *Helicobacter* colonization were similar in control and in neutrophil-depleted wt mice. In contrast, neutrophil depletion of *Helicobacter*-infected IL-10<sup>-/-</sup> mice decreased the severity of gastritis, modulated the *Helicobacter*-specific Th1 immune response, and delayed the clearance of bacteria from the stomach. These studies identify a role for neutrophils in the local and systemic immune response to gastric *Helicobacter* in IL-10<sup>-/-</sup> mice. *The Journal of Immunology*, 2003, 170: 3782–3789.

*Helicobacter pylori* is a human gastric pathogen that can cause gastritis, peptic ulcer disease, and gastric cancer. The organism was first associated with peptic ulcer disease in 1984 (1), and since this time there has been an exponential increase in our knowledge of the role of *Helicobacter* in disease. However, despite advances in the understanding of the biology of *H. pylori*, the factors that determine the outcome of the infection in an individual host remain poorly understood.

In recent years, evidence has accumulated to suggest that in both human patients and animal models, the host cellular immune response is an important determinant of the outcome of the infection. Indeed, *H. pylori*-infected individuals express proinflammatory cytokines in their gastric mucosa, and it has been shown that *Helicobacter* induce a Th1-type CD4<sup>+</sup> T cell immune response (2, 3). In addition to the T cell infiltrates, *Helicobacter* infection also induces a prominent neutrophilic infiltration as well, and it has been proposed that *H. pylori*-induced damage is related to neutrophil-mediated tissue injury. In support of this connection is the observation that the extent of mucosal injury is related to the degree of *H. pylori* infection and neutrophil infiltration (4, 5). Furthermore, extracts of *H. pylori* contain substances that elicit chemotactic activity in neutrophils and monocytes (6–11). In addition, it has been hypothesized that reactive oxygen intermediates, a

product of neutrophil activation, may also play a role in the pathogenesis of the disease (12).

Because of the difficulty of performing invasive studies in humans, much of our understanding of the immune basis of *H. pylori*-related disease comes from studies in animal models. Like humans infected with *H. pylori*, mice infected with *H. pylori* or the closely related *Helicobacter felis* (13) respond to gastric infection with infiltration of Th1-biased lymphocytes into the gastric mucosa and spleen (14). IFN- $\gamma$  becomes elevated in tissue and splenic Th cells respond in vitro to *H. pylori* by secreting high levels of IFN- $\gamma$  (15). IL-10, a potent anti-inflammatory and immune-regulatory cytokine, is also produced during *H. pylori* infection (16) and may down-regulate the host response to gastric *Helicobacter* infection. *H. felis* infection in mice with a targeted disruption of the IL-10 gene (IL10<sup>-/-</sup>) results in severe inflammation with metaplastic and dysplastic epithelial changes that occur within 4 wk of infection (17). *Helicobacter*-induced epithelial changes may take up to 1–2 years to develop in *H. felis*-infected wild-type (wt)<sup>3</sup> mice (18) and similar changes also occur in some patients chronically infected with *H. pylori*, usually over a period of decades.

In this study, we investigated the role of endogenous IL-10 on the development of a productive immune response to gastric *H. felis* infection. We observed that both wt and IL-10<sup>-/-</sup> mice developed a Th1-type T cell response to *H. felis* infection. However, the local and systemic immune response to *Helicobacter* was much greater in the absence of IL-10 and was associated with the eradication of gastric *Helicobacter* infection in the IL-10<sup>-/-</sup> mice. Our studies also show that the development of the anti-*Helicobacter* immune response and clearance of *Helicobacter* from the stomach are diminished in the absence of neutrophils.

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<sup>3</sup> Abbreviations used in this paper: wt, wild type; ROS, reactive oxygen species.

## Materials and Methods

### Mice

Healthy 6-wk-old IL-10<sup>-/-</sup> mice on a 129/EvSv background were used for this study (19). Wild-type 129/SvEv mice were purchased from Taconic Farms (Germantown, NY). Mice were maintained in microisolator cages under specific pathogen-free conditions at the animal care facility at the University of Iowa (Iowa City, IA). All mice were maintained in accordance with guidelines of the University of Iowa Animal Care and Use Committee.

### Bacteria

*H. felis* (ATCC 49179) was obtained from American Type Culture Collection (Manassas, VA). Bacteria were grown as described previously (20). Briefly, bacteria were grown on *Brucella* agar plates with TVP (Remel, Lenexa, KS) under microaerophilic conditions at 37°C for 2 days. Confluent plates of bacteria were harvested and the number of bacteria was determined by absorption at A<sub>450</sub>, with 1 OD unit corresponding to 10<sup>9</sup> bacteria. Bacteria were positively identified on the basis of morphology and presence of urease enzyme activity and via PCR amplification of the 16S ribosomal RNA gene using *Helicobacter*-specific primers (21).

### Infection with *H. felis*

*H. felis* (1 × 10<sup>8</sup> bacteria in 100 μl of PBS) were instilled by gavage using a 23-gauge feeding needle (Popper and Sons, New Hyde Park, NY). Mice received three inoculations over a period of 5 days, with 1 day separating each inoculation. Mice were fasted overnight before inoculations.

### Neutrophil depletion

To characterize the role of neutrophils during gastric *H. felis* infection, mice were depleted of neutrophils using mAb RB6.8C5, which is a rat anti-mouse IgG2b directed against Ly-6G, an Ag on the surface of mouse neutrophils. The RB6.8C5 cell line was a kind gift from Dr. R. Coffman (DNAX, Palo Alto, CA). Wild-type mice received 0.5 mg/mouse RB6.8C5 or control rat IgG (Sigma-Aldrich, St. Louis, MO) i.p. every other day, beginning 1 day before inoculation with *H. felis*. IL-10<sup>-/-</sup> mice received 1.0 mg/mouse Ab every other day for the duration of the experiment. This treatment resulted in >98% depletion of circulating neutrophils within 24 h. Mice were evaluated for colonization of *H. felis* 8 and 15 days after initiation of infection. The effect of neutrophil depletion on the immune response to *H. felis* was assessed on day 15.

### Quantification of neutrophil percentage in peripheral blood

Peripheral blood smears were prepared before treatment with mAb RB6.8C5 and at days 8 and 15. Smears were stained with Wright-Giemsa and 100 cell differentials were performed to determine the percentage of circulating neutrophils.

### Quantification of neutrophils in the stomach

The myeloperoxidase assay was used to quantify the degree of neutrophil infiltration in the stomachs of control and RB6.8C5-treated *H. felis*-infected IL-10<sup>-/-</sup> mice. The glandular portion of the stomach was weighed and subsequently homogenized in a solution of PBS with 0.5% hexadecyl trimethyl ammonium bromide. The samples were freeze-thawed three times and centrifuged at 10,000 × g for 20 min. The supernatants were diluted 1/2 in 50 mM NaPO<sub>4</sub> buffer, and 20 μl of sample was added to 180 μl of *o*-dianisidine HCl (0.2 mg/ml in NaPO<sub>4</sub> buffer), with or without 0.0006% H<sub>2</sub>O<sub>2</sub>. Plates were read at A<sub>450</sub>. Values were multiplied by 2.655 × 10<sup>-4</sup> to calculate the IU/sample (22) and normalized to the weight of the tissue.

### Gastric colonization by *H. felis*

To assess gastric colonization of *H. felis*, wt and IL-10<sup>-/-</sup> mice were inoculated with *H. felis* as described above. The first inoculation is referred to as day 1. Stomachs were assessed for colonization with *H. felis* on days 2, 4, 8, 15, 22, and 29. Twelve mice (6 wt and 6 IL-10<sup>-/-</sup> mice) were assessed at each time point.

### Assessment of colonization

The presence of *H. felis* was determined on histologic sections stained using a modified Steiner method (Sigma-Aldrich). Since *Helicobacter* colonization was focal at the early time points (days 2 and 4), eight longitu-

dinal cross-sections of the stomach from each wt or IL-10<sup>-/-</sup> mouse were examined. The number of infected glands and number of bacteria in each gland were counted in each section to determine the number of bacteria per section. The data presented are the mean number for all mice per group, using the value from the section with the highest number of bacteria. At later time points (1 wk and greater for wt mice, 1-wk time point for neutrophil-depleted IL-10<sup>-/-</sup> mice), gastric colonization was uniform and bacterial counts did not vary significantly between sections.

To further assess colonization, stomach DNA from infected wt and IL-10<sup>-/-</sup> mice was prepared using DNAzol (Life Technologies, Grand Island, NY) exactly per the manufacturer's instructions. The presence of bacteria was assessed via PCR amplification of the 16S ribosomal RNA gene using *Helicobacter*-specific primers (21).

For the reisolation of *H. felis*, the gastric tissue was rubbed over the surface of *Brucella* agar plates with TVP (Remel) and the plates were cultured under microaerophilic conditions at 37°C. Bacteria were positively identified on the basis of morphology and presence of urease enzyme activity.

### Histologic analysis

Stomachs from wt and IL-10<sup>-/-</sup> mice were fixed flattened in 95% ethanol, routinely processed, sectioned at 6 μm, and stained with H&E for light microscopic examination. For each stomach, 8–16 longitudinal sections extending from the junction of the squamous and glandular epithelium to the duodenum were examined and an overall score assigned. Sections were examined by the same pathologist (R.G.L.) without knowledge of the identity of the samples. Because lesions were multifocal and of variable severity, the grades given to any section of stomach took into account the number of lesions as well as their severity. A score from 0 to 6 was based on the following criteria: grade 0, no change from normal tissue; grade 1, unifocal mild cellular infiltration in the lamina propria usually located in the distal stomach or at the junction of the squamous and glandular epithelium; grade 2, few multifocal lesions of moderate inflammatory cell infiltrates in the lamina propria; grade 3, lesions involved a large area of the mucosa or were more frequent than grade 2; grade 4, lesions involved most of the section and were more severe than grade 3 lesions; and grade 5, inflammation was moderate and often involved the submucosa but was rarely transmural. Inflammatory cells consisted of a mixture of mononuclear cells as well as neutrophils, moderate epithelial metaplasia was also seen. Grade 6, inflammation was diffuse and severe. Transmural inflammation was present. Inflammatory cells consisted of a mixture of mononuclear cells as well as neutrophils. Epithelial metaplasia and ulcerations were present.

### Preparation of *H. felis* sonicate

Plate-grown *H. felis* (~10<sup>9</sup> bacteria) in 1 ml of PBS were sonicated with a Bradford sonifier at 4°C for two cycles of four 30-s bursts with 30-s rests. Intact bacteria were removed by centrifugation (5000 × g). Sonicates were sterile-filtered by passage through a sterile 45-μm filter. Protein concentration was measured using a commercial reagent based on bicinchoninic acid staining (Pierce, Rockford, IL) using BSA as an internal standard. Aliquots of Ag were stored at -80°C until further use.

### Cell culture protocols

Spleen cells from control or *H. felis*-infected wt or IL-10<sup>-/-</sup> mice (at day 15 postinoculation with *H. felis*) were cultured at 5 × 10<sup>6</sup> cells/ml in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 0.05 mM 2-ME, 100 U/ml penicillin, and 100 U/ml streptomycin in 12-well tissue culture plates (Costar, Corning, NY). Cells were incubated in medium alone or medium supplemented with *H. felis* sonicate at 1.0 μg/ml. Supernatants from triplicate cultures were harvested after 48 h and stored at -80°C before analysis for cytokine concentration.

In some cultures, spleen cells were depleted of T cells before incubation with *H. felis* Ag. Briefly, cell preparations were incubated with magnetic bead-conjugated anti-Thy 1.2 (Dyna, Oslo, Norway), and Ab-reactive cells were removed in a magnetic field. Populations were >98% depleted of T cells based on flow cytometric analysis for CD4<sup>+</sup> and CD8<sup>+</sup> T cells. T cell-depleted spleen cells were incubated at a concentration of 1 × 10<sup>6</sup> cells/ml in 96-well plates with medium alone (control) or medium supplemented with *H. felis* Ag. Culture supernatants were collected after 48 h of stimulation and frozen at -80°C until analysis of cytokine concentration by ELISA.

### Determination of cytokine levels

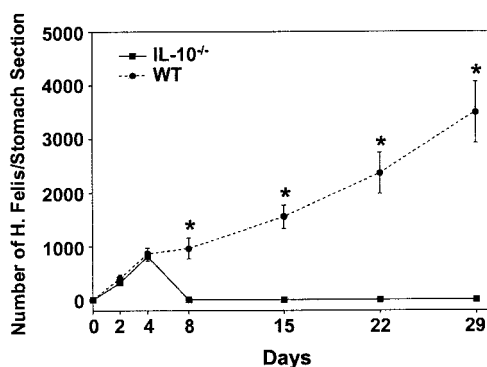
IL-4 and IFN- $\gamma$  concentrations in cell culture supernatants were measured using ELISA kits purchased from BD PharMingen (San Diego, CA) according to the manufacturer's directions.

## Results

### *H. felis* infection is rapidly eradicated in IL-10<sup>-/-</sup> mice

We previously reported that infection of IL-10<sup>-/-</sup> mice with *H. felis* resulted in rapid development of severe gastric inflammation, whereas *H. felis*-colonized wt mice developed only minimal inflammation (17). We therefore performed a longitudinal study to assess the effect of IL-10 deficiency on colonization of the stomach with *H. felis*. Wild-type and IL-10<sup>-/-</sup> mice were colonized with *H. felis* by gavage on days 1, 3, and 5 and stomachs were evaluated for *Helicobacter* colonization on days 2, 4, 8, 15, 22, and 29. At early time points (days 2 and 4), wt and IL-10<sup>-/-</sup> mice had similar levels of gastric colonization with *Helicobacter*. The level of *Helicobacter* colonization as assessed by Steiner stain steadily increased in wt mice examined at days 15, 22, and 29 (Figs. 1 and 2A). The presence of gastric *H. felis* infection in wt mice was confirmed by PCR analysis for the 16S rRNA gene of *Helicobacter* as well as by reisolation of the bacteria from cultures of the gastric tissue (data not shown). In contrast, although the level of *Helicobacter* colonization in IL-10<sup>-/-</sup> mice was similar to that of wt at days 2 and 4, IL-10<sup>-/-</sup> mice eradicated the gastric *Helicobacter* by day 8. No *Helicobacter* were detected in IL-10<sup>-/-</sup> mice at any of the subsequent time points by histochemistry (Figs. 1 and 2B) nor by PCR analysis for the 16S rRNA gene of *Helicobacter* or culture of gastric tissue (data not shown).

To clarify whether IL-10 was essential for colonization of the stomach by *Helicobacter*, wt and IL-10<sup>-/-</sup> mice were inoculated with *H. felis* on day 1 only, and the level of *Helicobacter* colonization was assessed at day 4 (3 days postinoculation). At day 4, there was no significant difference in the number of colonized bacteria in the stomachs of wt and IL-10<sup>-/-</sup> mice after one inoculation of *H. felis* (wt, 815  $\pm$  39 *H. felis*/section; IL-10<sup>-/-</sup>, 778  $\pm$  31 *H. felis*/section), indicating that presence of IL-10 was not necessary for gastric colonization by *Helicobacter*.



**FIGURE 1.** Longitudinal study of *H. felis* colonization in the stomachs of wt and IL-10<sup>-/-</sup> mice. Six-week-old IL-10<sup>-/-</sup> and wt mice were colonized with  $1 \times 10^8$  *H. felis* on days 1, 3, and 5. Wild-type (WT) and IL-10<sup>-/-</sup> mice were examined for *Helicobacter* colonization at days 2, 4, 8, 15, 22, and 29. A modified Steiner stain was performed on 8 longitudinal cross-sections of each stomach and the number of *H. felis* present on each section was determined. Data at each time point are expressed as mean  $\pm$  SD for all mice per group, using the value from the section with the highest number of bacteria. \*,  $p < 0.05$  as compared with IL-10<sup>-/-</sup> mice. Results are representative of three independent experiments.

### Induction of gastritis by *H. felis* in wt and IL-10<sup>-/-</sup> mice

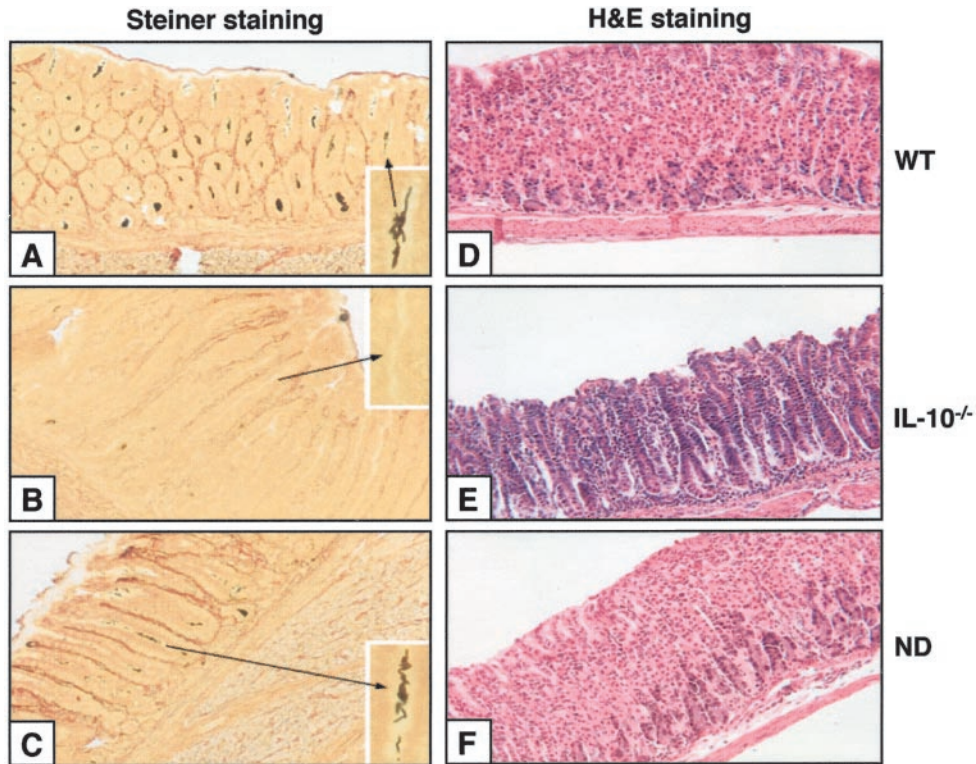
In our initial studies (17), we used a biphasic culture system to grow *Helicobacter* in vitro. Due to variable bacterial viability, we changed to a plate-grown bacterial system. Because we had modified our in vitro method of growing *H. felis*, we next assessed whether this modification would alter the development of *H. felis*-induced gastritis. As we had found previously (17), stomachs from wt mice colonized with plate-grown *H. felis* demonstrated minimal inflammation as compared with uninfected control wt mice (Figs. 2D and 3A). The infiltrates consisted of focal collections of neutrophils and mononuclear cells in the lamina propria. A small degree of inflammation was also seen at the junction of the squamous and glandular epithelium. Despite a progressive increase in the number of gastric *Helicobacter*, there was no increase in inflammation in wt mice at day 15 (Fig. 3A). In contrast, prominent inflammation was present in the stomachs from IL-10<sup>-/-</sup> mice (Fig. 2E). The character of the inflammation consisted mainly of focal collections of both neutrophils and mononuclear cells, mainly in the pylorus and at the junction of the squamous and glandular epithelium. The severity of the gastritis in IL-10<sup>-/-</sup> mice increased with time (Fig. 3A). At day 15, stomachs from the IL-10<sup>-/-</sup> mice had pathological lesions that consisted mostly of diffuse inflammation, primarily with mononuclear cells, but with a minor population of neutrophils present in the infiltrate. In addition, areas of altered gastric epithelium were prominent, with the loss of chief and parietal cells in the body of the stomach with replacement by a simple columnar epithelium (Fig. 2E). Gastritis remained severe at day 28 (pathologic score,  $4.9 \pm 0.7$ ). However, by 3 mo after initial infection, the gastritis in the IL-10<sup>-/-</sup> mice had completely resolved (data not shown). Thus, the modification of the in vitro bacterial culture protocol in the present investigations did not alter the type of inflammatory and epithelial changes induced in IL-10<sup>-/-</sup> mice. However, when plate-grown *H. felis* were used, the inflammatory changes occurred more quickly in the IL-10<sup>-/-</sup> mice as compared with *H. felis* grown in the biphasic culture system (17).

### *H. felis* infection induces a Th1 T cell response in wt and IL-10<sup>-/-</sup> mice

We next assessed the Th phenotype induced in *H. felis*-infected wt and IL-10<sup>-/-</sup> mice. Splenocytes from control and *H. felis*-infected wt and IL-10<sup>-/-</sup> mice were cultured with sonicated *H. felis* Ag, and culture supernatants were evaluated for the concentration of IFN- $\gamma$  and IL-4. Splenocytes from *H. felis*-infected wt mice produced low levels of IFN- $\gamma$  (Fig. 4A). In contrast, splenocytes from *H. felis*-infected IL-10<sup>-/-</sup> mice produced large amounts of IFN- $\gamma$  (500-fold that of wt mice, Fig. 4B). IL-4 was not detected in either wt or IL-10<sup>-/-</sup> spleen cell cultures (data not shown). To assess the cellular source of the IFN- $\gamma$  in IL-10<sup>-/-</sup> mice, we determined the effect of T cell depletion (Fig. 4). Minimal IFN- $\gamma$  was detected in T cell-depleted cultures from IL-10<sup>-/-</sup> mice, indicating that T cells were the main source of the IFN- $\gamma$ .

### Neutrophil depletion delays clearance of *H. felis* in IL-10<sup>-/-</sup> mice

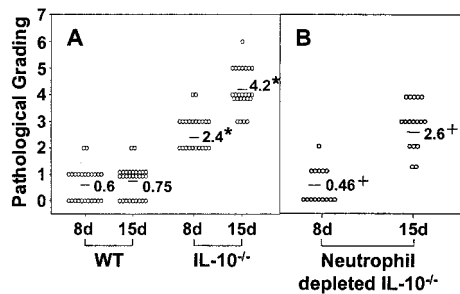
Review of the histologic sections demonstrates that neutrophils are a significant component of the *H. felis*-induced inflammatory infiltrate in both wt and IL-10<sup>-/-</sup> mice. Therefore, we depleted neutrophils from wt and IL-10<sup>-/-</sup> mice to assess their role in controlling colonization of the stomach with *Helicobacter*. Neutrophil depletion of wt mice had no effect on *H. felis* colonization at 8 or 15 days (data not shown). In contrast, neutrophil depletion delayed the clearance of *H. felis* from the stomachs of the IL-10<sup>-/-</sup> mice



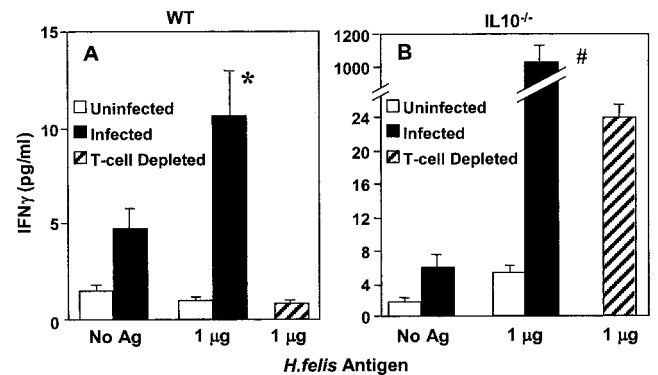
**FIGURE 2.** *H. felis* colonization and histopathology in *H. felis*-infected wt and IL-10<sup>-/-</sup> mice. Gastric sections from wt and IL-10<sup>-/-</sup> mice were stained using a modified Steiner method for the detection of *H. felis* or with H&E for light microscopic examination. The evaluation was performed 8 days after inoculation with *H. felis*. *A*, Steiner stain of *H. felis*-infected wt mouse (magnification, ×20); inset, ×100. Note dense colonization of glands with *H. felis*. *B*, Steiner stain of *H. felis*-infected IL-10<sup>-/-</sup> mouse (magnification, ×20); inset, ×100. Note absence of *H. felis*. *C*, Steiner stain of *H. felis*-infected, neutrophil-depleted IL-10<sup>-/-</sup> mouse (magnification, ×20); inset, ×100. Note dense colonization of glands with *H. felis*. *D*, H&E stain of *H. felis*-infected wt mouse (magnification, ×20); minimal infiltrate is noted. *E*, H&E stain of *H. felis*-infected IL-10<sup>-/-</sup> mouse (magnification, ×20); the lamina propria is infiltrated with both mononuclear cells and neutrophils. *F*, *H. felis*-infected, neutrophil-depleted IL-10<sup>-/-</sup> mouse (magnification, ×20); note absence of cellular infiltrate. WT, wild type; ND, neutrophil depleted.

(Fig. 5). At day 8, the number of *H. felis* in neutrophil-depleted IL-10<sup>-/-</sup> mice was nearly identical to the level of *H. felis* colonization in wt mice at day 8. Control Ab-treated IL-10<sup>-/-</sup> mice cleared the *Helicobacter* by day 8. By day 15, however, *Helicobacter* had been cleared from the neutrophil-depleted IL-10<sup>-/-</sup> mice (Fig. 5).

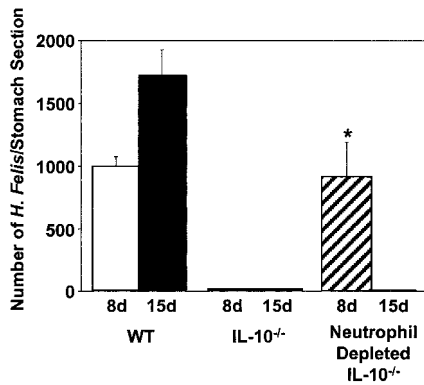
Serum complement levels were measured in IL-10<sup>-/-</sup> mice before the Ab treatment and at days 7 and 14 of neutrophil depletion to exclude complement depletion through activation by Gr1 as a



**FIGURE 3.** *A*, Histopathologic evaluation of *H. felis*-induced gastritis in wt and IL-10<sup>-/-</sup> mice. The evaluation was performed 8 and 15 days (d) after initiation of inoculation with *H. felis*. The average pathologic score is indicated for each group. \*, *p* < 0.05 as compared with wt mice. *B*, Effect of neutrophil depletion on *H. felis*-induced gastritis in IL-10<sup>-/-</sup> mice. Ab-mediated neutrophil depletion was initiated 1 day before inoculation with *H. felis*. Histopathologic evaluation was performed 8 and 15 days after initiation of inoculation with *H. felis*. Control wt and IL-10<sup>-/-</sup> mice and control mAb RB6.8C5-treated IL-10<sup>-/-</sup> mice had no evidence of gastritis (pathologic score, 0). Results are representative of three independent experiments. +, *p* < 0.05 as compared with control IL-10<sup>-/-</sup> mice.



**FIGURE 4.** Splenic lymphocytes from *H. felis*-infected wt (*A*) and IL-10<sup>-/-</sup> mice (*B*) produce IFN- $\gamma$ . *A*, Spleen cells (day 15) were cultured at  $5 \times 10^6$  cells/ml in complete medium or medium supplemented with *H. felis* Ag as described in *Materials and Methods*. After 48 h of culture, supernatants were harvested and analyzed for cytokine content (IFN- $\gamma$  and IL-4) by ELISA. No IL-4 was detected. In some cultures, T cells were depleted before culture with *H. felis* Ag. Data at each time point are expressed as mean  $\pm$  SD of observations from five mice per group; \*, *p* < 0.001 as compared with control uninfected wt mice; #, *p* < 0.0001 as compared with control uninfected IL-10<sup>-/-</sup> mice. Results are representative of three independent experiments. WT, wild type.



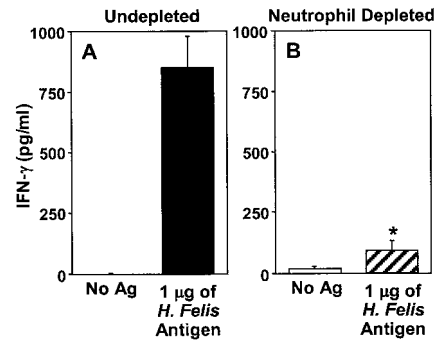
**FIGURE 5.** Effect of neutrophil depletion on the level of *H. felis* colonization in the stomachs of IL-10<sup>-/-</sup> mice. Anti-neutrophil Ab (RB6-8C5, 1 mg i.p., every other day) or isotype control Ab was administered to IL-10<sup>-/-</sup> mice beginning 1 day before inoculation with *H. felis* and continued for the duration of the experiment. Eight and 15 days (d) after inoculation, mice were assessed for the level of *H. felis* colonization. Results are expressed as the mean  $\pm$  SD of six mice within a given group; \*,  $p < 0.001$  as compared with control IL-10<sup>-/-</sup> mice. A representative experiment of five independent experiments is shown.

possible explanation for the detected effect of the Ab. There was no evidence of complement depletion in the control Ab or GR-1 Ab-treated IL-10<sup>-/-</sup> mice, indicating that the effect of the Ab was not mediated via complement depletion (data not shown).

Ab treatment effectively depleted neutrophils in IL-10<sup>-/-</sup> mice. The percentage of circulating neutrophils in the peripheral blood control Ab-treated *H. felis* infected IL-10<sup>-/-</sup> mice at day 8 was 32% ( $\pm 8\%$ ,  $n = 10$ ). In contrast, the percentage of circulating neutrophils in the peripheral blood at day 8 in the mAb-8C5-treated *H. felis*-infected IL-10<sup>-/-</sup> mice was 0.5% ( $\pm 0.5\%$ ,  $n = 10$ ). The percentage of circulating neutrophils in the peripheral blood control Ab-treated *H. felis*-infected IL-10<sup>-/-</sup> mice at day 15 was 44% ( $\pm 12\%$ ,  $n = 10$ ). In contrast, the percentage of circulating neutrophils in the peripheral blood at day 15 in the mAb-8C5-treated *H. felis*-infected IL-10<sup>-/-</sup> mice was 2.5% ( $\pm 0.5\%$ ,  $n = 10$ ). Myeloperoxidase assays on gastric tissue from *H. felis*-infected IL-10<sup>-/-</sup> mice were performed to further assess neutrophil infiltration into the stomach. The level of myeloperoxidase activity in gastric tissue from control Ab-treated *H. felis*-infected IL-10<sup>-/-</sup> mice at days 8 and 15 was 2060  $\pm$  74 IU/g tissue and 3385  $\pm$  112 IU/g tissue, respectively. In the mAb-8C5-treated *H. felis*-infected IL-10<sup>-/-</sup> mice, the myeloperoxidase activity levels of gastric tissue at days 8 and 15 were 514  $\pm$  23 IU/g tissue and 1293  $\pm$  86 IU/g tissue, respectively.

#### Neutrophil depletion decreases *H. felis*-induced gastritis in IL-10<sup>-/-</sup> mice

We next assessed the effect of neutrophil depletion on the development of *H. felis*-induced gastritis. Neutrophil depletion had no effect on the minimal gastritis that developed in wt mice (data not shown). In contrast, neutrophil depletion of IL-10<sup>-/-</sup> mice resulted in a marked decrease in gastric inflammation (Fig. 3B). The severity of inflammation at day 8 in neutrophil-depleted IL-10<sup>-/-</sup> mice was comparable to that of control *H. felis*-infected wt mice (Fig. 3B). By day 15, the severity of the inflammation in the neutrophil-depleted IL-10<sup>-/-</sup> mice had significantly increased; however, it was still less severe than that of the control-infected IL-10<sup>-/-</sup> mice at day 15.



**FIGURE 6.** Effect of neutrophil depletion on the T cell response to *H. felis* in *Helicobacter*-infected IL-10<sup>-/-</sup> mice. Spleen cells from isotype control Ab-treated *H. felis*-infected IL-10<sup>-/-</sup> mice and RB6-8C5-treated (neutrophil depleted), *H. felis*-infected IL-10<sup>-/-</sup> mice were cultured in vitro in medium alone or medium supplemented with *H. felis* sonicate (1  $\mu$ g/ml). Supernatants from triplicate cultures were harvested after 48 h. IFN- $\gamma$  concentrations in cell culture supernatants were measured by ELISA. Results represent the mean value of six mice within a given group in one of three independent experiments. \*,  $p < 0.005$  as compared with control (nondepleted) *Helicobacter*-infected IL-10<sup>-/-</sup> mice.

#### Increase in neutrophil numbers enhances clearance of *H. felis* in IL-10<sup>-/-</sup> mice

Since neutrophil depletion delayed clearance of *H. felis* in IL-10<sup>-/-</sup> mice, we treated IL-10<sup>-/-</sup> mice with G-CSF to increase neutrophil numbers and assessed the effect on *H. felis* colonization. IL-10<sup>-/-</sup> mice were treated with G-CSF for 7 days before inoculation with *Helicobacter* and for the ensuing 4 days postinoculation. G-CSF-treated mice had a 2-fold increase in absolute neutrophil counts at the time of initiation of the infection (control, 431.1  $\pm$  207.8 neutrophils/mm<sup>3</sup>; G-CSF-treated, 948.5  $\pm$  111 neutrophils/mm<sup>3</sup>,  $p < 0.001$ ). Increased neutrophil numbers enhanced the clearance of *H. felis* from IL-10<sup>-/-</sup> mice. At day 4 postinoculation, the number of *H. felis* in G-CSF-treated IL-10<sup>-/-</sup> mice was nearly 33% less than the level of control-infected IL-10<sup>-/-</sup> mice (control, 811  $\pm$  42 *H. felis*/gastric section vs. G-CSF-treated IL-10<sup>-/-</sup> mice, 537  $\pm$  23 *H. felis*/gastric section,  $p < 0.001$ ).

#### Neutrophil depletion decreases the T cell response to *H. felis* Ag in *Helicobacter*-infected IL-10<sup>-/-</sup> mice

Because neutrophil depletion resulted in persistent colonization with *Helicobacter* as well as decreased inflammation, we next assessed the effect of neutrophil depletion on the T cell response to *H. felis* in control and neutrophil-depleted IL-10<sup>-/-</sup> mice. Depletion of neutrophils led to nearly a 90% decrease in *H. felis*-induced IFN- $\gamma$  levels in spleen cell cultures (Fig. 6) as compared with control Ab-treated IL-10<sup>-/-</sup> mice. Neutrophil depletion did not appear to cause a switch to a Th2 T cell response, as no IL-4 was detected in the cultures. Flow cytometric analysis demonstrated that administration of anti-Gr1 did not alter the numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the spleens of the *H. felis*-infected, neutrophil-depleted IL-10<sup>-/-</sup> mice (data not shown).

## Discussion

This study clearly demonstrates that endogenous IL-10 limits the effectiveness of the immune response to gastric *Helicobacter* infection. In wt mice (which can produce IL-10), inoculation of the stomach with *H. felis* results in persistent colonization, with a progressive increase in bacterial load over the time points assessed. Histologically, the bacterial colonization of wt mice resulted in a mild gastritis, consisting of a mixed mononuclear and neutrophil

infiltration in the lamina propria of the pylorus and cardia. There were no alterations of gastric epithelial differentiation in wt mice colonized with *H. felis*. Although a systemic immune response to gastric *Helicobacter* was generated in wt mice, the level of IFN- $\gamma$  from *H. felis*-Ag-stimulated spleen cells was small, as compared with uninfected wt mice.

In contrast, colonization of IL-10<sup>-/-</sup> mice with gastric *Helicobacter* resulted in a robust immune response. IL-10<sup>-/-</sup> mice on a 129/SvEv background colonized with *Helicobacter* had gastric bacterial levels (at days 2 and 4) that were essentially the same as that of wt mice. However, by day 8 of infection, IL-10<sup>-/-</sup> mice had effectively cleared the organism. Histologically, concurrent with the increasing level of bacterial colonization in IL-10<sup>-/-</sup> mice, there was rapid development of a moderate to severe gastritis, consisting of a mixed mononuclear and neutrophil infiltration in the lamina propria of the pylorus and cardia. A pan-gastritis developed in some mice, with involvement of the entire body of the stomach. There were frequent alterations of gastric epithelial differentiation, with loss of normal glandular differentiation and replacement with simple columnar epithelium.

The systemic immune response to gastric *Helicobacter* in IL-10<sup>-/-</sup> mice was markedly greater than that which was generated in wt mice. The level of IFN- $\gamma$  from Ag-stimulated spleen cells of infected IL-10<sup>-/-</sup> mice was 500-fold that from spleen cells from infected wt mice. The IFN- $\gamma$  production was clearly from memory T cells, as in vitro T cell depletion essentially eliminated *Helicobacter*-Ag-stimulated IFN- $\gamma$  production. Thus, absence of IL-10 results in a strong Th1 response to the gastric luminal *Helicobacter* infection.

Our data suggest that endogenous IL-10 dampens the immune response to gastric *Helicobacter* via modulation of the Th1 CD4<sup>+</sup> T cell responses. It has been previously demonstrated that gastric *Helicobacter* infection (*felis* and *pylori*) induces a predominant Th1 CD4<sup>+</sup> T cell response in wt mice. Immunodeficient wt mice (SCID and RAG mice) are unable to clear gastric *Helicobacter*; however, transfer of CD4<sup>+</sup> T cells can effect clearance of the organism (23), further demonstrating the importance of the T cell immune response in eradication of gastric *Helicobacter*. The mechanism(s) underlying this phenomenon is not clear, as the *Helicobacter* is intraluminal (and noninvasive) whereas the T cells are located subepithelially in the lamina propria. Clearly, IL-10<sup>-/-</sup> mice were capable of generating a very strong Th1-mediated immune response and this is correlated with their ability to clear the organism. These data further support the concept that the T cell immune response is necessary for eradication of the gastric *Helicobacter* infection.

A key finding of our study was the effect of neutrophil-depletion on the anti-*Helicobacter* immune response in IL-10<sup>-/-</sup> mice. Neutrophil infiltration of the stomach in response to gastric *Helicobacter* infection has been documented in both humans and in animal models. Stomachs of individuals infected with *H. pylori* are known to have a prominent neutrophilic infiltration (24, 25), and the two reported cases of human gastric infection with *H. felis* revealed prominent neutrophil infiltration (26, 27). In the present study, neutrophils were a prominent component of the gastric infiltrate in response to *Helicobacter* infection in both wt and IL-10<sup>-/-</sup> mice. Neutrophil depletion of wt mice did not affect colonization of the stomach with *Helicobacter* or inflammation scores. In contrast, neutrophil depletion of IL-10<sup>-/-</sup> mice resulted in a delay in clearance of the gastric infection as well as decreased pathologic inflammation scores. Moreover, depletion of neutrophils resulted in a marked diminution of the Th1 immune response to *Helicobacter* infection. The *Helicobacter* ultimately were cleared in the neutrophil-depleted mice. This may have been due to

the incomplete ability to maintain the neutrophil-depleted state because there was a small increase in the percentage of circulating neutrophils and an increase in myeloperoxidase activity in the stomach at day 15. However, other immune effector mechanisms, complement for example (28), may be operative as well. Taken together, these observations suggest that neutrophils may play important roles in both the initiation and effector arms of the immune response to gastric *Helicobacter* in IL-10<sup>-/-</sup> mice.

Because neutrophils are capable of extravasating out of tissues, through epithelium and into the lumen of organs, they are prime candidates as effector cells of an immune response to an intraluminal infection. The primary importance, then, of the Th1 CD4<sup>+</sup> T cell immune response may be to produce IFN- $\gamma$  to prime the neutrophils, making them more effective killers of the bacteria.

An interesting finding of this study was the apparent dependence on neutrophils for the generation of the strong Th1 immune response to gastric *Helicobacter*. Ab-mediated neutrophil depletion has previously been reported to result in a switch from Th1 to Th2 immune response in *Candida* (29) and *Legionella pneumophila* infection (30). Neutrophils may favor induction of a Th1 response due to their ability to produce IL-12 (29). In our studies, neutrophil depletion of IL-10<sup>-/-</sup> mice did not alter the character of the immune response; rather the level of immune response was decreased. This may be due in part to increased production of IL-12 from other cell sources (e.g., dendritic cells) in the absence of IL-10. Neutrophils have also been implicated as key cellular inducers in both delayed-type hypersensitivity (31, 32) and contact hypersensitivity responses (33). Thus, neutrophils may have important roles in both the initiation of the immune response as well as serve as the final cellular effectors of the immune response.

This study also suggests that neutrophils may be mediators of the immunopathologic damage, since neutrophil-depleted IL-10<sup>-/-</sup> mice had less severe pathologic changes. Neutrophils may contribute to the immunopathologic damage seen with *Helicobacter* infection by their production of reactive oxygen species (ROS), which are key mediators of antimicrobicidal function as well as potential mediators of immunopathologic damage. IL-10 has previously been demonstrated to inhibit the production of ROS (34–37). Thus, absence of IL-10 may have resulted in neutrophils with increased capacity for ROS production. The ROS would presumably then induce epithelial damage. However, the mechanisms underlying the epithelial changes remain unknown. It is possible that ROS or other mediators are causing epithelial damage, resulting in cell death and subsequent replacement of the epithelium; however, we have not detected significant increases in epithelial apoptosis in this model (data not shown). Alternatively, the products of the immune response (cytokines, ROS) may be directly altering epithelial differentiation, leading to the observed epithelial changes.

Interestingly, epithelial pathology was not connected to the absolute number of gastric *Helicobacter*. It is well established that *Helicobacter* can produce bacterial products (e.g., VacA cytotoxin) that have cytotoxic effects on the epithelium (38, 39). In studies of T cell transfers into infected immunodeficient mice (40), the development of gastric pathology is clearly related to the immune response and the presence of the *Helicobacter*. In this study, the control IL-10<sup>-/-</sup> mice clear the *Helicobacter* infection within 1 wk. In contrast, neutrophil-depleted IL-10<sup>-/-</sup> mice continued to harbor the bacterium (at the same level as wt mice at this time point), yet the neutrophil-depleted mice had less histologic damage as well as less of a Th1-type immune response to the *Helicobacter*. This suggests that the immune response is the most important factor in the development of epithelial changes in *H. felis*-infected IL-10<sup>-/-</sup> mice.

The mAb RB6–8C5 recognizes the Ly6G Ag on the cell surface of both neutrophils and eosinophils, resulting in the depletion of these cell types *in vivo*. We hypothesize that the immunomodulatory effect of the anti-Ly6G Ab is secondary to depletion of neutrophils. Our finding that enhancement of neutrophil numbers decreased gastric colonization also supports our contention that neutrophils are mediators of the anti-*Helicobacter* immune response. However, the effect of the Ab treatment could potentially be due to depletion of other populations of cells. It has been reported that RB6–8C5 can recognize a small population of CD8<sup>+</sup> and CD4<sup>+</sup> T cells (41); however, it is not known whether Gr1<sup>+</sup> T cells have altered function compared with Gr1<sup>-</sup> T cells. Moreover, we could not detect any changes in CD4<sup>+</sup> and CD8<sup>+</sup> T cells numbers in anti-Gr1-treated mice. The Gr1 Ag has also been reported to be present on the surface of immature myeloid cells (42, 43). Since these cells have been reported to have immunosuppressive capabilities, one would have expected the Ab depletion study to enhance rather than diminish the immune response to *Helicobacter* infection. Importantly, it has recently been reported that dendritic cells with the plasmacytoid phenotype also express the Gr1 Ag (44). Depletion of a subset of dendritic cells that presents the gastrointestinal *Helicobacter* would be expected to decrease the immune response. However, we have found the percentage of splenic plasmacytoid dendritic cells (CD11c<sup>+</sup>B220<sup>+</sup>) was not altered in RB6–8C5-treated mice (data not shown). Nevertheless, we cannot rule out the possibility that Gr1 treatment may have altered the function or migration of this subset of dendritic cells.

In summary, our studies indicate that neutrophils have a pivotal role in the regulation of the immune and inflammatory response to gastric *Helicobacter* in IL-10<sup>-/-</sup> mice. We found that *Helicobacter*-infected IL-10<sup>-/-</sup> mice developed a prominent neutrophil infiltrate in the stomach and a strong Th1 immune response to *Helicobacter*. Moreover, IL-10<sup>-/-</sup> mice efficiently cleared the organism from the stomachs, whereas wt mice developed a persistent infection. Neutrophil depletion significantly delayed clearance of *Helicobacter* from IL-10<sup>-/-</sup>. Importantly, absence of neutrophils resulted in a decrease in immunopathologic damage and a markedly decreased immune response to *Helicobacter*. Taken together, these data indicate that neutrophils play a key role in the immune response to *Helicobacter*, both as an effector cell for elimination of the bacteria and as a regulator of the adaptive immune response to gastric *Helicobacter* infection.

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