Vaccine-Induced Immunity against *Helicobacter pylori* Infection Is Impaired in IL-18-Deficient Mice

Ali A. Akhiani, Karin Schön and Nils Lycke

*J Immunol* 2004; 173:3348-3356; doi: 10.4049/jimmunol.173.5.3348

http://www.jimmunol.org/content/173/5/3348

---

**References**

This article cites 69 articles, 39 of which you can access for free at:
http://www.jimmunol.org/content/173/5/3348.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

---

*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852

Copyright © 2004 by The American Association of Immunologists All rights reserved.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Vaccine-Induced Immunity against \textit{Helicobacter pylori} Infection Is Impaired in IL-18-Deficient Mice\textsuperscript{1}

Ali A. Akhiani,\textsuperscript{2} Karin Schön, and Nils Lycke

Protective immunity against \textit{Helicobacter pylori} infection in mice has been associated with a strong Th1 response, involving IL-12 as well as IFN-γ, but recent studies have also demonstrated prominent eosinophilic infiltration, possibly linked to local Th2 activity in the gastric mucosa. In this study we investigated the role of IL-18, because this cytokine has been found to be a coregulator of Th1 development as well as involved in Th2-type responses with local eotaxin production that could influence gastric eosinophilia and resistance to infection. We found that IL-18 \textsuperscript{-/-} mice failed to develop protection after oral immunization with \textit{H. pylori} lysate and cholera toxin adjuvant, indicating an important role of IL-18 in protection. Well-protected C57BL/6 wild-type (WT) mice demonstrated substantial influx of CD4\textsuperscript{+} T cells and eosinophilic cells in the gastric mucosa, whereas IL-18 \textsuperscript{-/-} mice had less gastritis, few CD4\textsuperscript{+} T cells, and significantly reduced numbers of eosinophilic cells. T cells in well-protected WT mice produced increased levels of IFN-γ and IL-18 to recall Ag. By contrast, unprotected IL-18 \textsuperscript{-/-} mice exhibited significantly reduced gastric IFN-γ and specific IgG2a Ab levels. Despite differences in gastric eosinophilic cell infiltration, protected WT and unprotected IL-18 \textsuperscript{-/-} mice had comparable levels of local eotaxin, suggesting that IL-18 influences protection via Th1 development and IFN-γ production rather than through promoting local production of eosinotaxin and eosinophilic cell infiltration. \textit{The Journal of Immunology}, 2004, 173: 3348–3356.

\textit{Helicobacter pylori} is a Gram-negative spiral bacterium that colonizes the human stomach, causing gastritis, peptic ulcer disease, and gastric cancer (1–3). \textit{H. pylori} infection results in strong local and systemic Ab production and an inflammatory infiltrate of neutrophils, eosinophils, lymphocytes, plasma cells, and macrophages in the gastric mucosa (4, 5). Despite the development of strong specific immunity, infection is usually life-long unless eradicated by antibiotic treatment. However, mice develop immune protection against \textit{H. pylori} infection after immunization with \textit{H. pylori} Ags given together with adjuvant. In most studies the protection is dependent on CD4\textsuperscript{+} T cells, but does not require specific Abs (4, 6–12). Infected patients as well as mice challenged with \textit{H. pylori} exhibit strong T cell infiltration with a typical Th1 phenotype in the gastric mucosa (13–16). Immunized and thus protected mice also demonstrate Th1 dominance, and IL-12 and perhaps IFN-γ are thought to play major roles in protection as well as being responsible for driving \textit{Helicobacter-induced} gastritis (17–19). Of note, however, conflicting data about the necessity of IFN-γ for protection have recently been reported (19). In contrast, Th2 cells do not appear to be needed for protection, but they may contribute to the severity of postimmunization gastritis, as suggested by observations made in IL-4-deficient mice (18, 20). Moreover, Th2 cells increase in number in well-protected mice over time after immunization, perhaps indicating a role in long term protection (6, 11). Thus, both Th1 and Th2 cells could be involved in the development of immune protection against \textit{H. pylori} (11, 21). These observations, in addition to the dominance of eosinophilic cells in well-protected gastric mucosa, suggest a common regulatory link between Th1- and Th2-dependent functions, promoting resistance to \textit{H. pylori} infection (6, 12). One factor that could fulfill such a role is IL-18, which is known to affect Th1 development/IFN-γ production as well as influence Th2-controlled functions (22, 23).

Originally named IFN-γ-inducing factor, IL-18 is both structurally and functionally related to the IL-1 family (24–26). It has been ascribed properties similar to those of IL-12, including its ability to stimulate IFN-γ production in T, NK, and B cells (27). Thus, IL-18 is a strong inducer of Th1 cell development through up-regulation of the IL-12R β\textsubscript{2}-chain and transactivation of the IFN-γ promoter (22, 28, 29). However, when IL-18 was combined with IL-2, there was a synergistic induction of the Th2 cytokine IL-13, suggesting that IL-18 can promote Th2 responses as well, depending on the cytokine milieu (23). Moreover, local IL-18 production stimulated lung tissue eosinophilia through the production of eotaxin from local cell populations, and accordingly, eotaxin \textsuperscript{-/-} mice were found to exhibit reduced airway eosinophilia (30). Thus, the presence of IL-18 systemically appears to enhance Th1-type responses and IFN-γ production, whereas local IL-18 production would be involved in Th2-type immunity by enhancing IL-13, IL-5, and eotaxin production. Previous studies have indicated that eotaxin exerts local control in the lung or the gastrointestinal tract of eosinophilic cell infiltration, whereas IL-5 has more generalized effects on eosinophilic cells (31–35). Therefore, IL-18 deficiency may directly affect local mucosal eosinophilia and influence the resistance to \textit{H. pylori} infection. What role, if any, eosinophilic cells have in protection against \textit{H. pylori} infection, however, is unknown.

We and others have found that eosinophilic infiltration is particularly pronounced in the postimmunization gastritis that develops in \textit{H. pylori}-immunized and protected mice (6, 12, 36). Previous studies have shown that IL-18 is increased in different inflammatory conditions, such as Crohn’s disease (37, 38), rheumatoid arthritis (39), and leprosy (40). In \textit{Helicobacter}-infected...
individuals, IL-18 mRNA expression was increased in the gastric mucosa (41, 42). The serum IL-18 level was significantly increased in patients with gastric carcinoma, which is frequently found to be associated with *H. pylori* infection (2, 43). Moreover, a limited study in mice addressing the role of IL-18 found no difference in resistance to infection between *H. pylori*-infected and wild-type (WT) mice (44). It was then assumed, but not assessed, that IFN-γ production was significantly reduced in IL-18−/− mice, and therefore, the result, showing normal protection, would favor the idea that IFN-γ is not essential for *H. pylori*-specific protection, in agreement with the report by Garhart et al. (19). However, more extensive investigations are needed to evaluate the functional roles of IL-18, eosinophilic cells, and IFN-γ in the development of immune protection and gastric inflammation in response to *H. pylori* infection. In the present study *H. pylori*-specific protective immunity and postimmunization gastritis were investigated in detail by comparing the immunopathology and resistance to infection in IL-18−/− mice with that in WT mice. In addition, we hoped to extend our knowledge about the need for IFN-γ in host resistance against *H. pylori* infection.

**Materials and Methods**

**Mice**

IL-18-deficient (IL-18−/−) (45) mice on a C57BL/6 background were bred in ventilated cages under pathogen-free conditions at the Laboratory for Experimental Biomedicine, University of Goteborg (Goteborg, Sweden). C57BL/6 WT mice were obtained from B&K Universal (Sollentuna, Sweden). All mice were sero-negative for *Helicobacter* spp. Ags before infection or immunization. Age- and sex-matched animals were used throughout the study.

**Immunization**

Groups of 8–10 mice were immunized orally with a blunting feeding needle (Popper & Sons, New Hyde Park, NY) four times at weekly intervals with 500 μg of *H. pylori* whole-cell lysate Ags and 10 μg of cholera toxin (CT) adjuvant (List Biological Laboratories, Campbell, CA) in 3% (w/v) NaHCO₃ in PBS in a total volume of 0.5 ml. Control mice received 10 μg of CT.

**H. pylori growth conditions and challenge**

*H. pylori* Sydney strain (SS1) cells were cultured on selective agar plates containing 5% sheep blood and antibiotics under microaerophilic conditions as described previously (18). The bacteria were then harvested and inoculated into Brucella broth (BD Biosciences, Mountain View, CA) supplemented with 5% heat-inactivated FCS (Biochrom, Berlin, Germany) as described previously (18). To assess protection after immunization, mice were challenged with 5 × 10⁶ CFU of *H. pylori* 2 wk after the last immunization and then killed 2 wk postchallenge. The gastric tissue was processed for urease activity, quantitative *H. pylori* culture, histopathology, and immunohistochemistry as described below.

**Preparation of *H. pylori* whole-cell lysate Ags**

*H. pylori* was grown on selective blood agar plates as described previously (18) and suspended in PBS. The cells were then washed three times in PBS by centrifugation at 6000 rpm for 10 min at 4°C before being disrupted by freeze-pressing with X-press (46). Briefly, the bacterial suspension was frozen at −35°C in the form of cylindrical rods that fit into the pressure chamber and were extruded by a piston forced back and forth by means of a hydraulic pump. After centrifugation (6000 rpm) to remove cell fragments, the preparation was filtered through a 0.2-μm pore size membrane filter (Schleicher & Schuell, Dassel, Germany). The protein content was determined by protein assay (Bio-Rad, Hercules, CA), and aliquots were frozen at −85°C until used.

**Gastric tissue analyses**

The stomach was dissected along the greater curve and divided into four longitudinal strips for assessment of urease activity, *H. pylori* culture, histopathology, and immunohistochemical analyses. For histopathology, longitudinal segments including the antrum and corpus plus a piece of attached intestine were fixed in 4% neutral buffered formaldehyde, embedded in paraffin, and sectioned at 3 μm by Histolab Products (Goteborg, Sweden). For immunohistochemistry, gastric segments were placed into Histocon (Histolab Products) at 4°C. The tissues were then placed in plastic chambers (Cryomold; Milex), filled with O.C.T. compound (Miles), and subsequently snap-frozen in isopentane in liquid nitrogen (N₂). For ~60 s. Frozen cross-sections (7 μm) were prepared on microslides using a cryostat-1720 (Leitz, Wetzlar, Germany) and frozen at ~85°C.

**Assessment of bacterial colonization**

The presence of *H. pylori* in gastric tissue was assessed by urease activity measured spectrophotometrically at 550 nm using a colorimetric assay (47). For quantitative assessment of *H. pylori* bacteria, longitudinal microsections of gastric tissue were homogenized in 0.5 ml of Brucella broth supplemented with 5% FCS, and replicate serial 10-fold dilutions were plated on *Helicobacter*-selective blood agar plates (47). The plates were incubated at 37°C under microaerobic conditions (10% CO₂, 5% O₂, and 85% N₂). Quantitation of CFU was performed 7 days later. In the microabscesses or cystic glands; or 4, epithelial changes throughout the lamina propria and into the submucosa, frequent microabscesses and prominent lymphoid aggregates.

**Epithelial changes**

For evaluation of epithelial changes, H&E-stained sections were scored based on the degree of infiltrating lymphocytes, plasma cells, and neutrophils (21). The scoring grades were defined as follows: 0, none; 1, a few leukocytes scattered in the deep mucosa; 2, moderate numbers of leukocytes in the deep to mid mucosa and occasional neutrophils in gastric glands (microabscesses); 3, dense infiltrates in the deep to mid mucosa, a few microabscesses, and one or two lymphoid aggregates; and 4, dense, diffuse infiltrates throughout the lamina propria and into the submucosa, frequent microabscesses, and prominent lymphoid aggregates.

**Immunohistochemistry of gastric tissue**

Frozen sections from gastric tissue were fixed in 50% acetone for 30 s, followed by 100% acetone for 5 min at 4°C. After washing in PBS, sections were blocked with avidin-biotin blocking reagents (Vector Laboratories, Burlingame, CA), and endogenous peroxidase activity was blocked with 3% H₂O₂ in PBS. Sections were probed overnight with 50 μg of mouse serum in 0.1% BSA (Sigma-Aldrich, St. Louis, MO)/PBS for 15 min in a humid chamber. Sections were labeled by incubation with biotinylated rat mAbs against mouse CD4 or CD8 (BD Pharmingen, San Diego, CA). For detection of CD3 cells, rat mAb against mouse CD3 (BD Pharmingen) followed by incubation with biotinylated rabbit anti-rat IgG (H+L) (Vector Laboratories) and HRP conjugated to an avidin-biotin complex were used (ABC-Elite Kit; DakoCytomation, Glostrup, Denmark). Cell-bound peroxidase was detected with 3-aminono-9-ethylcarbazole (Vector Laboratories) and H₂O₂. Sections were counterstained with hematoxylin. Control sections were incubated without specific mAb. Stained sections were scored from 0 to 4 based on the extent of infiltrating T cells (18). Scores were defined as follows: 0, none; 1, a few T cells scattered in the mucosa; 2, moderate numbers of T cells in the gastric mucosa; 3, dense infiltrates in the deep to mid mucosa; and 4, dense, diffuse infiltrates throughout the lamina propria and into the submucosa. A quantitative description of each score level was defined as follows: 1, 100 ± 20; 2, 200 ± 40; 3, 400 ± 80; and 4, 800 ± 160 cells/mm².

**Serum and gastric samples**

Blood was obtained from the axillary plexus of the mouse at death. Gastric secretions were collected with absorbent wicks positioned longitudinally in the gastric lumen (49) after extensive rinsing with PBS containing 0.2 mM 4-(2-aminoethyl)benzenesulfonil fluoride (Calbiochem, La Jolla, CA), 1 μg of aprotinin/ml, 10 mM leupeptin (Sigma-Aldrich), and 3.25 μM Bestatin (Roche, Indianapolis, IN) protease inhibitors. For extraction of gastric secretions from the wick, 0.5 ml of protease inhibitor containing 5% nonfat

---

*Abbreviations used in this paper: WT, wild type, AP, alkaline phosphatase; CT, cholera toxin.*
dry milk was added to each sample tube containing two wicks, vortexed extensively, and then frozen at −85°C until analyzed.

**Serum and gastric anti-H. pylori Ab determinations by ELISA**

Flat-bottom, 96-well microtiter plates (Nunc, Roskilde, Denmark) were incubated with 10 μg/ml H. pylori lysate in PBS. After washing and blocking with PBS containing 0.1% BSA, the wells were incubated with serial dilutions of sera or gastric secretions. The wells were then incubated with the appropriate dilutions of alkaline phosphatase (AP)-conjugated goat anti-mouse IgG1, anti-mouse IgG2a, or anti-mouse IgA (Southern Biotechnology Associates, Birmingham, AL), followed by the phosphate substrate p-nitrophenyl phosphate (Sigma-Aldrich) in ethanolamine buffer (pH 9.8), and the enzymatic reactions were read at 405 nm. Ab titers were defined on the linear portion of the curve as the interpolated dilution of a sample giving rise to an absorbance of 0.4 U above background.

**In vitro stimulation of spleen cells**

Spleen cells were obtained by mechanical dissociation and filtering through a nylon mesh. RBC were subjected to lysis by osmotic shock using hypotonic Tris-ammonium chloride. After washing in HBSS (Life Technologies, Paisley, U.K.), the cells were resuspended in Iscove’s medium (Biochrom, Berlin, Germany) supplemented with 10% heat-inactivated FCS (Biochrom), 50 μM 2-ME (Sigma-Aldrich), 1 mM l-glutamine (Biochrom), and 50 μg/ml gentamicin. Spleen cells (105 cells/well) were then cultured (8% CO2, 37°C) in round-bottom, 96-well microtiter plates (Nunc) for 3 days in the presence or the absence of varying concentrations of H. pylori lysate Ags. Cell-free supernatants were collected 96 h after incubation and stored at −85°C until assayed for cytokine concentration.

**Gastric tissue homogenate**

Gastric tissue from the whole stomach was homogenized on ice for 80 s in 1 ml of PBS containing 0.05% Triton X-100. The resulting supernatant was isolated after centrifugation (10,000 × g) and stored at −85°C until assayed for eotaxin and cytokine concentrations by ELISA.

**Cytokine assays**

The concentrations of IFN-γ, IL-4, IL-5, IL-10, IL-12, IL-13, IL-18, and eotaxin in the supernatants were assessed by ELISA. Briefly, 96-well, round-bottom microtiter plates (Dynatech Laboratories, Chantilly, VA) were incubated with 2.5 μg/ml rat anti-mouse IFN-γ (BD Pharmingen), 1.5–μg/ml anti-mouse IL-4 (Endogen, Woburn, MA), IL-5, IL-10 (BD Pharmingen), IL-13, IL-18 (R&D Systems, Abingdon, U.K.), or 0.2 μg/ml anti-mouse eotaxin (R&D Systems). The sample supernatants or recombinant mouse IFN-γ, IL-4, IL-5, IL-10, IL-13, IL-18, or eotaxin (R&D Systems) standards were then added to the appropriate wells. Bound cytokines were detected by sequential incubations with a polyclonal rabbit anti-IFN-γ antiserum or biotinylated mAb to mouse IL-4, IL-5, IL-10, IL-12, IL-13, IL-18, or eotaxin (R&D Systems), followed by AP-conjugated goat anti-rabbit Ig (Southern Biotechnology Associates) for IFN-γ assay; HRP-conjugated anti-biotin (Vector Laboratories) for IL-4, IL-5, and IL-10 assays; or HRP-conjugated streptavidin (R&D Systems) for IL-13, IL-18, and eotaxin assays. Finally, the AP substrate p-nitrophenyl phosphate (Sigma-Aldrich) in ethanolamine buffer (pH 9.8) or the HRP substrate H2O2 with ABTS (Sigma-Aldrich) in 0.1 M citrate buffer (pH 4.5) was added to each well, and the extent of the reaction was read at 405 nm using a Titer-Tek multiscan spectrophotometer. For IL-4, IL-5, and IL-10 cytokine development, the HRP substrate H2O2 with ABTS (Sigma-Aldrich) in ethanolamine buffer (pH 9.8) or the HRP substrate H2O2 with ABTS (Sigma-Aldrich) in 0.1 M citrate buffer (pH 4.5) was added to each well, and the extent of the reaction was read at 405 nm using a Titer-Tek multiscan spectrophotometer.

**Statistical analysis**

The Wilcoxon rank-sum test was used for independent samples for analysis of significance in all experimental groups, except for the cytokine values and Ab titers, which were compared with one-tailed Student’s t test and one-way ANOVA.
the gastric mucosa, whereas unprotected IL-18−/− had significantly lower CD4+ T cell counts (p < 0.05; Fig. 2). Thus, protection against H. pylori infection required IL-18 and was strongly associated with local infiltration of CD4+ T cells in the gastric mucosa.

**IL-18 influences postimmunization gastritis**

Next, we analyzed whether the ability to produce IL-18 can influence postimmunization gastritis, as seen after a challenge infection with H. pylori in orally immunized mice. Histological evaluation of gastritis was performed on H&E-stained sections of gastric mucosa from the immunized and challenged IL-18+/+ or WT mice. As shown in Figs. 3 and 4, protective immunity observed in the WT mice was associated with the development of pronounced eosinophilia in postimmunization gastritis (p < 0.0001). The gastritis in immunized, but unprotected, IL-18−/− mice was greatly reduced, and few eosinophilic cells were found (p < 0.05), indicating that IL-18 is associated with the development of postimmunization gastritis and gastric eosinophilia. Of note, the inflammatory changes in the protected WT mice were mostly confined to the fundic mucosa close to the border of the squamous epithelium (Fig. 4). Epithelial changes observed in the corpus region were parietal cell loss and hyperplasia of the epithelium, with a mean score of dysplasia of 1.7 ± 1.1, which was significantly different (p < 0.05) from that in the immunized, but unprotected, IL-18−/− mice (0.7 ± 0.96; Fig. 5).

**Immunogenicity of H. pylori Ags is unaffected in IL-18−/− mice**

Because IL-18−/− mice failed to develop protection and exhibited a milder postimmunization gastritis, we evaluated whether IL-18−/− mice were less responsive to oral immunization. However, we found that IL-18−/− mice developed high levels of anti-H. pylori IgG1 in their sera and gastric secretions, results not deviating from findings in immunized and well-protected WT mice (Fig. 6). As expected, IL-18−/− mice displayed reduced anti-H. pylori IgG2a titers in both sera (p < 0.05) and gastric secretions (p < 0.05; Fig. 6). Anti-H. pylori IgG2a titers were at least 16 times lower in sera and almost 4 times lower in gastric secretions compared with those in WT mice. Importantly, anti-H. pylori IgA titers in sera and gastric secretions were comparable in IL-18−/− and WT mice, indicating that the lack of IL-18 did not affect the overall immunogenicity of H. pylori Ags after oral immunization (Fig. 6).

IL-18−/− mice had lower specific IgG2a levels, suggesting that they also exhibited impaired IFN-γ responses to H. pylori Ags (50). We found that splenic T cells that were isolated 2 wk after challenge and cultured in the presence of recall H. pylori lysate Ags were poor producers of IFN-γ compared with T cells from protected WT mice (p < 0.05; Table I). Interestingly, we noted strong production of IL-18 to recall Ag in splenic T cell cultures of protected mice, whereas IL-18 was not detected in CT-treated control WT mice (Table I). No IL-18 was seen in cultures from IL-18−/− mice. Moreover, IL-13 production was comparable in the immunized challenged IL-18−/− and WT mice, corroborating the idea that IL-18 deficiency did not impair the overall immunogenicity of H. pylori Ags (Table I). The production of other Th2-type cytokines, IL-4, IL-5, and IL-10, was below the detection level in both strains (not shown). Thus, immune protection against

**FIGURE 2.** CD4+ T cell infiltration in immunized challenged IL-18−/− and WT mice. Groups of 8–10 mice were immunized with H. pylori lysate plus CT (●) or with CT alone (□) and challenged with H pylori bacteria. Two weeks after challenge, CD4+ T cells were detected in gastric tissue by immunohistochemistry. Sections were scored from 0 to 4 as described in Materials and Methods. The bars represent the mean ± SEM of pooled data from two experiments. *p < 0.05; †p = 0.001 (by Wilcoxon rank sum test).

**FIGURE 3.** Gastric inflammation in immunized challenged IL-18−/− (●) and WT (○) mice. Groups of 8–10 mice were immunized with H. pylori lysate plus CT or with CT alone and challenged with H. pylori bacteria. The extent of histological gastritis was assessed 2 wk after challenge as described in Materials and Methods. Data points pooled from two experiments are from individual mice. The number of mice per group is in parentheses. The bar represents the mean of each group. *p < 0.0001; †, p < 0.05 (by Wilcoxon rank-sum test).
H. pylori infection in WT mice was associated with increased T cell production of IFN-γ and IL-18 in response to recall Ag.

**Immunized unprotected IL-18−/− mice show poor gastric IFN-γ production, but unaltered eotaxin levels**

Detection of systemic immunity in protected WT or unprotected IL-18−/− mice was followed up by analyses of whole-stomach homogenates from immunized challenged mice for local gastric production of cytokines. As shown in Table II, we found strong production of IL-12p70, IL-18, and, in particular, IFN-γ in stomach homogenates of protected WT mice, whereas only low levels of IFN-γ and IL-12p70 were detected in IL-18−/− mice. Of note, the levels of IFN-γ and IL-12p70 in gastric tissue from unprotected IL-18−/− mice were, in fact, higher than those in immunized IL-18−/− mice (Table II). Gastric IL-13 production was of similar magnitude in both strains and did not change after immunization (Table II). Thus, well-protected WT mice exhibited strong IL-18 and IFN-γ production, supporting our previous observation of a prominent role of local IFN-γ for protection against H. pylori infection (18).

Because we found reduced levels of eosinophilic cells in unprotected IL-18−/− mice (Fig. 4), we also analyzed stomach homogenates for eotaxin (30). As determined by ELISA, gastric eotaxin levels were not significantly different in immunized WT and IL-18−/− mice, nor were they different in CT-treated control mice from either strain (Table II). Therefore, the local gastric eotaxin production did not appear to influence protection or the level of eosinophilic cells infiltrating the gastric mucosa.

**Discussion**

The present study is the first to analyze in detail the role of IL-18 in host resistance against H. pylori infection. We found that IL-18 is essential for the development of protective immunity against H. pylori infection and IL-18−/− mice developed poor systemic and local Th1 immunity after oral immunization with H. pylori Ags and CT-adjuvant. Protection in WT mice was associated with greatly enhanced T cell production of IFN-γ and IL-18 to recall Ags, whereas unprotected IL-18−/− mice failed to respond with significant IFN-γ production. The gastric mucosa of protected WT mice showed a massive infiltration of inflammatory cells (mostly eosinophils) in the upper submucosa and in the mucosa of protected WT mice (A and C) was observed, whereas only a mild inflammation was seen in unprotected IL-18−/− mice (B and D).
mice expressed elevated levels of IL-12p70, IL-18, and, in particular, IFN-γ compared with controls, whereas unprotected immunized IL-18−/− mice had low levels of these cytokines. Postimmunization gastritis and local infiltration of CD4+ T cells in the gastric mucosa were prominent in protected WT mice. By contrast, IL-18−/− mice had few CD4+ T cells and milder gastritis with few eosinophilic cells. Nevertheless, we observed comparable levels of gastric eotaxin in IL-18−/− and WT mice, suggesting that the eosinophilic cell infiltration in the gastric mucosa of protected WT mice was independent of eotaxin and IL-18. Therefore, our study supports that IL-18 is essential as a coregulator of H. pylori-specific Th1 immunity and critical for the development of strong IFN-γ production, whereas its effect on Th2 type of immunity, such as eotaxin and IL-13 production, appears not to be important for protection. Thus, vaccine-induced immunity against H. pylori infection is critically dependent on IL-18.

IL-18 has been shown to be important for development of immunity and host defense against many other microbial pathogens. For example, IL-18 was reported to play a major role in resistance against Yersinia, mycobacterial, cryptoccocal, Salmonella, Leishmania, herpes simplex virus type 1, feline leukemia virus, and vaccinia virus infections (51–58). Similar to H. pylori, these infectious agents elicit Th1-dominated immune responses (51, 52, 55, 57, 59). Given that IL-18 primarily is involved in protection as a coregulator of Th1 development together with IL-12 (61), it is not surprising that we found a strong dependence on IL-18 for the development of host resistance also against H. pylori infection. However, Panthel et al. (44) recently reported that IL-18−/− mice were as protected as WT mice after oral immunization with H. pylori sonicate and CT adjuvant. These authors used few animals (3–4) in each group and only two immunizations and evaluated the level of protection (day 63) 4 wk after challenge, whereas we performed several experiments, used larger groups and four oral immunizations, and assessed protection 2 wk after challenge. Although we used mice of the same genetic background, C57BL/6, derived from the same founders as Panthel et al., we arrived at opposite results (44). At present we cannot give a decisive explanation for this difference. We believe our study presents statistically significant and reproducible results, whereas the limited data provided by Panthel et al. (44) must be considered less reliable. Furthermore, the latter authors did not determine specific T cell or Ab responses; therefore, no conclusion about the role of IFN-γ could be drawn (44).

The exact mechanism by which IL-18 is important for protection against H. pylori infection is not known. It is likely, though, that IL-18 is critical for H. pylori-specific IFN-γ production, because IL-18−/− mice were found to have decreased levels of this cytokine. The low IFN-γ level in IL-18−/− mice is corroborated by several other studies, whereas overexpression of IL-18 in transgenic mice exhibits strongly up-regulated IFN-γ levels, clearly indicating a strong link between IL-18 and the ability to produce IFN-γ (60). As a coregulator, together with IL-12, of Th1 immunity, IL-18 can promote IFN-γ production, but it could also directly promote IFN-γ production by T and NK cells independently of IL-12 (61). In the present study IL-18−/− mice exhibited a reduction in splenic as well as gastric IFN-γ production. We and others have documented that well-protected WT mice have increased numbers of CD4+ T cells infiltrating the gastric mucosa (6, 8, 18, 47, 48). Therefore, one would hypothesize that local production of IFN-γ is a key element in T cell-mediated protection against H. pylori infection. In fact, gastric T cells in well-protected mice are known to contain IFN-γ (13). Alternatively, IL-18 itself could, of course, have a direct effect, independent of IFN-γ, because we found that IL-18 was increased in the gastric mucosa of immunized WT mice. Whether the gastric IL-18 was produced by T cells or, more likely, by macrophages or epithelial cells, perhaps under the influence of Ag-specific T cells, was not investigated. What kind of direct effect IL-18 has must be purely speculative at present, but it has been reported that IL-18 could affect apoptosis in hepatocytes (60), and in this respect it could help in eliminating epithelial or other cells and drive inflammation in the colonized mucosa. IL-18 could also directly augment IL-1, IL-6, and TNF cytokine production from, e.g., macrophages that would promote postimmunization gastritis and reduce bacterial counts (24, 62).

Several studies, including our own, have demonstrated that IL-12 is required for protection, whereas the need for IFN-γ is
currently being debated (18, 19). IFN-γ could affect the local expression of cytokines and chemokines/chemokine receptors, or it could up-regulate MHC class II, costimulatory, or other membrane molecules that could facilitate *H. pylori*-specific T cell priming or help focus specific T cells to the infected gastric epithelium (63, 64). The exact mechanism by which IFN-γ provides protection has yet to be established. We recently reported that IFN-γ−/− mice failed to develop protection after oral immunization (18). By contrast, Garhart et al. (19) failed to find protection in immunized IFN-γ−/− mice. Because there is wide agreement that Th1 cells are crucial for resistance against *Helicobacter* infection, the question of what these cells provide other than IFN-γ is critical (11, 65, 66). Whether there actually exists an IFN-γ-independent, IL-12 p40-dependent Th1 effector function mediated by TNF-α, GM-CSF, or IL-8, as suggested by Garhart et al. (19), is unknown. The present study does not preclude the possibility that other factors, such as IL-12, could be important for protection, but it clearly extends the information about IFN-γ, which is a key component associated with *H. pylori*-specific protection. Whether IL-12 is crucial for host resistance against *H. pylori* infection as a coregulator of Th1 differentiation together with IL-12 or has an augmenting effect on IFN-γ production independently of IL-12 has yet to be investigated. Of note, IL-12p70 levels were up-regulated in well-protected gastric mucosa of WT mice, but not in unprotected IL-18−/− mice, corroborating previous findings of an important role for IL-12 in host protection against *H. pylori* (18).

In our study local IL-18 production did not correlate to the presence of eosinophils in the gastric mucosa, because eosinophil levels were similar in WT and IL-18−/− mice (30). Yet, we observed that immunized, but unprotected, IL-18−/− mice exhibited reduced gastric inflammation and eosinophilia compared with well-protected WT mice. Notwithstanding the fact that IFN-γ is particularly important for the development of gastric inflammation, eosinophilic cells may be involved in driving the postimmunization gastritis observed in *H. pylori*-infected and protected WT animals (6). Whether postimmunization gastritis is a prerequisite for protection is currently being debated (6, 12, 18, 19, 67). In any case, eosinophilic cells may contribute to protection (67, 68). However, it should be remembered that another cytokine, IL-5, is better known to play a key regulatory role for eosinophilic cells (31–33, 69). Thus, in *H. pylori* infection, IL-5 might be even more critical than IL-18 for the tissue eosinophilia. Indeed, immunized and challenged IL-5-deficient mice have been found to have 5- to 6-fold fewer eosinophilic cells in the gastric mucosa, but demonstrated protection comparable to that of WT mice (12). Therefore, based on the findings in IL-5−/− and IL-18−/− mice, we cannot say whether eosinophilic cells per se are important for host resistance to *H. pylori* infection.

<table>
<thead>
<tr>
<th>Mice</th>
<th>Immunization</th>
<th>IFN-γ (pg/ml)</th>
<th>IL-12 (pg/ml)</th>
<th>IL-13 (pg/ml)</th>
<th>Eotaxin (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-18−/−</td>
<td>Lysate + CT</td>
<td>12.5 ± 5</td>
<td>42.5 ± 20a</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>10 ± 6</td>
<td>30 ± 30</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>PBS (naive)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>C57BL/6 WT</td>
<td>Lysate + CT</td>
<td>21 ± 11</td>
<td>171 ± 80a</td>
<td>35 ± 12</td>
<td>396.8 ± 23.8</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>12 ± 8</td>
<td>35 ± 21</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>PBS (naive)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IS IL-18 DEPENDENT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: AD, IL-12, could be important for protection, but it clearly extends the study does not preclude the possibility that other factors, such as IL-12, could be important for protection, but it clearly extends the information about IFN-γ, which is a key component associated with *H. pylori*-specific protection. Whether IL-12 is crucial for host resistance against *H. pylori* infection as a coregulator of Th1 differentiation together with IL-12 or has an augmenting effect on IFN-γ production independently of IL-12 has yet to be investigated. Of note, IL-12p70 levels were up-regulated in well-protected gastric mucosa of WT mice, but not in unprotected IL-18−/− mice, corroborating previous findings of an important role for IL-12 in host protection against *H. pylori* (18).

Table II. Cytokine production in gastric mucosa of immunized challenged IL-18−/− and WT mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>Immunization</th>
<th>IFN-γ (pg/ml)</th>
<th>IL-12p70 (pg/ml)</th>
<th>IL-18 (ng/ml)</th>
<th>IL-13 (pg/ml)</th>
<th>Eotaxin (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-18−/−</td>
<td>Lysate + CT</td>
<td>1720 ± 330a</td>
<td>275.3 ± 53.9a</td>
<td>ND</td>
<td>8961 ± 2384</td>
<td>3242.8 ± 434.4a</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>1150 ± 700</td>
<td>220.3 ± 21.8a</td>
<td>ND</td>
<td>9278 ± 1050</td>
<td>2809.5 ± 328.8</td>
</tr>
<tr>
<td></td>
<td>PBS (naive)</td>
<td>22 ± 11</td>
<td>252.5 ± 166.6</td>
<td>ND</td>
<td>2882 ± 1384</td>
<td>687.2 ± 335</td>
</tr>
<tr>
<td>C57BL/6 WT</td>
<td>Lysate + CT</td>
<td>3170 ± 1300a</td>
<td>580.5 ± 132.5a</td>
<td>405.3 ± 3.2b</td>
<td>8148 ± 1239</td>
<td>3984.4 ± 1161.2</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>2410 ± 860</td>
<td>396.5 ± 153.8</td>
<td>289.5 ± 51.6</td>
<td>8204 ± 1725</td>
<td>2722.4 ± 1138.3</td>
</tr>
<tr>
<td></td>
<td>PBS (naive)</td>
<td>54 ± 10</td>
<td>343.3 ± 250.6</td>
<td>138.5 ± 64.5</td>
<td>3939 ± 1509</td>
<td>1408.4 ± 409.2</td>
</tr>
</tbody>
</table>

Note: ND, not detected. Groups of mice were immunized orally with *H. pylori* lysate plus CT or CT alone and then challenged with *H. pylori* bacteria. At 2 wk after challenge, stomachs were homogenized, and the resulting supernatants were analyzed by ELISA in triplicate for the concentration of cytokines. Values are expressed as the mean ± SD for five mice per group. One representative experiment of two is shown. Data were also subjected to a one-way ANOVA, and a similar significant difference was found for IFN-γ (*p = 0.05*) and IL-12 (p = 0.001) in protected WT vs unprotected IL-18−/− mice.


