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

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

Ali A. Akhiani, Karin Schön, and Nils Lycke

Protective immunity against *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 eosinophil production that could influence gastric eosinophilia and resistance to infection. We found that IL-18-/- mice failed to develop protection after oral immunization with *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+ T cells and eosinophilic cells in the gastric mucosa, whereas IL-18-/- mice had less gastritis, few CD4+ 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-/- mice exhibited significantly reduced gastric IFN-γ and specific IgG2a Ab levels. Despite differences in gastric eosinophilic cell infiltration, protection was evident in IL-18-/- mice, which is known to affect Th1 development and IFN-γ production as well as Th2 cell development.

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 β2-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-/- 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 *H. pylori* infection. What role, if any, eosinophilic cells have in protection against *H. pylori* infection, however, is unknown.

We and others have found that eosinophilic infiltration is particularly pronounced in the postimmunization gastritis that develops in *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 *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 immunized IL-18−/− 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 sex-matched and immunized. Age- and sex-matched animals were used throughout the study.

**Immunization**

Groups of 8–10 mice were immunized orally with a blunt 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 × 10⁷ 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 curvature 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 vials (Cryomold; Miles East, IN) filled with O.C.T. (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 microsegments 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), and quantitation of CFU was performed 7 days later. In the microabscesses or cystic glands; or 4, epithelial changes throughout the mucosa plus four or more microabscesses or cystic glands.

**Histopathology**

For evaluation of gastritis, 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 plus four or more 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.

**Epithelial changes**

For evaluation of epithelial changes, H&E-stained sections were scored based on the degree of epithelial destruction in the corpus, including parietal cell loss and hyperplasia of the surface epithelium as described by Ermak et al. (48). Briefly, epithelial scores were defined as follows: 0, none; 1, small, focal areas of parietal cell loss in the corpus and/or hyperplasia of the surface epithelium; 2, epithelial changes throughout 75% of the mucosa; 3, epithelial changes throughout the mucosa plus one to microabscesses or cystic glands; or 4, epithelial changes throughout the mucosa plus four or more microabscesses or cystic glands.

**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 then incubated with 5% normal 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-amino-9-ethylcarbazole (Vector Laboratories) and HRP conjugated to an avidin-biotin complex. Cell-bound peroxidase was detected with 3-amino-9-ethylcarbazole (Vector Laboratories) and HRP conjugated to an avidin-biotin complex. Cell-bound peroxidase was detected with 3-amino-9-ethylcarbazole (Vector Laboratories) and HRP conjugated to an avidin-biotin complex. Cell-bound peroxidase was detected with 3-amino-9-ethylcarbazole (Vector Laboratories) and HRP conjugated to an avidin-biotin complex.

**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)benzenesulfonfonyl 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

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*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 $-85^\circ$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 $\mu$g/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 antimouse 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 $\mu$M 2-ME (Sigma-Aldrich), 1 mM t-glutamine (Biochrom), and 50 $\mu$g/ml gentamicin. Spleen cells (10$^3$ cells/well) were then cultured (8% CO$_2$, 37°C) for IL-4, IL-5, IL-10, IL-12, IL-13, IL-18, and eotaxin development, the HRP substrate H$_2$O$_2$ with citrate buffer (pH 4.5) was added to each well, and the extent of the reaction was measured with an enzymatic spectrophotometer. For IL-18, 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 $-85^\circ$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 $\times$ g) and stored at $-85^\circ$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 $\mu$g/ml rat anti-mouse IFN-γ (BD Pharmingen), 1–$\mu$g/ml 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 $\mu$g/ml mouse 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 (Endogen), IL-5, IL-10 (BD Pharmingen), 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 H$_2$O$_2$ 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 Titertek multispec microphotometer. For IL-13, IL-18, and eotaxin development, the HRP substrate H$_2$O$_2$ with 3,3',5,5'-tetramethylbenzidine (Sigma-Aldrich) in phosphate citrate buffer (pH 5.0) was added to appropriate wells, followed by the addition of 1 M H$_2$SO$_4$ as stop solution, and the extent of reaction was read at 450 nm within 30 min. The cytokine concentrations in stimulated culture supernatants were estimated from the standard curves generated with each cytokine.

**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.

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**Results**

**IL-18$^-/-$ mice fail to develop protection against H. pylori infection**

We recently reported that protective immunity against H. pylori infection in mice is associated with a proinflammatory Th1 cell response, involving the production of IL-12 and IFN-γ (18). However, the role of IFN-γ has been disputed (19). Because IL-18 not only promotes IFN-γ and Th1 development, but, in addition, has a significant regulatory role in local inflammation and tissue eosinophilia, the role of IL-18 in resistance against H. pylori infection was reinvestigated (22, 29, 30). To this end groups of IL-18$^-/-$ and C57BL/6 WT mice were immunized orally with H. pylori lysate and CT adjuvant, and subsequently challenged with live H. pylori organisms. As shown in Fig. 1, immunized IL-18$^-/-$ mice failed to develop protection, whereas immunized WT mice were significantly protected against challenge with live H. pylori bacteria, as assessed by urease activity (data not shown) and quantitative culture. In three experiments, immunized and challenged WT mice exhibited at least a 13-fold reduction in bacterial load in the gastric tissue with a geometric mean log$_{10}$ value of 3.97 ± 1.6 vs 5.1 ± 1.2 in the control mice receiving CT alone (p < 0.001; Fig. 1). By contrast, H. pylori colonization was unaltered in immunized IL-18$^-/-$ mice compared with that in the controls. The geometric mean log$_{10}$ value was 5.1 ± 1.2 for immunized mice vs 4.98 ± 1.4 for the control mice. Moreover, immunized and protected WT mice exhibited a strong infiltration of CD4$^+$ T cells in the gastric tissue with a geometric mean log$_{10}$ value of 3.97 ± 1.6 vs 5.1 ± 1.2 for immunized mice vs 4.98 ± 1.4 for the control mice.
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
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 control WT 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 immunized 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 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, cryptococcal, 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, 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

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**FIGURE 6.** Anti-H. pylori Ab titers in sera and gastric secretions of immunized challenged IL-18−/− and WT mice. Sera (A) and gastric secretions (B) from IL-18−/− and WT mice immunized with lysate plus CT were analyzed for H. pylori lysate-specific IgG1 (■), IgG2a (■), and IgA (□) 2 wk after challenge using ELISA. The results are given as the mean SEM of 8–10 mice/group. *, p = 0.022; †, p = 0.049 (by Student’s t test, compared with lysate- plus CT-immunized WT mice). One representative experiment of two is shown.
PROTECTION AGAINST *H. pylori* IS IL-18 DEPENDENT

**Table I. Cytokine production by spleen cells from the immunized challenged IL-18−/− and WT mice**

<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>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Medium</td>
<td>Lysate</td>
<td>Medium</td>
<td>Lysate</td>
</tr>
<tr>
<td>IL-18−/−</td>
<td>Lysate + CT</td>
<td>12.5 ± 5</td>
<td>42.5 ± 20p</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>10 ± 6</td>
<td>30 ± 30</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>PBS (naive)c</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 ± 80q</td>
<td>35 ± 12</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>12 ± 8</td>
<td>35 ± 21</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>PBS (naive)c</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* p = 0.032, by Student’s t test compared with CT (IL-18−/−) and *p* = 0.028 compared with lysate plus CT (WT).

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>
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<tbody>
<tr>
<td></td>
<td>Medium</td>
<td>Lysate</td>
<td>Medium</td>
<td>Lysate</td>
<td>Medium</td>
<td>Lysate</td>
</tr>
<tr>
<td>IL-18−/−</td>
<td>Lysate + CT</td>
<td>1720 ± 330p</td>
<td>2753 ± 53.9q</td>
<td>ND</td>
<td>8961 ± 2384</td>
<td>3242.8 ± 434.4*</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>1150 ± 700</td>
<td>220.3 ± 21.8</td>
<td>ND</td>
<td>9278 ± 1050</td>
<td>2809.5 ± 328.8</td>
</tr>
<tr>
<td></td>
<td>PBS (naive)c</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>1370 ± 1300p</td>
<td>580.5 ± 132.5</td>
<td>405.3 ± 3.2*</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>2727.4 ± 1138.3</td>
</tr>
<tr>
<td></td>
<td>PBS (naive)c</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>

* p = 0.0007 compared with lysate plus CT (WT).

Table data 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-dependent 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 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-12 production did not correlate to the presence of eotaxin in the gastric mucosa, because eotaxin 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.
References


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The Journal of Immunology 3355


