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J Immunol 2010; 184:5121-5129; Prepublished online 29 March 2010;
 doi: 10.4049/jimmunol.0901115
<http://www.jimmunol.org/content/184/9/5121>

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Helicobacter pylori-Induced Th17 Responses Modulate Th1 Cell Responses, Benefit Bacterial Growth, and Contribute to Pathology in Mice

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CD4⁺ T cell responses are critical for the pathogenesis of *Helicobacter pylori* infection. The present study evaluated the role of the Th17 subset in *H. pylori* infection. *H. pylori* infection induced significant expression of IL-17 and IFN- γ in mouse gastric tissue. IL-23 and IL-12 were increased in the gastric tissue and in *H. pylori*-stimulated macrophages. Cell responses were examined by intracellular staining for IFN- γ , IL-4, and IL-17. Mice infected with *H. pylori* developed a mixed Th17/Th1 response; Th17 responses preceded Th1 responses. Treatment of mice with an anti-IL-17 Ab but not a control Ab significantly reduced the *H. pylori* burden and inflammation in the stomach. *H. pylori* colonization and gastric inflammation were also lower in IL-17^{-/-} mice. Furthermore, administration of recombinant adenovirus encoding mouse IL-17 increased both *H. pylori* load and inflammation. Further analysis showed that the Th1 cell responses to *H. pylori* were downregulated when IL-17 is deficient. These results together suggest that *H. pylori* infection induces a mixed Th17/Th1 cell response and the Th17/IL-17 pathway modulates Th1 cell responses and contributes to pathology. *The Journal of Immunology*, 2010, 184: 5121–5129.

Helicobacter *pylori* is a Gram-negative, microaerophilic bacterium that resides extracellularly in the gastric mucosa and infects >50% of the population worldwide. *H. pylori*-induced chronic inflammation is the cause of gastritis and peptic ulcers and a risk factor for gastric cancer (1, 2). *H. pylori* infection causes severe local inflammation in the gastric mucosa. CD3⁺CD4⁺ T cells are increased in infected gastric lamina propria and play important roles in the pathogenesis of persistent *H. pylori* infection (3). Traditionally, CD4⁺ T cells are classified into two main classes: Th1 and Th2, on the basis of their cytokine secretion and immune regulatory function. Th1 cells secrete IFN- γ , IL-2, and IL-12 and regulate cellular immunity, whereas Th2 cells produce IL-4, IL-5, and IL-13 and mediate humoral responses. To date, studies of immune responses to *H. pylori* have largely focused on Th1 and Th2 cells, and it is generally accepted that *H. pylori* infection results in a Th1-dominant

response and that gastric inflammation largely depends on Th1 cell responses (3–6); however, IFN- γ secretion alone is insufficient to induce gastritis (3). Thus, the detailed mechanism of pathogenesis is not clear. A novel subset of effector T cells, identified by secretion of IL-17, has been defined as Th17 cells. Th17 cells are distinct from Th1 and Th2 cells in their differentiation and function (7, 8). TGF- β and IL-6 from activated macrophages/dendritic cells are required for Th17 cell differentiation in murine systems (9), whereas IL-12 and IFN- γ promote Th1 cell development and IL-4 primes Th2 cell differentiation. The expansion and survival of Th17 cells are promoted by IL-23 (9), a heterodimeric cytokine composed of a unique p19 subunit and a p40 subunit shared with IL-12 (10). The identification of Th17 cells necessitates a re-evaluation of Th cell responses in *H. pylori* infection.

Th17 cells are clearly implicated in the pathogenesis of autoimmune diseases by promoting chronic inflammation (11). However, the protective and pathogenic functions of IL-17-producing Th cells were both reported in infections (12). Given the growing number of reports on the association of Th17 cells with a variety of other infections and the increased expression of IL-17 in *H. pylori*-infected gastric mucosa (13–15), it is likely that Th17 cells are involved in responses to *H. pylori*. However, the characteristics of Th cell responses, including those of Th1, Th2, and Th17 cells, remain unclear, and the role of Th17 cell responses in *H. pylori* infection has not been fully elucidated.

In the current study, we characterized Th cell responses, especially Th17 cell responses, to *H. pylori* postinfection (p.i.) in mouse model and attempted to elucidate the role of Th17 cells during the early stage of *H. pylori* infection.

Materials and Methods

H. pylori culture and Ag preparation

The VacA⁺/CagA⁺ *H. pylori* strain CCS9803 (China Chongqing Strain 9803) was recovered from a patient who suffered from chronic antrum gastritis in Chongqing, China, in 1998. The isolated strain was adapted to colonize the

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Received for publication April 16, 2009. Accepted for publication February 21, 2010.

This work was supported by grants from the National Natural Science Foundation of China (30801044), National Basic Research Program of China (2007CB914801), National Outstanding Young Scientist Award of NSFC (30725015), and Chongqing Science and Technology Commission (2007BB5034).

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Abbreviations used in this paper: AdmIL-17, recombinant adenovirus encoding mouse IL-17; AdLuc, recombinant adenovirus encoding luciferase; β 2-M, β 2-microglobulin; MFC, mouse forestomach carcinoma; mIL-17, mouse IL-17; MLN, mesenteric lymph node; MMP, matrix metalloproteinase; p.i., postinfection; PLN, paragastric lymph node; WCP, whole cell protein; WT, wild-type.

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gastric mucosa of BALB/c mice by five passages in vivo to obtain a good mouse-adapted strain (CCS9803/B5). The *H. pylori* CCS9803/B5 were grown on brain–heart infusion plates containing 10% rabbit blood and amplified in *Brucella* broth with 5% FBS with gentle shaking at 37°C under microaerobic conditions. After culture for 1 d, live bacteria were collected and adjusted to 10⁹ CFU/ml *Brucella* broth prior to inoculation. To prepare *H. pylori* whole cell protein (WCP) as Ag, *H. pylori* were sonicated, and the supernatants were collected.

Infection of mice with *H. pylori*

Specific pathogen-free female BALB/c mice and C57BL/6 mice (6- to 8-wk-old) were purchased from the Experimental Animal Center of the Third Military Medicine University. IL-17^{-/-} mice (C57BL/6 background) were kindly provided by Richard A. Flavell (Yale University School of Medicine, New Haven, CT). All of the animal experiments were approved by the Animal Ethical and Experimental Committee of Third Military Medical University. The mice were fasted overnight and orogastrically inoculated twice at a 1 d interval with 5 × 10⁸ CFU *H. pylori*. Age-matched control mice were mock-inoculated with *Brucella* broth. Four to six mice per group per time point were used for studies.

Evaluation of inflammation and bacterial load

The mice were sacrificed at the indicated times p.i. The greater curvature of the stomach was cut to perform H&E staining. The intensity of inflammation was evaluated in a blinded fashion by two pathologists, and each section was given a score of 0–5 according to established criteria (16). The remaining stomach was cut into three parts for DNA, RNA, and protein extraction, respectively. The density of *H. pylori* colonization was quantified by real-time PCR, detecting *H. pylori*-specific 16S rDNA as previously described using a specific primer and probe (17). The amount of mouse β2-microglobulin (β2-M) DNA in the same specimen was measured to normalize the data. The primers and probe for detection of mouse β2-M DNA were as follows: forward, 5'-GAAATCCAAATGCTGAAGAAGC-3'; reverse, 5'-GGAAGAACTTGAGGCTTACCC-3'; probe, 5'-TGGCCGAGCCCAAGACCGTCTAC-3'. The density of *H. pylori* in the samples was expressed as the number of bacterial genomes per nanogram of host genomic DNA according to a previous report (18).

Real-time PCR

Total RNA was isolated from the stomachs or cells by TRIzol extraction (Invitrogen, Carlsbad, CA) and reverse-transcribed to cDNA using ReverTra Ace (TOYOBO, Osaka, Japan). Real-time PCR was performed on a Rotor-Gene 6000 instrument (Corbett Life Science, Sydney, Australia). Cytokine expression was determined by the TaqMan method using primers and probes previously reported or designed as follows: IL-17, forward, 5'-GAATGTGAAGGTCAACCTCAAAGTC-3', reverse, 5'-TTCCAGATCACAGAGGGATATCT-3', probe, 5'-TCAACCGTTCACGTCACCCCTGG-3'; IFN-γ, forward, 5'-GATCCTTTGGACCTCTGACTT-3', reverse, 5'-TGACTGTGCCGTGGCAGTAA-3', probe, 5'-CAATGAACGCTACACACTGCATCTTGGC-3'; IL-4, forward, 5'-GAGCTGCAGAGACTCTTTCG-3', reverse, 5'-ACTCATTCATGGTGCAGCTTA-3', probe, 5'-GCTTTTCGATGCCTGGATTCATCG-3'; T-bet, forward, 5'-ACCA-GAGCGCAAGTGGG-3, reverse, 5'-TGGACATATAAGCGGTTCCCTG-3', probe, 5'-FAM-CAGTGTGGAAAGGCAGAAGGCAG-TAMRA-3'. Previously described primers and probes were used for IL-23 p19, IL-12/IL-23 p40, IL-12 p35 (19), IL-6 (20), and TGF-β (21). All of the probes were labeled with the reporter dye FAM at the 5' and the quencher dye TAMRA at the 3' end. Matrix metalloproteinases (MMPs) and chemokines were measured by the SYBR Green method using the following primers: CXCL1, forward, 5'-GCACCCAAACCGAAGTCAT-3', reverse, 5'-GAAGCCACGCTTACCAGA-3; MMP-2, forward, 5'-GCCTCATAACAGCGTCAACTT-3', reverse, 5'-CGGTTTATTGGCGGACAGT-3'; MMP-3, forward, 5'-TCCACAGACTTGTCCCGTTTC-3', reverse, 5'-AGGGTGCTGACTGCATCAAA-3'; MMP-7, forward, 5'-GCAGAATACTCACTAATGCCAAAC-3', reverse, 5'-GCAGTCCCCCACTAACCT-3'; MMP-9, forward, 5'-GCCCTGGAACCTCACACGACA-3', reverse, 5'-TTGGAAACTCACAGCCAGAAG-3'. The primers for CCL2, CCL5, CCL20, and CCL25 were as previously described (22, 23). Mouse β2-M served as the normalizer, and uninfected stomach served as the calibrator (8). The relative gene expression was expressed as fold change calculated by the ΔΔCt method.

ELISA for cytokines in gastric tissue

Gastric tissues from *H. pylori*-infected and uninfected mice were collected, homogenized in 1 ml sterile PBS, and centrifuged. The supernatants were analyzed for IFN-γ, IL-4, IL-17, IL-12, or IL-23 production using ELISA

kits (eBioscience, San Diego, CA) according to the manufacturer's instructions. Total protein was measured by the Lowry method. The cytokine concentrations in stomach were expressed as picograms per milligrams of total protein.

Cytokine production

Splenic lymphocytes (1.6 × 10⁶ lymphocytes per milliliter) from *H. pylori*-infected and noninfected BALB/c mice on day 21 p.i. were cultured in RPMI 1640 medium with or without *H. pylori* WCP (2.5 μg/ml). The production of IFN-γ, IL-4, and IL-17 in the supernatants was measured by ELISA (eBioscience) at 48 h.

Intracellular cytokine staining

The lymphocytes isolated from spleen, mesenteric lymph node (MLN), and paragastric lymph node (PLN) were stimulated with PMA (50 ng/ml; Sigma-Aldrich, St. Louis, MO) and ionomycin (1 μg/ml; Sigma-Aldrich) for 6 h or with *H. pylori* WCP (2.5 μg/ml) for 24 h. BD GolgiStop was added at the final 6 h (BD Pharmingen, San Diego, CA). Standard intracellular cytokine staining was performed as follows. The cells were first stained extracellularly with FITC-conjugated anti-CD4 (RM4-5) and PE-Cy5-conjugated anti-CD3 (17A2), then fixed and permeabilized with BD Cytofix/Cytoperm solution (BD Pharmingen). The cells were then separately stained intracellularly with PE-conjugated anti-IFN-γ (XMG1.2), anti-IL-4 (11B11), or anti-IL-17 (TC11-18H10) (all from BD Pharmingen). Samples were acquired on a FACSCalibur (BD Biosciences, San Diego, CA) and gated with CD3. Data were analyzed using BD CellQuest Pro software (BD Biosciences).

Preparation of splenic macrophages

Mouse spleens were passed through meshed steel sieves to obtain single-cell suspensions. Erythrocytes were lysed hypotonically, and the remaining cells were washed in PBS. The cells were resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin to yield a final concentration of 1.0 × 10⁷ cells per milliliter. The cell suspensions were plated in 24-well culture plates in 2 ml and incubated at 37°C for 3 h. Nonadherent cells were then washed off three times with PBS, and the remaining adherent cells, consisting of 95% macrophages, were used for subsequent assays.

Cell culture and treatment

Splenic macrophages and the mouse forestomach carcinoma (MFC) cell line (24) (purchased from the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, Beijing, China) were used in the experiments. MFC cells were derived from epithelial cells and confirmed by staining with Ab against CK-18 (epithelial cell marker). The cells were cultured in RPMI 1640 with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO₂. The cells were seeded in six-well plates at a density of 2.5 × 10⁶ cells per well and stimulated with recombinant mouse IL-17 (mIL-17) (100 ng/ml; R&D Systems, Minneapolis, MN) or *H. pylori* (multiplicity of infection, 100). Cells were collected at the indicated times for analysis of cytokine mRNA expression. Unstimulated cells were used as a control.

In vivo blockade of IL-17

The recombinant mIL-17–GST fusion protein expressed in *Escherichia coli* using the pGEX-4T-1 vector (Amersham Pharmacia Biotech, Uppsala, Sweden) was used to immunize the rabbits to produce anti-mIL-17 polyclonal Ab. The rabbit IgG was purified on a Sepharose A column (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The neutralizing activity of the rabbit anti-mIL-17 Ab was determined by blocking secretion of IL-6 from MFC cells after IL-17 stimulation in vitro. One hour before infection with *H. pylori*, 500 μg anti-mIL-17 IgG or normal rabbit IgG was administered to each mouse i.p., and the administration was repeated every 72 h until the mice were sacrificed at indicated time. The colonization of *H. pylori* and the inflammation of the stomach were measured.

Delivering mIL-17 to gastric tissue by oral administration of recombinant adenovirus encoding mIL-17

Recombinant adenovirus encoding mouse IL-17 (AdmIL-17) and recombinant adenovirus encoding luciferase (AdLuc) were kindly provided by Dr. Jay K. Kolls (Children's Hospital of Pittsburgh, Pittsburgh, PA). Mice received 5 × 10⁹ PFU of AdmIL-17 or AdLuc (as a control) orally and were infected with *H. pylori* 5 d later. The mice were sacrificed at the

indicated time, and the inflammation and colonization with *H. pylori* in the stomach were measured.

Statistical analysis

Student *t* test was generally used to analyze the differences between two groups, but when the variances differed, the Mann-Whitney *U* test was used. Bacterial colonization data in the adenovirus treatment experiment were analyzed by the Mann-Whitney *U* test. Differences were considered as significant when $p < 0.05$. All of the experiments were repeated at least twice.

Results

Dynamics of gastric cytokine expression during *H. pylori* infection

To analyze immune responses to *H. pylori*, a reliable animal model was established by infecting BALB/c mice with *H. pylori*, and gastric tissues were collected at the indicated times to evaluate cytokine expression. Real-time PCR showed that the levels of IFN- γ and IL-4 in *H. pylori*-infected stomach were elevated on day 35 p.i. ($p < 0.05$; Fig. 1A, 1B). IL-17 expression showed a significant increase on day 28 and peaked on day 35 p.i. ($p < 0.05$), then decreased (Fig. 1C). Levels of IFN- γ and IL-17 protein were also significantly increased on day 35 p.i. ($p < 0.05$; Fig. 2A), whereas IL-4 expression was not significantly changed at the protein level. The induction of IL-17 and IFN- γ at both RNA and protein levels suggests that Th1 and Th17 cells might participate in the *H. pylori*-induced immune responses. We also detected the expression of cytokines regulating the Th1 and Th17 cell responses. IL-12 p35 and IL-12/IL-23 p40 were increased on day 28 and peaked on day 35 p.i. (Fig. 1D, 1E). IL-23 p19 mRNA expression was elevated from days 21 to 35 with a peak on day 28 p.i. (Fig. 1F). These data suggest that the Th17 cell regulating cytokine IL-23 and Th1 cell regulating cytokine IL-12 were also increased during *H. pylori* infection and might be involved in Th17 and Th1 cell differentiation.

H. pylori induced specific Th1/Th17 cell responses

To correlate the expression of cytokines in the gastric mucosa with lymphocyte-mediated responses, IFN- γ , IL-4, and IL-17 in the supernatants of cultured splenic lymphocytes were tested following *H. pylori* WCP stimulation in vitro for 48 h. Lymphocytes from the *H. pylori*-infected mice showed increased IL-17, IFN- γ , and IL-4 secretion in response to stimulation with the WCP ($p < 0.05$; Fig. 2B), but lymphocytes from control mice did not (Fig. 2B). Further flow cytometry was used to detect the frequency of cytokine-producing CD4⁺ T cells, which as the main source of cytokine production play an essential role in the pathogenesis of *H. pylori* infection. Splenic lymphocytes from infected mice showed significantly higher Th1 and Th17 cell responses than those from uninfected mice when stimulated with PMA and ion-

omycin, whereas Th2 cell responses were not significantly changed (Fig. 3A). The results indicated that *H. pylori* infection induces Th1 and Th17 cell responses. When the WCP was used as Ag, the splenic lymphocytes from control mice displayed a minor response to the stimulation, but the cells from infected mice reacted significantly, as shown by the increased Th1 and Th17 cell responses (Fig. 3B), suggesting that specific Th1 and Th17 cell responses were induced in *H. pylori* infection.

The murine macrophages isolated from spleen were adopted to determine whether *H. pylori* could induce the expression of cytokines that regulate Th17 and Th1 cell differentiation. When splenic macrophages were cocultured with *H. pylori*, gene expression of IL-6, TGF- β , IL-23 p19, IL-12/IL-23 p40, and IL-12 p35 were all upregulated significantly at 6 h (Fig. 4). The cytokines secreted by macrophages may partially explain the increased IL-23 and IL-12 expression in the stomach. *H. pylori* may promote Th17 and Th1 cell responses by inducing the secretion of regulatory cytokines.

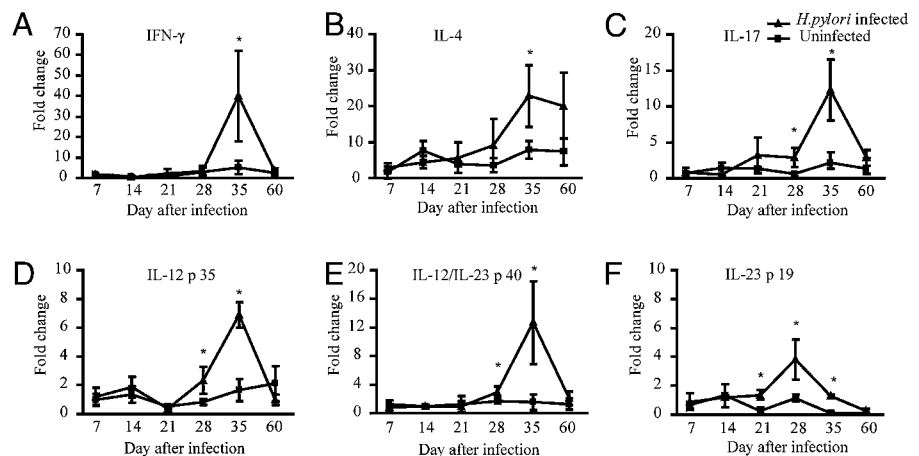
Kinetics of Th cell responses in MLN, spleen, and PLN

To characterize the CD4⁺ T cell immune responses to *H. pylori* more fully, we determined the dynamics of Th cell responses in MLN, spleen, and PLN. Because it is difficult to isolate lymphocytes from the stomach of uninfected mice, we used the detection of CD4⁺ T cell response in the PLN to reflect the situation in the stomach. The results showed that Th1, Th2, and Th17 cell responses in MLN were primed only on day 7 p.i. (Fig. 5A). In the spleen, Th17 cells were expanded on day 14 and peaked on day 21 p.i., and Th1 cells were induced from days 21 to 28 p.i., whereas Th2 showed no significant change during the course of infection (Fig. 5B). The Th cell responses were delayed in PLN, reaching detectable levels on day 28 p.i. (Fig. 5C). The Th cell responses in spleen and PLN showed a dynamic change; Th17 cell responses preceded Th1 cell responses. The time course of Th cell responses in different organs suggests that MLN may be an earlier site of T cell priming during *H. pylori* infection.

In vivo blockade of IL-17 significantly reduced *H. pylori* colonization and inflammation in the stomach

The function of Th1 cells in *H. pylori* infection has been well established, so the current study focused mainly on the role of Th17 cells. CD4⁺ T cells exert most of their effector functions through secretion of cytokines. Therefore, we focused on the effect of IL-17, the main effector of these cells, to evaluate the role of Th17 cells in *H. pylori* infection. Rabbit anti-mIL-17 polyclonal Ab was produced, and its neutralizing activity was confirmed by blocking IL-6 secretion from MFC cells stimulated with

FIGURE 1. Expression of cytokine mRNAs in gastric tissues. Total RNA was extracted from the stomachs of *H. pylori*-infected and time-matched uninfected mice and subjected to real-time PCR. Mouse β 2-M served as the normalizer, and uninfected stomach on day 7 served as the calibrator. Data, expressed as fold change, were calculated by the $\Delta\Delta$ Ct method and are expressed as mean \pm SD for (A) IFN- γ , (B) IL-4, (C) IL-17, (D) IL-12 p35, (E) IL-12/IL-23 p40, and (F) IL-23 p19. * $p < 0.05$ compared with uninfected mice; $n = 4-6$ per group per time point.



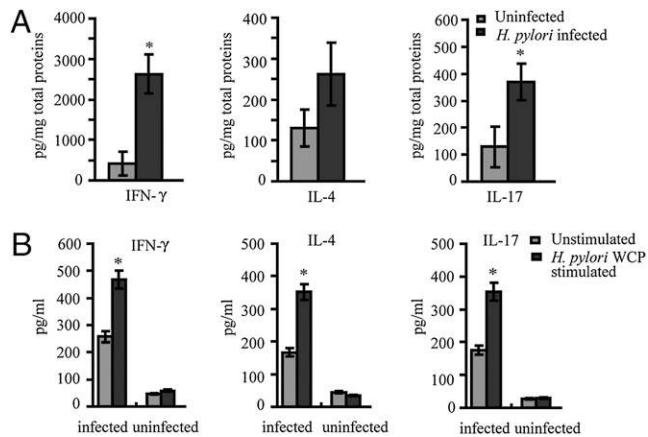


FIGURE 2. Cytokine contents of stomach tissue and supernatants from cultured lymphocytes. *A*, The supernatants of the gastric tissue homogenates from *H. pylori*-infected and uninfected mice (35 d p.i.) were analyzed for IFN- γ , IL-4, and IL-17 production by ELISA. Results are expressed as mean \pm SD. $*p < 0.05$ compared with uninfected mice. *B*, Splenic lymphocytes isolated from *H. pylori*-infected and uninfected mice (21 d p.i.) were restimulated with or without *H. pylori* WCP (2.5 μ g/ml). Supernatants were collected 48 h after stimulation, and cytokines were measured by ELISA. Results are expressed as mean \pm SD. $*p < 0.05$ compared with unstimulated control; $n = 4-6$.

mIL-17, using the commercial anti-mIL-17 mAb for comparison. Rabbit anti-mIL-17 Ab or normal rabbit IgG was administered before and during *H. pylori* infection. The colonization of *H. pylori* in the anti-IL-17 Ab-treated mice was significantly lower than that in the control IgG-treated mice on day 28 ($p < 0.05$; Fig. 6A), and the inflammation of gastric tissue in the anti-IL-17 Ab-treated mice was significantly lower than that in the control mice ($p < 0.05$; Fig. 6B). There was mild inflammatory cell infiltration in the gastric lamina propria of mice treated with IL-17 Ab (Fig. 6C), whereas mice treated with control IgG showed

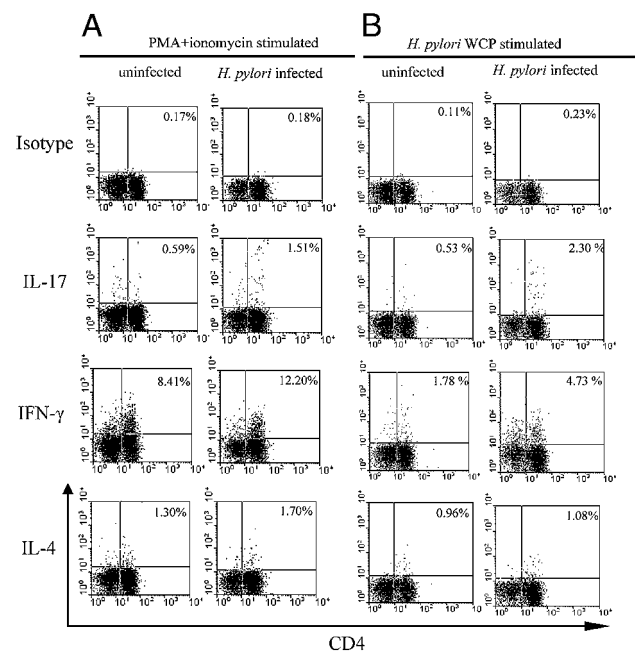


FIGURE 3. Th cell responses induced by *H. pylori* infection. Spleen cells were isolated from *H. pylori*-infected and uninfected mice on day 21 p.i. Th1, Th2, and Th17 cell responses stimulated with PMA and ionomycin (*A*) or *H. pylori* WCP (*B*) were examined by intracellular staining. Cells were gated on CD3⁺ cells. The results are representative of three independent experiments.

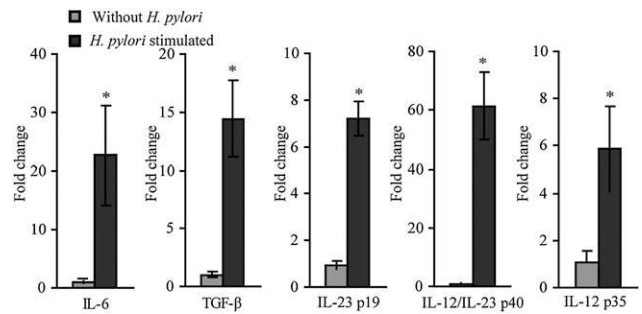


FIGURE 4. Effects of *H. pylori* on cytokine expression in splenic macrophages. The murine macrophages isolated from spleen cells were cocultured with *H. pylori* for 6 h, and the expression of IL-6, TGF- β , IL-23 p19, IL-12/IL-23 p40, and IL-12 p35 was examined by real-time PCR. Data (fold change) are expressed as mean \pm SD ($n = 3$). $*p < 0.05$ compared with culture without *H. pylori*.

moderate infiltration of inflammatory cells in the submucosa and lamina propria (Fig. 6D). The results indicate that neutralization of IL-17 activity contributes to bacterial clearance and prevents inflammation.

To confirm these results, we infected IL-17^{-/-} mice with *H. pylori* and evaluated bacterial colonization and inflammation. The results were consistent with those using IL-17 Ab; IL-17^{-/-} mice infected with *H. pylori* showed decreased bacterial colonization and gastric inflammation (Fig. 7). These results suggest that the Th17/IL-17 pathway does not favor the host defense against *H. pylori* infection.

Effect of IL-17 overexpression on *H. pylori* infection

To confirm the effect of IL-17 on *H. pylori* infection, we used AdmIL-17 to transfer the mIL-17 gene to the mouse stomach and then infected the mice with *H. pylori*, and the colonization was assayed on day 28 p.i. IL-17 expression in mice treated with AdmIL-17 was significantly greater than that in mice treated with AdLuc or PBS (Fig. 8A). Mice treated with AdmIL-17 showed more copies of *H. pylori* in the stomach than those treated with AdLuc on day 28 p.i. ($p < 0.01$), whereas the copies of *H. pylori* in AdLuc-treated mice were not different from those in PBS-treated mice ($p > 0.05$; Fig. 8B). In addition, mice treated with AdmIL-17 showed significantly more gastric inflammation than those treated with AdLuc ($p < 0.0125$; Fig. 8C), and the inflammatory response in AdLuc-treated mice was similar to that in PBS-treated mice ($p > 0.05$; Fig. 8C). In AdmIL-17-treated mice, diffuse inflammatory cell infiltration was seen in most of the submucosa and mucosa, and the mucosal lesions were severe (Fig. 8D). In contrast, mice treated with AdLuc and PBS often showed only mild to moderate inflammatory cell infiltration in the gastric lamina propria and submucosa (Fig. 8E, 8F). These results indicate that Th17/IL-17 might increase the host susceptibility to *H. pylori* infection.

IL-17^{-/-} mice infected with *H. pylori* exhibit decreased Th1 cell responses

Th1 cell responses have been shown to be important in the pathogenesis of *H. pylori* infection (5, 6). Our results showed that Th17 cell responses, which precede Th1 cell responses, also contribute to pathogenesis. The association between Th17 and Th1 cell responses was further evaluated. We found that IFN- γ production by *H. pylori*-infected gastric tissue was not significantly different in the IL-17 blocking and overexpression models, whereas IFN- γ production was significantly lower in *H. pylori*-infected IL-17^{-/-} mice than that in *H. pylori*-infected wild-type (WT) mice (Fig. 9A). Consistent with the decreased IFN- γ secretion, expression of T-bet and IL-12 in *H.*

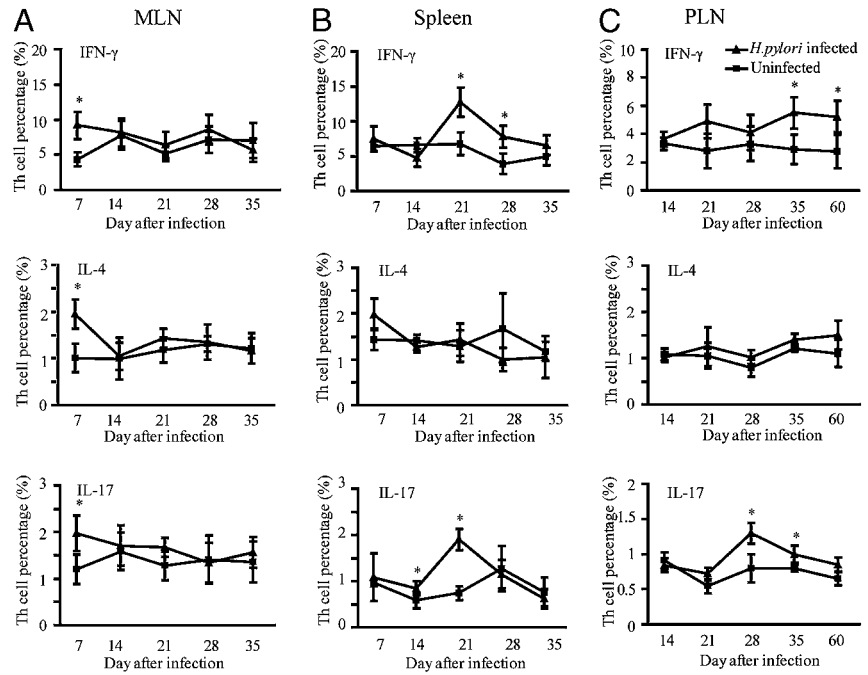


FIGURE 5. Dynamic change of Th cell responses in MLN, spleen, and PLN following *H. pylori* infection. Lymphocytes from MLN (A), spleen (B), and PLN (C) were isolated from *H. pylori*-infected and uninfected mice ($n = 5-6$) at the indicated times. Th1, Th2, and Th17 cell responses were examined by intracellular staining. Cells were gated on CD3 cells. Data are expressed as mean \pm SD. * $p < 0.05$ compared with uninfected mice.

pylori-infected gastric tissue was also downregulated significantly in IL-17^{-/-} mice (Fig. 9B, 9C). Th1 cell frequencies in the spleens of *H. pylori*-infected IL-17^{-/-} mice were also lower (Fig. 9D). These results indicate that the Th17 cell response may regulate the Th1 cell response during *H. pylori* infection.

Effect of mIL-17 on expression of MMPs and chemokines

MMP and chemokine expression can be regulated by IL-17, and this might be important in promoting IL-17-induced inflammation or cell responses, including Th1 cell responses. We therefore examined whether the gastritis regulated by IL-17 in *H. pylori* infection was associated with the production of MMPs and certain

chemokines. The mouse gastric MFC cells were used to study the effect of IL-17 on gastric epithelial cells. MMP-2, MMP-3, MMP-7, MMP-9, and the chemokines CCL2, CCL5, CCL20, CCL25, and CXCL1 were more highly expressed when MFC cells were stimulated with IL-17 (Fig. 10A). To determine whether these factors are associated with the role of IL-17, we detected their expression in the *H. pylori* infection model pretreated with IL-17 Ab or AdmIL-17 and in IL-17^{-/-} mice. IL-17 Ab pretreatment reduced MMP-9 and CCL25 expression in *H. pylori*-infected stomachs (Fig. 10B). The expression of MMP-3, MMP-9, CCL5, and CCL25 was significantly lower in *H. pylori*-infected IL-17^{-/-} mice than that in WT mice ($p < 0.05$; Fig. 10C). AdmIL-17 treatment increased MMP-2, MMP-9, and CCL25 expression ($p < 0.05$; Fig. 10D). The results indicate that the Th17/IL-17 pathway

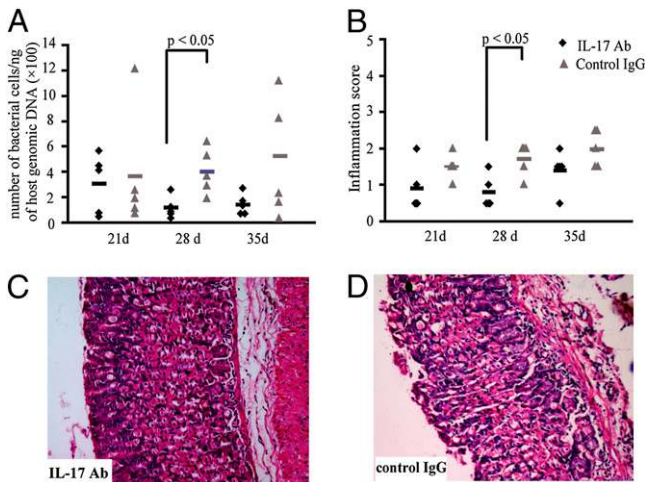


FIGURE 6. Effects of neutralization of IL-17 on *H. pylori* infection. One hour before *H. pylori* infection, mice were treated with rabbit anti-mIL-17 or control IgG, and the administration was repeated every 72 h ($n = 5-6$). A and B, The mice were sacrificed at the indicated time to assay *H. pylori* colonization in the stomach (A) and histological scores of gastritis (B). C, Representative gastric histopathology in mice pretreated with IL-17 Ab on day 28 p.i., with only mild inflammatory cell infiltration of the lamina propria. D, Representative gastric histopathology in mice pretreated with control IgG on day 28 p.i., showing moderate inflammatory cell infiltration of the submucosa and lamina propria. C and D, H&E staining, original magnification $\times 200$.

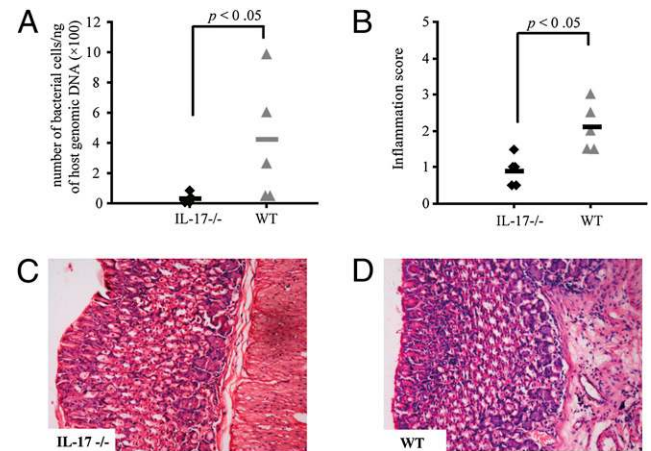


FIGURE 7. *H. pylori* colonization and gastric inflammation in IL-17^{-/-} mice. IL-17^{-/-} and WT mice were infected with *H. pylori*. A and B, The bacteria colonization in the stomach (A) and histological scores of gastritis (B) were assayed on day 28 p.i. C, Representative gastric histopathology in IL-17^{-/-} mice showed a mild inflammatory cell infiltration of the lamina propria. D, Representative gastric histopathology in WT mice, with moderate inflammatory cell infiltration of the submucosa and lamina propria. C and D, H&E staining, original magnification $\times 200$.

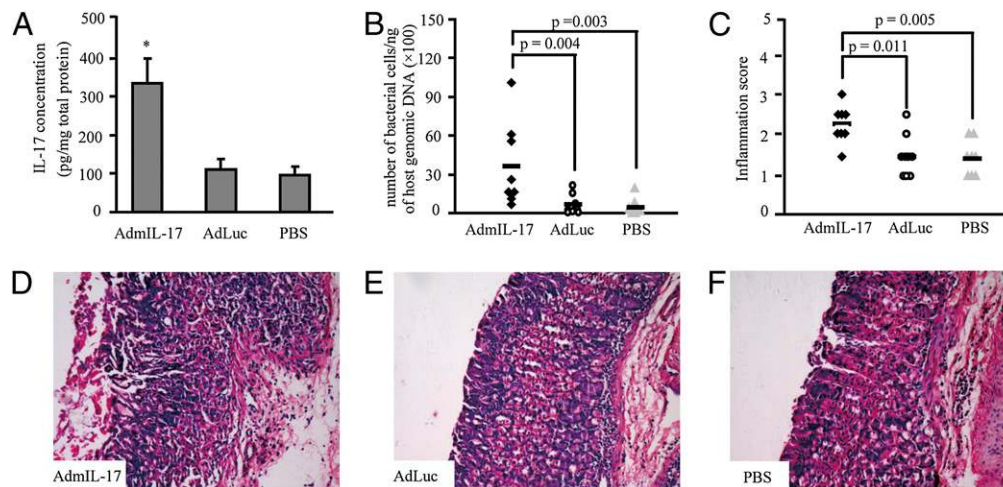


FIGURE 8. Evaluation of *H. pylori* colonization and inflammation after AdmIL-17 treatment. Mice were pretreated with 5×10^9 PFU of AdmIL-17 or AdLuc before *H. pylori* infection ($n = 8-10$). **A**, The expression of recombinant adenovirus-transduced IL-17 in gastric tissue was analyzed by ELISA. $*p < 0.05$ compared with mice treated with AdLuc or PBS. **B** and **C**, *H. pylori* colonization of the stomach (**B**) and gastric inflammation (**C**) were examined on day 28 p.i. **D-F**, Representative gastric histopathologies in mice treated with AdmIL-17 (**D**), AdLuc (**E**), and PBS (**F**) are shown. **D-F**, H&E staining, original magnification $\times 200$.

may exert its effect on regulation of the inflammatory milieu in stomach by enhancing MMP-9 and CCL25 production.

Discussion

In view of the discovery of Th17 cells, we have re-examined the character of Th cell responses to *H. pylori* infection and found that

H. pylori infection mainly leads to specific Th17/Th1 immune responses. Th2 cell responses were only transiently induced in MLN. The results are consistent with previous reports that Th1 but not Th2 cell responses are induced in *H. pylori*-infected mice (3, 5). The increased production of IL-17 in the stomach is also consistent with previous studies (13-15). Furthermore, we found that the numbers of *H. pylori*-specific Th17 cells were increased during infection. *H. pylori* dramatically induced IL-6, TGF- β , IL-23, and IL-12 production in splenic macrophages. In addition, IL-23 and IL-12 expression in the stomach was also elevated. The secretion of these inflammatory mediators creates a cytokine milieu that facilitates the polarization of Th17/Th1 responses to *H. pylori*. We also detected Th cell responses in the MLN, spleen, and PLN, and the time course of immune responses induced in different lymphoid organs suggests that the MLN is an earlier priming site of the anti-*H. pylori* immune responses (25) than the spleen or PLN. Recently, it has been reported that the small intestine Peyer's patches are major induction sites of the *H. pylori*-induced immune responses (26). We speculate that *H. pylori*-specific T cells may be primed in Peyer's patches and migrate to MLN, then subsequently are trafficked to the gastric mucosa.

Previous studies have focused on Th1 and Th2 cell responses in *H. pylori* infection. It has been determined that Th1 cells contribute to inflammation and play a role in pathogenesis of *H. pylori* infection (6), but the role of Th17 cells has not been fully elucidated. Previous studies have shown that Th17 cells may play important roles in host protection against mucosal Gram-negative bacteria, such as *Klebsiella pneumoniae*, by secreting IL-17 (27). In addition to extracellular bacteria, IL-17 is also involved in host defense against *Mycoplasma pulmonis* (28) and *Pneumocystis carinii* (29). So it is reasonable to suppose that Th17 cells might also mediate mucosal host defense against *H. pylori* (30). Unexpectedly, we found that the *H. pylori* burden and inflammation were both reduced when IL-17 activity was blocked in vivo or IL-17 $^{-/-}$ mice were used. These findings are consistent with a very recent study on IL-17 $^{-/-}$ mice (31). The present results also showed that administration of AdmIL-17 increased host susceptibility to *H. pylori* infection. Our data from these complementary methods provide substantial evidence that the Th17/IL-17 pathway plays a pathogenic role in *H. pylori* infection by promoting mucosal inflammation and contributing to bacterial colonization. A number of reports also indicate that the Th17/IL-17 pathway

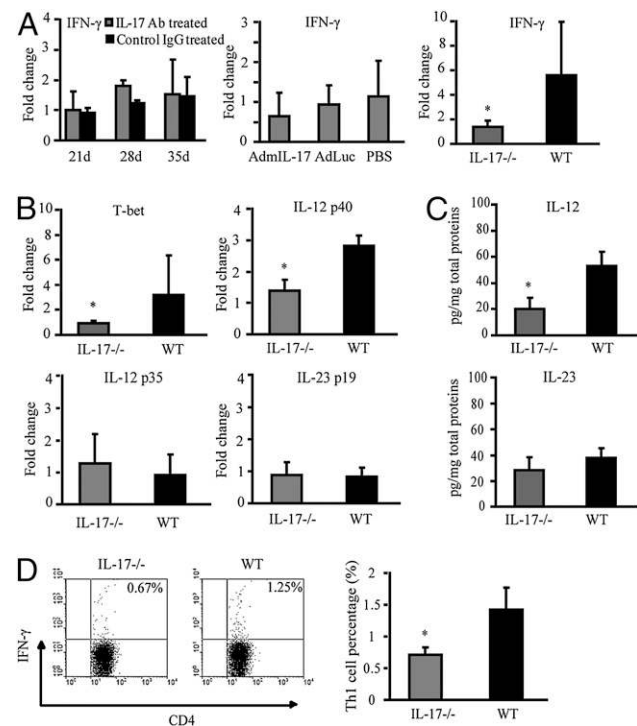


FIGURE 9. IL-17 $^{-/-}$ mice infected with *H. pylori* exhibit a decreased Th1 cell response. **A**, IFN- γ expression in *H. pylori*-infected gastric tissue was detected in mice pretreated with IL-17Ab at indicated time or AdmIL-17 on day 28 p.i. and in IL-17 $^{-/-}$ mice on day 28 p.i. **B-D**, IL-17 $^{-/-}$ and WT mice were infected with *H. pylori* for 28 d. **B**, The mRNA expression of T-bet, IL-12 p40, IL-12 p35, and IL-23 p19 in the gastric tissue was detected by real-time PCR. **C**, IL-12 and IL-23 production in the gastric tissue was detected by ELISA. **D**, Splenic lymphocytes were stimulated with *H. pylori* WCP, and IFN- γ -expressing Th cells were detected by intracellular staining. The cells were gated on CD4 cells. The results are expressed as mean \pm SD ($n = 5-6$). $*p < 0.05$ compared with WT mice.

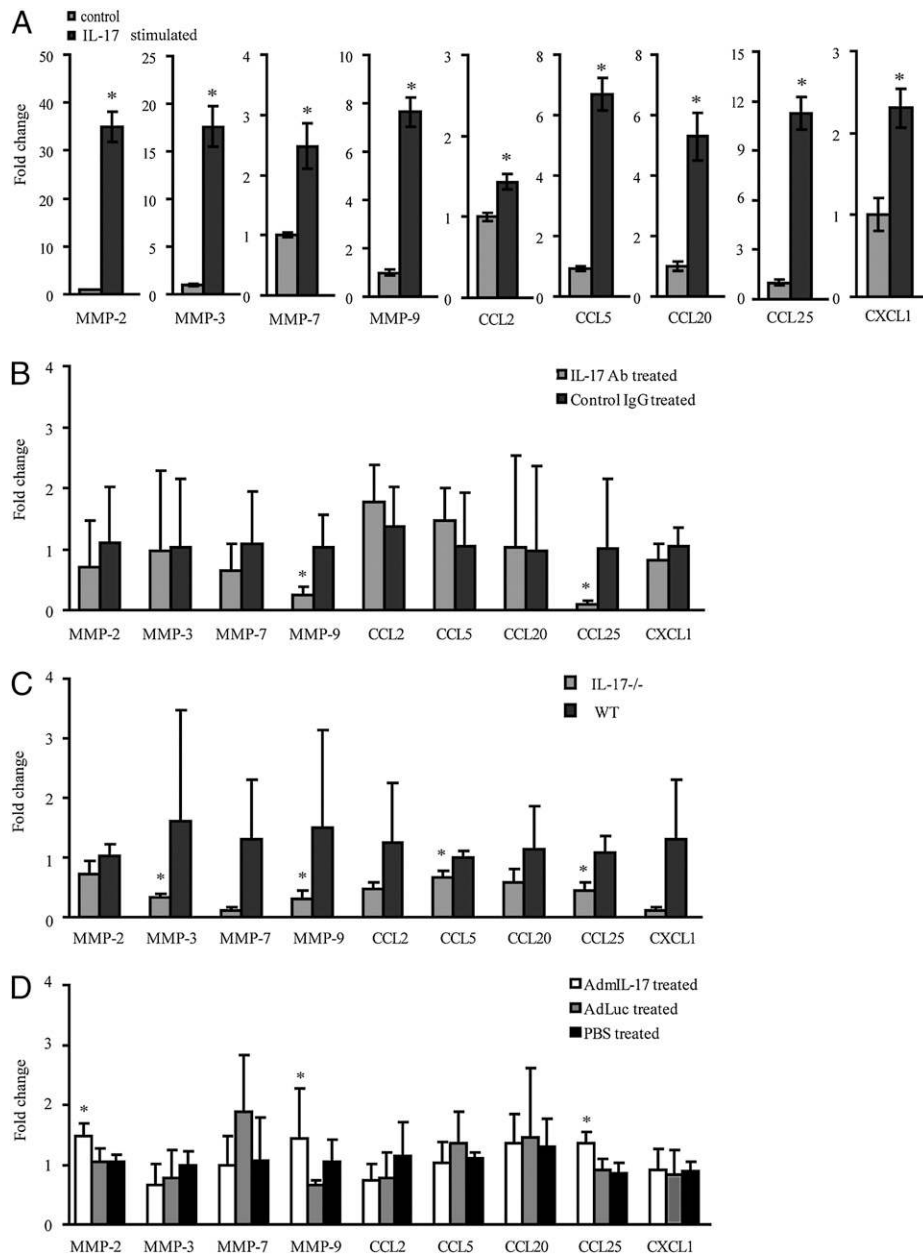


FIGURE 10. MMP and chemokine expression regulated by IL-17. *A*, MFC cells were stimulated with mIL-17 for 24 h. The expression of MMPs and chemokines was determined by real-time PCR. Unstimulated cells were used as a control. **p* < 0.05 compared with control; *n* = 3. *B*, Mice were treated as in Fig. 6, and the expression of MMPs and chemokines in the stomach was detected by real-time PCR. **p* < 0.05 compared with control IgG-treated mice. *C*, IL-17^{-/-} and WT mice were infected with *H. pylori*, and the expression of MMPs and chemokines in the stomach was detected by real-time PCR. **p* < 0.05 compared with WT mice. *D*, Mice were treated as in Fig. 8, and the expression of MMPs and chemokines in the stomach was detected by real-time PCR. **p* < 0.05 compared with AdLuc control.

promotes inflammation and susceptibility in mucosal *Candida albicans* and *Aspergillus fumigatus* infections (32) and that Th17 cells are involved in the immunopathology induced by *Schistosoma mansoni* (33, 34). Some reports also indicate that IL-17 is necessary for the development of intra-abdominal abscesses induced by *Bacteroides fragilis* (35) and shown to contribute to arthritis in vaccinated IFN- γ ^{-/-} mice challenged with *Borrelia burgdorferi* (36). Thus, the role of Th17 cells in infectious diseases remains an open question. They might exhibit a protective or a detrimental effect depending on the pathogen and the infection conditions (11). Infection with *K. pneumoniae* or *M. pulmonis* (28) may represent an acute inflammatory reaction in which Th17/IL-17 may exert a protective role in host defense by enhancing neutrophil re-

cruitment and activating macrophages. In contrast, mucosal infection with *H. pylori*, *Candida*, *S. mansoni*, or *B. burgdorferi* results in chronic and persistent infection, in which the Th17/IL-17 pathway possibly contributes to chronic inflammation and tissue damage and thus favors pathogen infection. The role of the Th17/IL-17 pathway in different infections therefore needs further investigation.

Thus, Th1 and Th17 cells both mediate mucosal inflammation in *H. pylori* infection. The dynamics of Th cell immune responses to *H. pylori* show that Th17 cell responses are induced earlier than Th1 cell responses, implying that Th17 and Th1 cells may promote inflammation at different stages. Currently, the interaction between Th17 and Th1 cells in *H. pylori* infection is not clear. In

this study, we found that Th1 cell responses to *H. pylori* were significantly reduced in IL-17^{-/-} mice but not significantly different in the IL-17 blockade and IL-17 overexpression models. The difference in results from the IL-17^{-/-} mice and IL-17 blocking model might be because IL-17 activity is not completely blocked when IL-17 Ab is used. Our results suggested that the Th17/IL-17 pathway modulates Th1 cell responses and Th17 and Th1 cells may act synergistically to induce gastritis during *H. pylori* infection.

IL-17 might stimulate epithelial cells and fibroblasts to release proinflammatory factors and chemokines (27), recruiting inflammatory cells that infiltrate the gastric tissue and induce gastritis. In support of this, we found that IL-17 stimulated gastric epithelial cells to produce MMPs and some chemokines. In addition, MMP-9 and CCL25 expression in the stomach following *H. pylori* infection were significantly reduced when IL-17 is deficient or blocked, whereas AdmIL-17 increased MMP-2, MMP-9, and CCL25 expression. CCL25 is a chemotactic factor for macrophages, activated monocytes, and dendritic cells and is linked to the homing of dendritic cells, T cells, and leukocytes. MMP expression is likely to contribute to tissue damage during *H. pylori*-associated gastritis and may accelerate an oncogenic progression by disrupting epithelial organization and increasing invasion (37). It has also been reported that MMP-9 enhances the host susceptibility to pulmonary infection with *Francisella tularensis* (38). We therefore deduce that the Th17/IL-17 pathway may lead to gastritis by triggering the recruitment of inflammatory cells including Th1 cells into the gastric mucosa by inducing chemokines and may destroy the tissue by inducing MMP production, favoring subsequent pathogen dissemination and persistent infection. However, other factors and mechanisms involved in the IL-17-mediated effect in *H. pylori* infection need further study.

Many studies have suggested an important role of *H. pylori*-induced chronic gastritis in the development of gastric cancer (2). Th17 cells have been reported to contribute to gastric cancer pathogenesis (39), implying that *H. pylori*-induced Th17 responses may also be involved in the pathogenesis of *H. pylori*-associated gastric cancer. The Th17/IL-17 pathway has been suggested as a target for therapeutic intervention in treatment of autoimmune and chronic inflammatory disorders associated with Th17 (40). Because *H. pylori* infection leads to chronic gastritis and persistent infection, Th17 cells and IL-17 may be potential targets for immunotherapy of *H. pylori*-related diseases. IL-17 antagonistic strategies may be an alternative for controlling the *H. pylori* load, because a neutralizing Ab reduces the load and exerts protective effects. In addition, modulation of Th17 differentiation using anti-IL-23 Ab may have potential in preventing *H. pylori*-induced diseases.

In conclusion, *H. pylori* infection mainly induces mixed Th17/Th1 cell responses. The induction of Th17/Th1 cell responses contributes to gastric inflammation and *H. pylori* colonization. Further study is required to elucidate the mechanisms by which *H. pylori* interacts with Th17 cells and how these cells facilitate infection. A better understanding of the nature, regulation, and function of Th cell responses to *H. pylori* may help to explore novel and effective immunotherapies for gastric diseases induced by this organism.

Acknowledgments

We thank Dr. Jay K. Kolls for kindly providing the adenovirus; Professor Zhongmin Zou (Third Military Medical University), Dr. Xue-jie Yu (University of Texas Medical Branch, Galveston, TX), and Dr. Weisan Chen (University of Melbourne, Melbourne, Australia) for helpful discussions and for polishing this manuscript; and Xin Li for technique assistance to perform flow cytometry.

Disclosures

The authors have no financial conflicts of interest.

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