Published in final edited form as:

Helicobacter. 2008 December ; 13(6): 518–524. doi:10.1111/j.1523-5378.2008.00629.x.

IL-17 is involved in *Helicobacter pylori*-Induced Gastric Inflammatory Responses in a Mouse Model

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Abstract

Background—*Helicobacter pylori* (*H. pylori*) is the major cause of chronic active gastritis and peptic ulcer disease. Recent studies have shown that *H. pylori* produces various cytokines that are related to neutrophil or mononuclear cell accumulation. Interleukin-17 (IL-17) is the founding member of an emerging family of inflammatory cytokines whose biological activities remain incompletely defined. In this study, the contributions of IL-17 to the induction of gastric inflammation and to the protection from *H. pylori* infection were investigated using IL-17 gene-knockout (IL-17^{-/-}) mice.

Materials and methods—IL-17^{-/-} and wild-type C57BL/6 mice were challenged with *H. pylori* CPY2052 (2×10^8 CFU/ml) and the histological and microbiological evaluation were carried out at specified times. IL-17 and myeloperoxidase (MPO) protein levels in tissues were assayed in duplicate using ELISA kits.

Results—In wild-type mice, IL-17 was undetected at baseline, however, the protein expression of IL-17 was induced after infection with *H. pylori*. A severe infiltration of neutrophils appeared in the submucosa and the lamina propria in wild-type mice. In contrast, the degree of neutrophil infiltration in IL-17^{-/-} mice was significantly lower than that in wild-type mice. Although wild-type mice infected with *H. pylori* showed drastically higher MPO activity compared with uninfected wild-type mice, any significant increase in the enzyme activity was not revealed in infected IL-17^{-/-} mice. The number of *H. pylori* colonized in the stomach of IL-17^{-/-} mice was significantly lower than that of wild-type mice from 1 to 6 months after infection.

Conclusions—These results suggest that IL-17 may play an important role in the inflammatory response to the *H. pylori* infection and ultimately influence the outcome of the *H. pylori* associated disease.

Keywords

Helicobacter; interleukin 17; inflammation; neutrophil; mice

Introduction

Helicobacter pylori (*H. pylori*), a microaerophilic, Gram-negative bacterium that selectively colonizes the human stomach, is the major cause of chronic active gastritis and peptic ulcer disease [1]. Recent studies have shown that *H. pylori* produces various cytokines that are related to neutrophil or mononuclear cell accumulation, such as interleukin-1 β (IL-1 β), interferon(IFN)- γ , IL-6, IL-8, tumor necrosis factor (TNF)- α and MIP-1 α [2-4]. We previously investigated the cytokine expression patterns of human gastric mucosal biopsy specimens by the reverse-transcription polymerase chain reaction method [5-7]. In particular, since the results showed that the expression of IL-8 mRNA was significantly higher in *H. pylori*-positive gastritis than in *H. pylori*-negative controls and there was a close correlation between the expression of IL-8 mRNA and the severity of gastritis, we suggested that IL-8 could play important roles in gastric mucosal inflammation [5].

IL-17 is the founding member of an emerging family of inflammatory cytokines whose biological activities remain incompletely defined [8]. IL-17 is a T cell-derived cytokine produced predominantly by the T memory compartment. IL-17 has pleiotrophic activities including the induction of proinflammatory cytokines such as TNF- α , IL-1 β , and IL-6, and chemokines like IL-8 and monocyte chemoattractant protein 1 on various cell types. In addition, IL-17 is involved in the induction of inducible nitric oxide synthase and cyclooxygenase 2 in chondrocytes, induction of prostaglandin E2-mediated osteoclast differentiation factor expression in osteoblasts, up-regulation of intracellular adhesion molecule 1 and HLA-DR expression in keratinocytes, promotion of stem cell factor and granulocyte-colony stimulating factor-mediated granulopoiesis, promotion of tumor rejection by natural killer cell activation, and enhancement of alloreactivity via promotion of dendritic cell maturation. IL-17 stimulates IL-8 release by gastric epithelial cells and facilitates the chemotaxis of neutrophils through an IL-8-dependent mechanism, and contributes to the enhancement of IL-8 levels in *H. pylori*-colonized gastric mucosa [9].

Recent studies reported that IL-17 stimulates IL-8 release by gastric epithelial cells and facilitates the chemotaxis of neutrophils through an IL-8-dependent mechanism, and contributes to the enhancement of IL-8 levels in *H. pylori*-colonized gastric mucosa.[10,11]

However, in vivo studies using IL-17 gene-knockout mice has not reported to investigate the role of IL-17 in *H. pylori* infection. In the present study, we designed a *H. pylori*-infected IL-17 gene-knockout mouse model and investigated the role of IL-17 in the development of gastric inflammation.

Methods

Animals

IL-17^{-/-} and wild-type C57BL/6 mice used in this study were specific pathogen-free 6-week-old males. The wild-type C57BL/6 mice were purchased from Charles River Japan (Atsugi, Japan). The IL-17^{-/-} were generated by a targeted mutation of the IL-17 gene as previously described [12]. Briefly, the targeting vector containing the disrupted IL-17 gene was transfected into A3-1 embryonic stem cells. These cells were injected into the blastocysts from superovulated C57BL/6 females, and then the embryos were transferred to the uteri of pseudopregnant ICR recipients. The male chimeras were crossed with C57BL/6 females,

and then embryonic stem cell-derived pups were backcrossed to C57BL/6 mice. The homozygous IL-17^{-/-} mice were produced by intercrossing the heterozygous mice. The mice were all maintained in a barriered animal facility under pathogen-free conditions at the Kyoto Prefectural University of Medicine. All equipments and supplies including cages, water bottles, wooden chips and food pellets were sterilized. Under these conditions, the IL-17^{-/-} mice and wild-type mice grew normally and healthily. All animal studies were approved by the Animal Care and Use Committee of Kyoto Prefectural University of Medicine.

Bacteria

The following *H. pylori* strains were used in this study. *H. pylori* CPY2052 was generously supplied by Dr. M. Karita (Houfu Onsen Hospital, Japan). KP142 strain was isolated from human gastric biopsy specimens at the Kyoto Prefectural University of Medicine. Both strains were Gram-negative spiral rods and were positive for *cagA* and *vacA* genes.

Preparations of bacterial inoculum were previously described [13]. Briefly, frozen CPY2052 and KP142 strains were thawed and streaked on Helicobacter-selective brain heart infusion (BHI) agar plates containing 7% (vol/vol) horse blood, vancomycin (10 mg/ml), polymyxin B (2.5 U/ml), trimethoprim (5mg/ml), and amphotericin B (2 mg/ml) (HSBHI agar plate) (Eiken Chemical Co., Tokyo, Japan), and incubated for 5 days at 37°C with an Aeropack System charged with a gas mixture consisting of 80% N₂, 15% CO₂, and 5% O₂ (Mitsubishi Gas Chemical Co., Tokyo, Japan). One milliliter of PBS was added to each incubated plate, and then the plate was rinsed. Two hundred microliters of the rinse solution was added to 5 ml of BHI broth containing 4% (vol/vol) fetal bovine serum (FBS) in a 20-ml test tube and cultivated for 24 h at 37°C under a gas mixture consisting of 80% N₂, 15% CO₂, and 5% O₂ on a reciprocal shaker (200 rpm). At this time the bacterial growth had reached the late logarithmic phase. After we confirmed that the bacterial concentration of this culture was 2.0×10^8 to 3.0×10^8 CFU/ml by measuring the optical density at 550 nm with a spectrophotometer (UV-120-01; Shimadzu Co., Kyoto, Japan), we used this culture as the inoculum preparation for the mouse infection experiments.

Mice were challenged only once at the beginning of the experiment, after 4-h fasting, with 0.2 ml of *H. pylori* (2×10^8 CFU/ml) by use of an oral stainless steel catheter. The mice were then maintained on the fast for 4 h and housed as described in the previous section. The colonization to gastric mucosa was assessed at specified times. Each experimental group was comprised of 5 to 10 mice.

Microbiological evaluation and identification of *H. pylori*

Mice were sacrificed after 4-h fasting and their stomachs were resected. The resected stomachs were divided longitudinally into two halves and weighed. One half of the resected stomach was added to 1 ml of PBS and homogenized using a tissue grinder (Pellet Pestle Mixer, Kimble/Kontes, USA). A 100 µl portion of the homogenized stomach was plated on an HSBHI agar plate and incubated for 5 days at 37 °C using an Aeropack System as described in the previous section. The colonies on the plate were counted and expressed as CFU/g tissue.

For the determination of whether the organisms of the colonies were *H. pylori*, a PCR was carried out with the DNA preparation of the colonies as previously described[14]. The primers for the Helicobacter genus were 5'- AGGGAATATTGCTCAATGGG-3' (sense) and 5'- TCGCCTTCGCAATGAGTATT-3' (antisense), and the primers for Hp1/2 gene were 5'- CTGGAGAGACTAAGCCCTCC -3' (sense) and 5'- ATTACTGACGCTGATTGTGC -3' (antisense). PCR was performed for 35 cycles, each

cycle consisted of 1 min at 95 °C for denaturation, 1 min at 50 °C for annealing and 1 min at 72 °C for extension. Ten µl of each PCR product was analyzed by electrophoresis on a 1.5% agarose gel containing ethidium bromide, and the bands were visualized under UV light.

Histological evaluation

The other half of each resected stomach was used for the histological evaluation. Longitudinal sections from the gastroesophageal junction to the gastroduodenal junction were fixed in neutral buffered 10% formalin, embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin. The degrees of inflammation were assessed by microscopic observation without knowledge of the experimental groups and was expressed as follows: none (score 0), normal appearance of scattered inflammatory cells on the lamina propria (the same degree as in uninfected control mice); mild (score 1), moderate infiltration of inflammatory cells in the lamina propria and the submucosa and no erosions in the epithelium; moderate (score 2), moderate infiltration of inflammatory cells in the lamina propria and the submucosa and erosions in some parts of the epithelium; and severe (score 3), severe infiltration of inflammatory cells in the lamina propria and the submucosa and erosions in many parts of the epithelium. The numbers of neutrophils and mononuclear cells infiltrating the lamina propria were counted in five high-power fields ($\times 200$ magnification). Density of the mononuclear cells and polymorphonuclear leukocytes in the lamina propria or submucosal layer was also scored using updated Sydney system[15].

IL-17 and myeloperoxidase assay

The homogenized stomach was centrifuged at 15,000 rpm for 10 min at 4 °C to precipitate the insoluble cellular debris, and supernatant was stored at -80 °C until used. IL-17 and myeloperoxidase (MPO) protein levels in supernatants were assayed in duplicate using ELISA kits specific for IL-17 (R&D Systems, Minneapolis, MN, USA) and MPO (HyCult biotechnology, PB Uden, The Netherlands) according to the manufacturer's instructions.

Statistics

Colonization and inflammation score were expressed as means and standard errors. Sample means were compared by the Mann-Whitney U test. A p value of <0.05 was considered to be significant.

Results

Induction of IL-17 in stomachs of mice after infection with *H. pylori*

To determine whether IL-17 is induced as a response cytokine after oral challenge with *H. pylori* CPY2052, IL-17 protein levels were measured in the gastric mucosa of wild-type and IL-17^{-/-} mice. In wild-type mice, gastric mucosal IL-17 was undetected at baseline, however, the IL-17 levels were significantly induced at 2 months after infection with *H. pylori*. IL-17 levels were gradually increased up to 12 months after infection (Fig. 1). On the other hand, IL-17 levels in IL-17^{-/-} mice were undetectable irrespective of the infection.

Histology of the gastric mucosa of wild-type and IL-17^{-/-} mice after infection

To investigate the role of IL-17 in the gastric inflammation induced by *H. pylori* infection, the histological changes were examined in the gastric mucosa of mice infected with *H. pylori* CPY2052. In wild-type mice, a moderate infiltration of neutrophils and mononuclear cells was observed in the submucosa and the lamina propria 2 months after infection. Twelve months after infection, a severe infiltration of neutrophils appeared in the submucosa and the lamina propria (Fig.2A and 2B). In contrast, in the CPY2052-infected IL-17^{-/-} mice, we observed a very weak inflammatory findings even 12 months after

infection (Fig. 2C and 2D). To confirm that IL-17 is involved in *H. pylori* induced an inflammatory response, we carried out the same infection study with KP142 strain of *H. pylori*. As shown in Table 1, the gastric inflammation of IL-17^{-/-} mice at 1 and 6 months after infection with KP142 was significantly suppressed as compared with wild-type mice.

To determine which types of cells were infiltrated in inflammation site in *H. pylori*-infected gastric mucosa, we investigated the scores of neutrophils and mononuclear cells in the body and the antrum. As shown in Fig. 3, the infiltration of neutrophils was observed in both the body and the antrum in wild-type and IL-17^{-/-} mice. However, the degree of neutrophil infiltration in IL-17^{-/-} mice was significantly lower than that in wild-type mice.

The infiltration of mononuclear cells was also observed in the body and the antrum in wild-type and IL-17^{-/-} mice (Fig. 4). Interestingly however, there were no significant differences in the mononuclear cell infiltration score between wild-type mice and IL-17^{-/-} mice.

MPO activity in stomachs of wild type mice and IL-17^{-/-} mice

Because the expression of MPO has been commonly used for quantification of neutrophils in the gastric mucosa [15], the gastric mucosa of mice was homogenized and MPO activity in the supernatant was measured. As shown in Figure 5, wild-type mice infected with *H. pylori* showed drastically higher MPO activity compared with uninfected wild-type mice, consistent with the severe stomach inflammation in these animals. In contrast, any significant increase in the enzyme activity was not revealed in infected IL-17^{-/-} mice, confirming that the genetically modified animals do not develop gastritis. The data were consistent with quantitative scoring of neutrophils on representative stomach sections.

Colonization of *H. pylori* in wild-type and IL-17^{-/-} mice

To examine whether IL-17 are involved in protection against *H. pylori* infection, the number of colonized *H. pylori* in the gastric mucosa was determined at 1, 4 and 6 months after infection (Fig. 6). The number of *H. pylori* in IL-17^{-/-} mice was significantly lower than that in wild-type mice from 1 to 6 months after infection. These results suggest that IL-17 may not play a protective role against *H. pylori* infection.

Discussion

It is well known that IL-17 plays a key role in the pathogenesis of a number of chronic inflammatory diseases. For example, bioactive IL-17 has been detected in rheumatoid arthritis (RA) and osteoarthritis synovial fluids [16-18]. Indeed, IL-17 appears to play a causative role in arthritis, as blocking TNF- α and IL-17 in a mouse model of RA is more effective in controlling synovial inflammation and bone resorption than blocking TNF- α alone [19]. In addition, collagen-induced arthritis is suppressed in IL-17-deficient mice [20]. Furthermore, IL-17 plays a role in inflammatory pulmonary diseases that are mediated by the recruitment of neutrophils. Specifically, IL-17 levels are increased in the lungs of patients with asthma and in airways inflamed as a result of exposure to organic dust [21,22]. In this study, we confirmed that gastric mucosal IL-17 levels were increased in mice after *H. pylori* infection.

Several lines of evidence implicate IL-17 in host immunity to infection. Elevated levels of IL-17 have been found in the mucosa of *H. pylori*-infected patients [10]. In addition, IL-17 and IL-8 activities in organ cultures of biopsy specimens from the antrum are substantially higher in *H. pylori*-positive patients than in *H. pylori*-negative patients, and that among *H. pylori*-infected gastric ulcer patients, the gastric mucosa at the ulcer sites contains significantly more IL-17 and IL-8 than the antral mucosa [9,23].

The most convincing evidence of a role for IL-17 in host defense comes from studies in IL-17 receptor-deficient (IL-17R^{-/-}) mice. These mice are highly sensitive to intranasal *Klebsiella pneumoniae* infection, showing enhanced mortality in comparison with wild-type controls [24,25]. One mechanism of this mortality is a significant delay in neutrophil recruitment, as a result, in part, of a reduction of granulocyte-colony stimulating factor (G-CSF) and MIP-2. In the present study, we showed that the inflammation scores and the number of colonized bacteria in the gastric mucosa of IL-17^{-/-} mice were significantly decreased in comparison with wild-type mice. These results suggest that IL-17 may be a key mediator in inflammation and host defense against bacterial organisms. Proposed models suggest that during infection, bacterial products directly induce a subset of T cells to secrete IL-17, which then acts on the microenvironment of infected tissues to release secondary mediators such as chemokines and G-CSF, which in turn activate and recruit neutrophils [26]. In addition, IL-17 works in conjunction with many innate immune system cytokines such as TNF- α and IL-1 β to amplify the immune response and the inflammatory process. However, the full spectrum of molecular mechanisms that controls inflammation and neutrophil recruitment and activation remains incompletely defined.

Furthermore, IL-17R^{-/-} mice display less severe tissue damage in response to *T. gondii* infection [27], and the mIL-17A/mIL-17AR system is required for normal fungal host defense in vivo [28]. It is reported that IL-17A could have potential as a therapeutic cytokine for systemic *C. albicans* infections in immunocompromised patients with cancer or advanced acquired immunodeficiency syndrome.

IL-17 is an active recruiter of neutrophils to inflammatory sites [25,29,30] and IFN- γ regulates the induction of Th17 cells [13,31]. These facts may explain the damaging inflammatory response seen during mycobacterial infection of IFN- γ -deficient mice [32] in that IFN- γ and IL-17 may counterregulate each other during chronic mycobacterial infection. Cruz et al. reported that an IL-17-producing CD4 T cell population can be generated by mycobacterially activated APC and that IFN- γ regulates the generation of these cells. Their data lend further support to the hypothesis that IFN- γ is required to control IL-17-producing cells and thus the severe pathology that is seen in IFN- γ -deficient mice with experimental autoimmune encephalitis and collagen-induced arthritis or mycobacterial infection [14,32-35]. However, our previous study showed that *H. pylori*-induced gastritis was suppressed in IFN- γ -deficient mice [3]. Further studies are necessary to test whether counterregulation between IFN- γ and IL-17 occurs during *H. pylori* infection.

In conclusion, we report an increased expression of T cell-derived IL-17 in the gastric mucosa of mice and an impaired mucosal neutrophil infiltration in IL-17^{-/-} mice after *H. pylori* infection, suggesting that this cytokine may play an important role in the inflammatory response to the *H. pylori* colonization and ultimately influence the outcome of the *H. pylori*-associated disease.

Acknowledgments

This work was supported by Grant-in-Aid for Scientific Research(C) 19590696 from Japan Society for the Promotion of Science (JSPS) and also supported in part by National Institutes of Health grant through DK 62813 (to Y.Y). The authors have no competing interests.

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Fig. 1.

Induction of IL-17 in the gastric mucosa of mice after infection with *H. pylori*. Wild-type and IL-17^{-/-} mice were challenged with 0.2 ml of *H. pylori* CPY2052 (2×10^8 CFU/ml) by use of an oral stainless steel catheter. Mice were sacrificed and their stomachs were resected. Levels of IL-17 in stomachs were measured by ELISA. Data are presented as means and standard deviations of 5 mice. * $p < 0.05$, ** $p < 0.01$

Fig. 2. Histology of the stomachs of wild-type and IL-17^{-/-} mice at 12 months after *H. pylori* infection. In wild-type mice (A and B), a severe infiltration of neutrophils and mononuclear cells appeared in the submucosa and the lamina propria. In contrast, the degree of neutrophil infiltration in IL-17^{-/-} mice was significantly lower than that in wild-type mice (C and D). Hematoxylin-Eosin stain: original magnification $\times 100$ (A and C), $\times 200$ (B and D).

Fig. 3.

Infiltration of neutrophils in the body (A) and the antrum (B). The numbers of neutrophils infiltrating the lamina propria were counted in five high-power fields ($\times 200$ magnification). Density of polymorphonuclear leukocytes in the lamina propria or submucosal layer was scored as 0 (none), 1 (mild), 2 (moderate) and 3 (marked) in a manner analogous to the updated Sydney system. Data are presented as means and standard deviations of 5 mice. * $p < 0.05$

Fig. 4. Infiltration of mononuclear cells in the body (A) and the antrum (B). The numbers of mononuclear cells infiltrating the lamina propria were counted in five high-power fields ($\times 200$ magnification). Density of mononuclear cells in the lamina propria or submucosal layer was scored as 0 (none), 1 (mild), 2 (moderate) and 3 (marked) in a manner analogous to the updated Sydney system. Data are presented as means and standard deviations of 5 mice. NS, not significant.

Fig. 5. MPO activity in stomachs of wild-type and IL-17^{-/-} mice. The stomach of mice at 6 months after infection with CPY2052 was homogenized and MPO activity in the supernatant was measured by ELISA. Data are presented as means and standard deviations of 5 mice. * p<0.05

Fig. 6. Colonization of *H. pylori* in wild-type and IL-17^{-/-} mice. Mice were orally infected with *H. pylori* CPY2052, and the number of colonized bacteria in the stomach was determined at 1, 4 and 6 months after infection. Data are presented as means and standard deviations of 5 mice. * p<0.05

Table 1

Histopathological inflammation score in wild-type and IL-17^{-/-} mice after infection with CPY2052 and KP142 strains of *H. pylori*

Strain	Mice	0M		Time after infection (months)			
CPY2052	Wild-type	0.0 ± 0.0		1M		6M	
	IL-17 ^{-/-}	0.0 ± 0.0	N.S	0.7 ± 0.2		1.1 ± 0.1	
KP142	Wild-type	0.0 ± 0.0		0.1 ± 0.1	p<0.005	0.2 ± 0.1	p<0.001
	IL-17 ^{-/-}	0.0 ± 0.0	N.S	0.8 ± 0.1		1.2 ± 0.1	
		0.0 ± 0.0		0.1 ± 0.1	p<0.005	0.2 ± 0.1	p<0.001

Data were expressed as means ± S.D. (n=10)