Evaluation of Serum Interleukin-18 levels in *Helicobacter Pylori*-infected Peptic Ulcer Patients and its Association with Bacterial CagA Virulence Factor

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ABSTRACT

Background: *Helicobacter pylori* infection is one of the most common gastrointestinal infections worldwide. Predominant T-helper 1 (Th1) responses with increased gamma interferon (IFN- γ) levels have been proposed to play an important role in *H. pylori*-induced peptic ulcer. However, bacterial factors contributing to the initiation of Th1 polarization of *H. pylori*-specific immune responses have not been characterized. **Objective:** Comparing serum concentrations of IL-18 in *H. pylori*-infected peptic ulcer (PU) patients, *H. pylori*-infected asymptomatic (AS) carriers and healthy control group and its association with bacterial virulence factor CagA. **Methods:** Thirty *H. pylori*-infected PU patients (20 patients were positive for anti-CagA antibody and 10 patients were negative for anti-CagA antibody), 30 *H. pylori*-infected (AS) carriers (15 subjects with positive test for anti-CagA antibody and 15 subjects with negative test for anti-CagA antibody) and 20 healthy uninfected subjects were included in this study. Serum concentration of IL-18 was measured by ELISA method. **Results:** The mean serum levels of IL-18 in PU patients (333.2 pg/ml ± 158), was significantly higher than those found in AS (146.5 pg/ml ± 90.1; $P<0.001$) and healthy control (82.2 pg/ml ± 45.7; $P<0.0001$). In both PU and AS groups, mean serum IL-18 levels in subjects with positive test for anti-CagA antibody were significantly higher than those observed in subjects with negative test for anti-CagA antibody. No significant difference was observed between serum IL-18 levels of healthy uninfected control and AS carriers with negative test for anti-CagA antibody. **Conclusion:** The results of the present study showed higher serum concentrations of IL-18 in peptic ulcer patients compared with H.Pylori carriers and healthy controls. This difference in cytokine levels may be explained by differential expression of H.Pylori CagA gene during the course of the infection.

Keywords: Helicobacter pylori, Peptic Ulcer, Interleukin-18

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INTRODUCTION

H. pylori is one of the most common chronic bacterial infections in humans. It occurs throughout the world and has a major etiologic role for several gastroduoedenal diseases, including gastric ulcer, duodenal ulcer, gastric MALT lymphoma, and distal gastric cancer (1). In 1994, the World Health Organization has categorized *H. pylori* as a group I carcinogen (2). Despite significant advances in understanding the biology of *H. pylori*, factors that determine the outcome of infection are still poorly understood. The host immune response to *H. pylori* infection might be important with regard to the outcome of infection by this organism, e.g., to explain why only a proportion of infected subjects develop peptic ulcers. In addition to the host factors, bacterial factors seem to influence the inflammatory response and the development of a more severe pathology. Bacterial virulence factors such as cytotoxin-associated protein (CagA) and vacuolating cytotoxin (VacA) are associated with enhanced inflammation and cancer development (3, 4). In recent years, accumulating evidence shows that host's cellular immune response is an important determinant of the outcome of infection. The T helper cell response towards *H. pylori* is generally considered to have Th1 phenotype leading to a cell-mediated immune response (5). There is increasing evidence that the *H. pylori* induced Th1 response also contributes to gastric cancer development (6). Down-regulation of the Th1 response in mice was shown to protect against the development of *H. pylori*-induced pathogenesis (7, 8). However, factors influencing the extent of the *H. pylori* induced Th1 response are currently unknown. Important cytokines characterizing Th1 mediated immune responses are IFN-γ, TNF-α and IL-1, all of which become up-regulated during chronic *H. pylori* infection (9-11). The cytokine interleukin-18 (IL-18) a recently identified cytokine (originally termed IFN-γ inducing factor) induces production of IFN-γ by activated T lymphocytes and promotes a Th1 profile (12). In this study we analyzed serum IL-18 levels in *H. pylori*-infected peptic ulcer (PU) patients, asymptomatic carriers (AS) as well as healthy, uninfected subjects, to evaluate whether there is any association between increased levels of this cytokine and peptic ulcer disease and to determine whether IL-18 levels differ in subjects infected with *Cag*-positive and *Cag*-negative *H. pylori* strains.

SUBJECTS AND METHODS

Subjects. We conducted a descriptive study for determination of serum concentrations of IL-18 in *H. pylori*-infected PU patients, *H. pylori*-infected AS carriers and healthy control group and its association with bacterial factor CagA. Thirty *H. pylori*-infected PU patients (mean age, 40.5 years; range from 18 to 65 years; 17 men and 13 women), 30 *H. pylori*-infected asymptomatic carriers (AS) (mean age, 38.6 years; range from 18 to 60 years; 18 men and 12 women) were included in our study. The AS carriers were positive by serological tests for *H. pylori*-specific antibodies are described below. For comparison, 20 healthy, uninfected (*H. pylori*-negative) volunteers (mean age, 40 years; range from 18 to 60 years; 11 men and 9 women) were included in our study. The AS carriers as well as the uninfected healthy subjects were chosen from blood donors and interviewed with regard to gastrointestinal symptoms (e.g., dyspepsia), and none of them had any history of gas-

trointestinal or any other relevant disease. AS and healthy controls did not undergo endoscopy, were basically healthy with no acute or chronic illnesses. Indeed individuals with a history of chronic or acute disease and history of any drug were excluded from the study. Endoscopy confirmed PU in the patients and none of the subjects were on any medications including nonsteroidal anti-inflammatory drugs at the beginning of the study. *H. pylori* infection was confirmed by biopsy-based tests (rapid biopsy urease test) and presence of serum anti-*H. pylori* IgG and IgA antibodies using commercial enzyme-linked immunosorbent assay (ELISA) kits. Blood samples were obtained from the participants and the sera were separated and stored at –20°C until analysed.

Determination of *H. pylori*-specific Antibodies in Serum. The levels of anti-*H. pylori* immunoglobulin A and G were measured using the commercial enzyme-linked immunosorbent assay (Equipar, Italy) according to the manufacturer's guidelines. The sensitivity and specificity of assay were > 99%. Serum anti-CagA IgG antibody levels were also assayed by the ELISA method using a commercial kit (Diagnostic Bioprobes, Italy).

Cytokine Assay. The serum concentrations of IL-18 were quantified by sandwich ELISA using a commercial kit (Bender Medsystems, Austria). The analyses were performed in duplicates and according to the manufacturer's procedure. The detection limit of the ELISA kit for IL-18 was 4 pg/ml.

Statistical Analysis. Data were expressed as mean ± SD. Differences in variables were analyzed using Mann-Whitney U-test for comparison of two groups. For comparison of more than two groups, the Kruskal-Wallis (for skewed variance) and ANOVA (for the homogeneity of variance) tests were used. The p-values less than 0.05 were considered significant.

RESULTS

Serum concentrations of IL-18 in PU patients, AS carriers and the healthy controls are summarized in figures 1-2. Statistical analysis of serum IL-18 concentration showed that there were significant differences among PU patients (333.2 pg/ml ± 158), AS carriers (146.5 pg/ml ± 90.1) and the healthy control (82.2 pg/ml ± 45.7; $p<0.00001$). The mean serum IL-18 levels in PU patients was significantly higher compared to the level found in the uninfected control ($P<0.0001$). Moreover, when the serum IL-18 levels in PU patients and AS group were compared, significantly higher levels of IL-18 were found in the PU patients ($P<0.001$). Furthermore, when the serum IL-18 levels for AS carriers and healthy uninfected subjects were compared, the level of IL-18 was significantly higher in AS carriers in comparison to healthy subjects ($P<0.01$).

Comparison of serum IL-18 levels within each group and between groups according to anti-CagA status is summarized in table 1. According to anti-CagA status, the PU patients were subdivided into two groups; $CagA^+$ and $CagA^-$. Our results showed that the mean serum IL-18 level in patients with positive test for anti-CagA antibody (358 pg/ml ± 206) was significantly higher than those observed in other groups, except the PU patients with negative test for anti-CagA antibody (Table 1, Figure 1). Similarly, the mean serum IL-18 level in PU group without anti-CagA antibody (283.6 pg/ml ± 128.9) was significantly higher than those observed in AS group with anti-CagA

antibody (180.5 pg/ml ± 104.2; p<0.03), AS group without anti-CagA antibody (112.4 pg/ml ± 58.8; P<0.002) and control group (82.2 pg/ml ± 45.7; p<0.0001). Moreover, in AS carriers it was found that mean serum IL-18 levels in subjects with positive anti-CagA antibody (180.5 pg/ml ± 104.2) was significantly higher than that observed in AS carriers with negative anti-CagA antibody (112.4 pg/ml ± 58.8; p<0.01). However, no significant difference was observed for mean serum IL-18 levels between healthy uninfected control and AS carriers with negative anti-CagA antibody (Table 1).

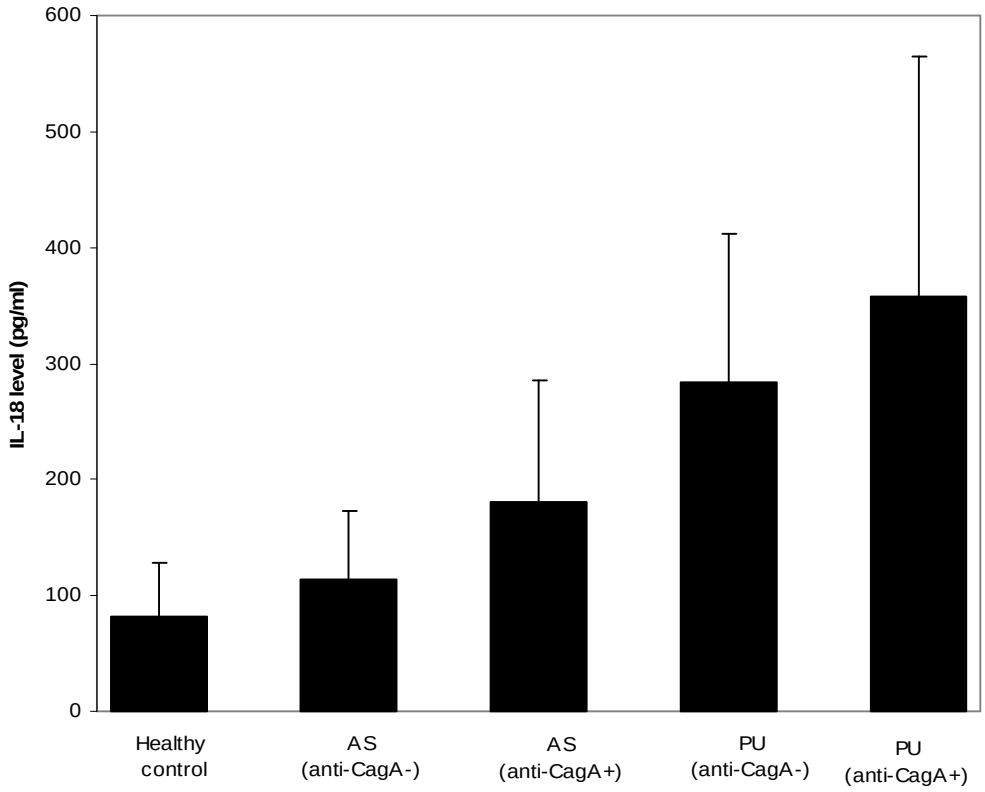

Figure 1. Serum IL-18 levels in uninfected healthy control, H.pylori- infected AS and PU groups with or without anti-CagA antibody. The mean serum IL-18 level in PU group with anti-CagA (358 pg/ml ± 206) was significantly higher than those observed in AS group with anti-CagA (180.5 pg/ml ± 104.2; p<0.008), AS group without anti-CagA (112.4 pg/ml ± 58.8; P<0.00005) and control group (82.2 pg/ml ± 45.7; p<0.000001).Similarly, The mean serum IL-18 level in PU group without anti-CagA (283.6 pg/ml ± 128.9) was significantly higher than those observed in AS group with or without anti-CagA and control group.

Table 1. Statistical comparison of serum IL-18 levels between PU, AS and uninfected control groups according to anti-CagA status

Groups	PU (anti-CagA$^+$)	PU (anti-CagA$^-$)	AS (anti-CagA$^+$)	AS (anti-CagA$^-$)
Uninfected control	0.000001*	0.0001	0.0007	0.09
AS (anti-CagA$^-$)	0.00005	0.002	0.01	-
AS (anti-CagA$^+$)	0.008	0.03	-	0.01
PU (anti-CagA$^-$)	0.36	-	0.03	0.002

* The symbol represents p-values.

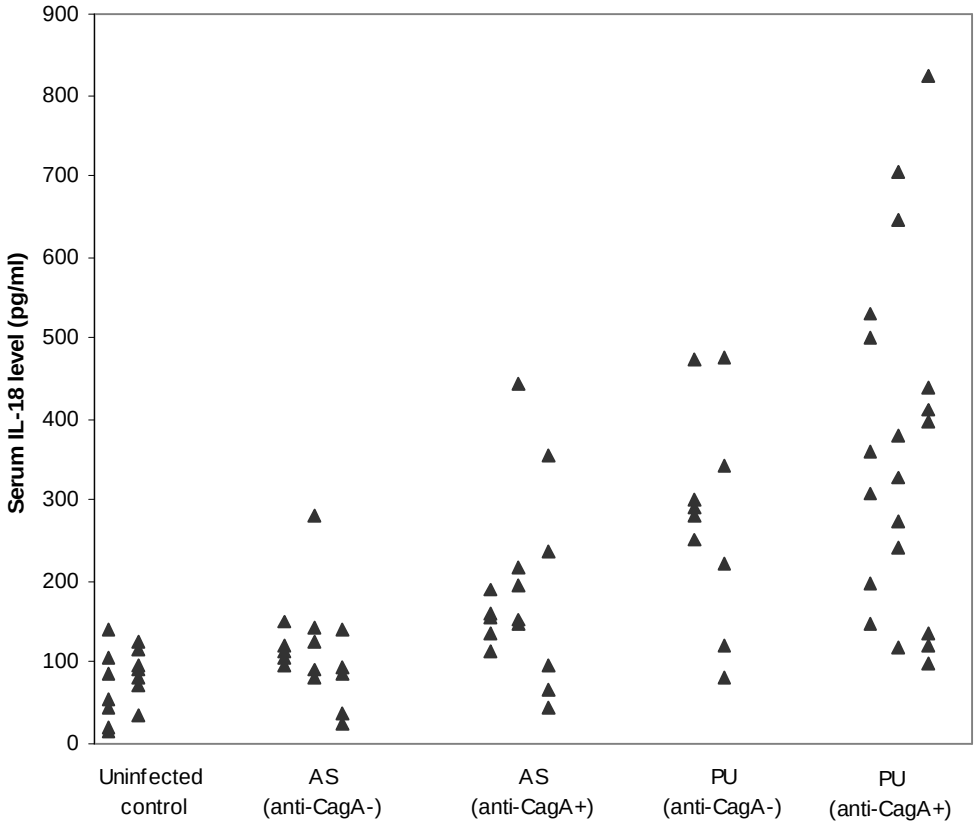

Figure 2. Distribution of serum IL-18 levels in healthy control, AS subjects and PU patients. Higher levels of serum IL-18 were observed in anti-CagA^{+} compared to anti-CagA^{-} subjects showing that CagA influences the release of IL-18.

DISCUSSION

Previous studies have shown that IL-18 increases in different inflammatory conditions, such as Crohn's disease (13), rheumatoid arthritis (14), and tuberculoid leprosy (12). Our results demonstrated increased serum IL-18 levels in *H. pylori*–infected PU patients. Moreover, the level of serum IL-18 was markedly elevated in subjects infected with *cagA*-positive *H. pylori* strains compared to those infected with *cagA*-negative strains. Studies in both humans (15) and primates (16) have demonstrated that the T cell response to *H. pylori* has a Th1 profile. Like humans infected with *H. pylori*, mice infected with *H. felis* respond to infection with infiltration of Th1 lymphocytes into the gastric mucosa and spleen. The promotion of cell-mediated responses in the gastric mucosa is likely to be an important contributor to tissue injury. Furthermore, the importance of T cell responses in the generation of *Helicobacter*-induced gastric pathology has been recently demonstrated in T cell–deficient RAG-1 $^{-/-}$ mice (17).

H. pylori has been shown to induce a strong cytokine response in both human gastric epithelial cells (18) and gastric epithelial cell lines (19). Chronic infection with *H. pylori* is associated with gastric IFN-γ–producing T cells (15) and increased mucosal IL-12, indicating a predominant Th1 response (20). IL-12 mRNA levels are increased in infection with *cag*-positive *H. pylori* (21). This results in up-regulation of the ex-

pression of IL-18 receptors in both Th1 and NK cells (22). It seems that in chronic *H. pylori* infection, mucosal production of IL-18, together with IL-12, would be important in promoting Th1 responses and IFN-γ secretion. Moreover, it has been demonstrated that Th cells respond to *H. pylori* antigen by secreting high levels of IFN-γ and undetectable levels of IL-4. Furthermore, IFN-γ-deficient knockout mice, when infected with *H. felis*, fail to develop any gastritis (5,23), while IL-10 knockout mice develop gastritis which is much more severe than that of wild-type mice (24). Finally, splenic lymphocytes from nonresponder mouse strains that do not develop gastritis, express IL-10, but not IFN-γ in response to *H. pylori* Ag (25). These data suggest that Th1-based cellular immune responses largely determine the outcome of gastric *Helicobacter* infection.

The immunoregulatory and proinflammatory cytokines induced by *H. pylori* may influence the nature of the local T-cell response. The local Th cell response in *H. pylori* infection is generally considered to be Th_1 response since the levels of IFN-γ, but not IL-4 and IL-5, have been shown to increase in *H. pylori*-induced gastritis (26).

In this study higher serum concentrations of IL-18 were found in peptic ulcer patients. In other studies it has been reported that the serum IL-18 level was significantly increased in patients with gastric carcinoma, which is frequently found to be associated with *H. pylori* infection (27). It has been demonstrated that in *H. pylori*-infected individuals, IL-18 mRNA expression and IL-18 production were increased in the gastric mucosa (28,29). It seems 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, whereas over-expression 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 (30). As a co-regulator, together with IL-12, 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 (31). It has been shown that IFN-γ could affect the local expression of cytokines and chemokines/chemokine receptors, or it could up-regulate MHC class II, co-stimulatory or other membrane molecules that could facilitate *H. pylori*-specific T cell priming and activation (32, 33). Furthermore, IL-18 could also directly augment IL-1, IL-6, and TNF cytokine production from macrophages that would promote gastritis and reduce bacterial counts (34). The functional role of IL-18 in Helicobacter-induced gastric inflammation and the contribution of different cell populations to produce IL-18 require further investigation. *Helicobacter* infection of IL-18–deficient mice could determine the in vivo role of IL-18 in gastric immune responses.

Some bacterial factors may influence cytokine production. The cytotoxin-associated protein (CagA) is one of the most extensively studied virulence factors of *H. pylori* and has been associated with cytokine expression (21). CagA is thought to be the major *H. pylori* virulence factor involved in the pathogenesis of *H. pylori* disease produced by only a subset of *H. pylori* strains, defined as *H. pylori* type I. Bacterial strains which do not express CagA are termed *H. pylori* type II. *H. pylori* strains carrying CagA are more commonly isolated from PU patients and infection with these strains has been found to be correlated with increased IL-18 production both in vivo and in vitro (21,35). Accordingly, considerably higher levels of serum cytokine observed in PU patients and AS subjects could also be explained by differences in the infecting bacterial strains. It seems that the differences in cytokine expression levels observed in our study could be partly explained by differences in CagA expression. Thus IL-18 response was dependent on CagA status of infecting strains. These find-

ings indicate that serum IL-18 levels are positively influenced by *H. pylori*, particularly by CagA-positive strains. However, since a proportion of AS carriers and PU patients are CagA negative, various host genetic factors may also be important for the development of PU (36).

In conclusion, the present study indicates that serum IL-18 levels are increased in *H. pylori* infection. CagA seems to represent a bacterial virulence factor influencing the release of IL-18 that is necessary for the initiation of the inflammatory and the Th1 responses.

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REFERENCES

1. Siavoshi F, Malekzadeh R, Daneshmand M, Ashktorab H, Helicobacter pylori endemic and gastric disease. Dig Dis Sci. 2005;50:2075-80.
2. Bourke B. Will treatment of Helicobacter pylori infection in childhood alter the risk of developing gastric cancer? Can J Gastroenterol. 2005;19:409-11.
3. Figueiredo C, Machado JC, Yamaoka Y. Pathogenesis of Helicobacter pylori Infection. Helicobacter. 2005;10:14-20.
4. Lu H, Yamaoka Y, Graham DY. Helicobacter pylori virulence factors: facts and fantasies. Curr Opin Gastroenterol. 2005;21:653-9.
5. Smythies LE, Waites KB, Lindsey JR, Harris PR, Ghiara P, Smith PD. Helicobacter pylori-induced mucosal inflammation is Th1 mediated and exacerbated in IL-4, but not IFN-gamma, gene-deficient mice. J Immunol 2000;165:1022-9.
6. Ernst P. Review article: the role of inflammation in the pathogenesis of gastric cancer. Aliment Pharmacol Ther. 1999;13:13-8.
7. Fox JG, Beck P, Dangler CA, Whary MT, Wang TC, Shi HN et al Concurrent enteric helminth infection modulates inflammation and gastric immune responses and reduces helicobacter-induced gastric atrophy. Nat Med. 2000;6:536-42.
8. Fox JG, Sheppard BJ, Dangler CA, Whary MT, Ihrig M, Wang TC. Germ-line p53-targeted disruption inhibits helicobacter-induced premalignant lesions and invasive gastric carcinoma through down-regulation of Th1 proinflammatory responses. Cancer Res. 2002;62:696-702.
9. D'Elios MM, Amedei A, Benagiano M, Azzurri A, Del Prete G. Helicobacter pylori, T cells and cytokines: the "dangerous liaisons". FEMS Immunol Med Microbiol. 2005;44:113-9.
10. Yamaoka Y, Kita M, Kodama T, Sawai N, Kashima K, Imanishi J. Induction of various cytokines and development of severe mucosal inflammation by cagA gene positive Helicobacter pylori strains. Gut. 1997;41:442-51.
11. Rad R, Gerhard M, Lang R, Schoniger M, Rosch T, Schepp W et al. The Helicobacter pylori blood group antigen-binding adhesin facilitates bacterial colonization and augments a nonspecific immune response. J Immunol. 2002;168:3033-41.
12. Garcia VE, Uyemura K, Sieling PA, Ochoa MT, Morita CT, Okamura H et al. IL-18 promotes type 1 cytokine production from NK cells and T cells in human intracellular infection. J Immunol. 1999;162:6114-21.
13. van Den Brink GR, ten Kate FJ, Ponsioen CY, Rive MM, Tytgat GN, van Deventer SJ et al. Expression and activation of NF-kappa B in the antrum of the human stomach. J Immunol. 2000;164:3353-9.
14. Gracie JA, Forsey RJ, Chan WL, Gilmour A, Leung BP, Greer MR et al. A proinflammatory role for IL-18 in rheumatoid arthritis. J Clin Invest. 1999;104:1393-401.
15. Bamford KB, Fan X, Crowe SE, Leary JF, Gourley WK, Luthra GK et al. Lymphocytes in the human gastric mucosa during Helicobacter pylori have a T helper cell 1 phenotype. Gastroenterology. 1998;114:482-92.
16. Mattapallil JJ, Dandekar S, Canfield DR, Solnick JV. A predominant Th1 type of immune response is induced early during acute Helicobacter pylori infection in rhesus macaques. Gastroenterology. 2000;118:307-15.
17. Roth KA, Kapadia SB, Martin SM, Lorenz RG. Cellular immune responses are essential for the development of Helicobacter felis-associated gastric pathology. J Immunol. 1999;163:1490-7.
18. Lindholm C, Quiding-Jarbrink M, Lonroth H, Hamlet A, Svennerholm AM. Local cytokine response in Helicobacter pylori-infected subjects. Infect Immun. 1998;66:5964-71.
19. Crowe SE, Alvarez L, Dytoc M, Hunt RH, Muller M, Sherman P et al. Expression of interleukin 8 and CD54 by human gastric epithelium after Helicobacter pylori infection in vitro. Gastroenterology. 1995;108:65-74.
20. Yun CH, Lundgren A, Azem J, Sjoling A, Holmgren J, Svennerholm AM et al. Natural killer cells and Helicobacter pylori infection: bacterial antigens and interleukin-12 act synergistically to induce gamma interferon production. Infect Immun. 2005;73:1482-90.
21. Hida N, Shimoyama T Jr, Neville P, Dixon MF, Axon AT, Shimoyama T Sr et al. Increased expression of IL-10 and IL-12 (p40) mRNA in Helicobacter pylori infected gastric mucosa: relation to bacterial cag status and peptic ulceration. J Clin Pathol. 1999;52:658-64.

22. Yoshimoto T, Takeda K, Tanaka T, Ohkusu K, Kashiwamura S, Okamura H et al. IL-12 up-regulates IL-18 receptor expression on T cells, Th1 cells, and B cells: synergism with IL-18 for IFN-gamma production. J Immunol. 1998;161:3400-7.
23. Sawai N, Kita M, Kodama T, Tanahashi T, Yamaoka Y, Tagawa Y et al. Role of gamma interferon in Helicobacter pylori-induced gastric inflammatory responses in a mouse model. Infect Immun. 1999;67:279-85.
24. Berg DJ, Lynch NA, Lynch RG, Lauricella DM. Rapid development of severe hyperplastic gastritis with gastric epithelial dedifferentiation in Helicobacter felis-infected IL-10(-/-) mice. Am J Pathol. 1998 ;152:1377-86.
25. Sutton P, Kolesnikow T, Danon S, Wilson J, Lee A Dominant nonresponsiveness to Helicobacter pylori infection is associated with production of interleukin 10 but not gamma interferon. Infect Immun. 2000;68:4802-4.
26. Karttunen R, Karttunen T, Ekre HP, MacDonald TT. Interferon gamma and interleukin 4 secreting cells in the gastric antrum in Helicobacter pylori positive and negative gastritis. Gut. 1995;36:341-5.
27. Kawabata T, Ichikura T, Majima T, Seki S, Chochi K, Takayama E et al. Preoperative serum interleukin-18 level as a postoperative prognostic marker in patients with gastric carcinoma. Cancer. 2001;92:2050-5.
28. Tomita T, Jackson AM, Hida N, Hayat M, Dixon MF, Shimoyama T et al. Expression of Interleukin-18, a Th1 cytokine, in human gastric mucosa is increased in Helicobacter pylori infection. J Infect Dis. 2001;183:620-7.
29. Day AS, Su B, Ceponis PJ, Jones NL, Yau E, Sieveking D et al. Helicobacter pylori infection induces interleukin-18 production in gastric epithelial (AGS) cells. Dig Dis Sci. 2004;49:1830-5.
30. Akhiani AA, Schon K, Lycke N. Vaccine-induced immunity against Helicobacter pylori infection is impaired in IL-18-deficient mice. J Immunol. 2004;173:3348-56.
31. Kohno K, Kataoka J, Ohtsuki T, Suemoto Y, Okamoto I, Usui M et al. IFN-gamma-inducing factor (IGIF) is a costimulatory factor on the activation of Th1 but not Th2 cells and exerts its effect independently of IL-12. J Immunol. 1997;158:1541-50.
32. Engstrand L, Scheynius A, Pahlson C, Grimelius L, Schwan A, Gustavsson S. Association of Campylobacter pylori with induced expression of class II transplantation antigens on gastric epithelial cells. Infect Immun. 1989;57:827-32.
33. Ye G, Barrera C, Fan X, Gourley WK, Crowe SE, Ernst PB et al. Expression of B7-1 and B7-2 costimulatory molecules by human gastric epithelial cells: potential role in CD4+ T cell activation during Helicobacter pylori infection. J Clin Invest. 1997;99:1628-36.
34. Puren AJ, Fantuzzi G, Gu Y, Su MS, Dinarello CA. Interleukin-18 (IFNgamma-inducing factor) induces IL-8 and IL-1beta via TNFalpha production from non-CD14+ human blood mononuclear cells. J Clin Invest. 1998;101:711-21.
35. Yamaoka Y, Kita M, Kodama T, Sawai N, Imanishi J. Helicobacter pylori cagA gene and expression of cytokine messenger RNA in gastric mucosa. Gastroenterology. 1996;110:1744-52.
36. Garcia-Gonzalez MA, Lanas A, Santolaria S, Crusius JB, Serrano MT, Pena AS. The polymorphic IL-1B and IL-1RN genes in the aetiopathogenesis of peptic ulcer. Clin Exp Immunol. 2001;125:368-75.