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The cytokine response in THP-1 (monocyte) and HL-60 (neutrophil-differentiated) cells infected with different genotypes of *Helicobacter pylori* strains

STOMACH

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Original Article

ABSTRACT

Background/Aims: *Helicobacter pylori (H. pylori)* is a microaerophilic bacterium related with peptic ulcer and gastric cancer. Its virulence factors include cytotoxin-associated gene A (CagA) and vacuolating cytotoxin gene A (VacA) proteins. Cytokine release inducted by *H. pylori* colonization has an important role in pathogenesis of *H. pylori*. The severity of gastric pathologies depends on the *H. pylori* genotypes found in different geographical regions. We aimed to determine the relationship between different *H. pylori* genotypes and their effects on the cytokine release levels.

Materials and Methods: *ureC, cagA, vacAs1/s2, vacAm1/m2*, and blood group antigen-binding adhesion protein A2 (*babA2*) virulence related genes were investigated in 21 *H. pylori* strains. Genotyping of 21 strains were made due to the presence of *cagA, vacAs1/s2, vacAm1/m2*, and *babA2* genes. The *H. pylori* strains were cultured together with THP-1 and neutrophil-differentiated Human promyelocytic leukemia cells (HL-60) cells. The levels of cytokines interleukin (IL)-1β, IL-6, IL-8, IL-12, tumor necrosis factor-alpha (TNF-α), and IL-10 in these cells were measured after co-culturing with *H. pylori* strains.

Results: The following five different genotypes were detected: Genotype1: *cagA* and *vacAs1m2*; Genotype2: *cagA* and *vacAs1m1*; Genotype3: *cagA*, *vacAs1m2*, and *babA2*; Genotype4: *vacAs2m2*; and Genotype5: *cagA* and *vacAs2m2*. All these genotypes significantly induced the levels of IL-1β, IL-6, IL-8, IL 10, and TNF-α in THP-1 cells. Genotype 5 caused higher amounts of IL-1β, IL-6, TNF-α, and IL-10, whereas genotype 1 induced the highest levels of IL-8. In neutrophil-differentiated HL-60 cells, genotype 4 increased IL-6 levels and genotype 3 and 4 elevated IL-8 levels significantly.

Conclusion: These results suggested that cytokine response of the host varies depending on the specific immune response of the host against different *H. pylori* strains.

Keywords: *H. pylori*, *cagA*, *vacA*, *babA*, cytokine response

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a gram-negative, microaerophilic bacterium which colonizes the gastric mucosa of human (1-3) and is related with gastritis, peptic ulcer, gastric cancer, and mucosa-associated lymphoid tissue lymphoma (MALT) (4). In developing countries, *H. pylori* prevalence is high and approximately 70% of the people have *H. pylori*, while 25%–50% of the population in the developed countries is infected by *H. pylori*. One of the possible modes of transmission of *H. pylori* may be by saliva. Chronic *H. pylori* infections of the stomach are a major risk factor for the development of gastroduodenal diseases. *H. pylori* is regarded as Class 1 carcinogen by the International Agency for Cancer Research (2,5).

H. pylori urease enzyme has an important role in the colonization of *H. pylori* in the gastric mucosa. Other bacterial virulence factors, such as the cytotoxin-associated gene (Cag A) and the vacuolating cytotoxin gene (Vac A) products, are related with the occurrence of gastric

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pathologies like peptic ulcers and gastric carcinoma (GC). The highly polymorphic EPIYA (glutamic acid-proline-isoleucine-tyrosine-alanine) motif located in the C-terminal end of the CagA protein is related with the severity of the infection in geographically different regions (6). The VacA protein has important roles in the vacuolization and apoptosis of the gastric epithelial cells. The blood group antigen-binding adhesion protein (BabA), an *H. pylori* adhesion virulence factor, is involved in colonizing and transferring the major virulence factors (like CagA) to the gastric epithelial cells (7).

Following *H. pylori* colonization, T lymphocytes, plasma cells, mononuclear phagocytes, and neutrophils infiltrate the gastric tissue, and pro-inflammatory cytokines [like interleukin-8 (IL-8), IL-1 β, tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ)] are secreted from the immune cells (7-9). *H. pylori* generally causes a persistent bacterial infection. The failure of *H. pylori* clearance is caused by its virulence factors, escaping strategies from the immune defense of the host, and inadequate immune response (7). Various histopathological changes may be seen by the virulence factors of *H. pylori* like *cagA, vacA,* and *babA* in the host (6,7). Geographic differences in gastric pathology incidence may be due to the differences in the genotypes of *H. pylori* strains (6,10).

We isolated our study strains from patients who reside in İstanbul and its neighborhoods. We aimed to classify isolated *H. pylori* strains into various genotypes as per the virulence factors, *cagA, vacAs1/s2, vacAm1/m2*, and *babA2*, and to determine the inflammatory cytokine (IL-1β, IL-6, IL-8, IL-12, TNF-α, and IL-10) responses of the neutrophil and monocyte cell lines infected with various *H. pylori* strains, and also to examine the relationship between *H. pylori and* gastric pathogenesis with respect to bacterial genotypes and cytokine responses.

MATERIALS AND METHODS

Patients

This study was planned as an experimental study. Our subjects were selected from the patients with dyspeptic symptoms who were applied to the endoscopy unit of Gastroenterology department of Cerrahpaşa Faculty of Medicine. The working range of this study was between November 2011 and December 2012. The mean age of the patients was 46 years (20–70 years). Twenty-one *H. pylori* strains were isolated from the antrum and corpus biopsy of the patients.

Our patient selection criteria included the following exclusions: to be under 18 years and individuals who had gastric surgery and treatment *for H. pylori* eradication, a history of bleeding and/or coagulation disorders, consumption of antibiotics in the previous month; or consumption of anti-secretory drugs, bismuth salts, or sucralfate in the last 2 weeks. The ethics committee of Cerrahpaşa Medical Faculty approved this study and all patients gave their informed consent to this study.

H. pylori culture

H. pylori strains were isolated from the corpus and antrum biopsies of the patients. A gram staining was employed from the homogenized biopsies. *H. pylori* was cultured on *Helicobacter* agar (Salubris, İstanbul, Turkey) at 37°C for 5-7 days in a 5% CO_2 incubator (Sanyo Panasonic, Tokyo, Japan) under microaerophilic conditions. *H. pylori* fluid cultures were obtained from *Brucella* broth (BD BBL, New Jersey, United States) containing 10% fetal bovine serum (FBS) (Lonza; Verviers, Belgium) with shaking in a microaerophilic atmosphere at 37°C. *H. pylori* colonies were identified by Gram staining and oxidase, catalase, and urease reactions.

Polymerase chain reaction analysis

Genomic DNA was extracted with the Real Genomics Genomic DNA Extraction kit (RBC Bioscience; Taipei, Taiwan) according to the manufacturer's instructions. *H. pylori ureC* gene was determined by real-time polymerase chain reaction (PCR) reaction using *H. pylori*–QLS 1.0 *H. pylori* DNA detection kit (Fluorion; Istanbul, Turkey) (11). *H. pylori* genotyping was determined by PCR using specific primers for *cagA, vacAs1/s2, vacAm1/m2*, and *babA2* genes. All of the study primers were listed in Table 1 (12).

Cell lines

THP-1 (ATCC TIB-202™) and HL-60 (ATCC CCL-240™) cell lines were obtained from the American Cell Culture Collection (ATCC). THP-1 cell are frequently used cells as seen as a model system for monocytes in various studies. They respond with

Table 1. PCR primers for amplification of cagA, vacA, and babA2 gene sequences

Gene	Primer	Primer sequence (5′-3′)	PCR product (bp)
*cagA**	Forward	GAT AAC AGG CAA GCT TTT GAG G	349
	Reverse	CTG CAA AAG ATT GTT TGG CAG A	
*vacAs1/s2***	Forward	ATG GAA ATA CAA CAA ACA CAC	259 (s1)
	Reverse	CTG CTT GAA TGC GCC AAA C	286 (s2)
vacAm1/m2	Forward	CAA TCT GTC CAA TCA AGC GAG	570 (m1)
	Reverse	GCG TCT AAA TAA TTC CAA GG	645 (m2)
*babA2****	Forward	AAT CCA AAA AGG AGA AAA AGT ATG AAA	832
	Reverse	TGT TAG TGA TTT CGG TGT AGG ACA	

*cag: cytotoxin-associated gene
**vac: vacuolating cytotoxin gene
***bab: blood group antigen-binding adhesin

alike transcriptional pattern as peripheral blood mononuclear cells derived macrophages (13-17). In addition, HL-60 cells can differentiate into neutrophils in response to various stimuli like dimethyl sulfoxide, retinoic acid, and dibutyryl cyclic adenosine monophosphate (Bt_2-cAMP). Due to the differentiation potential of these cells, neutrophil differentiated HL-60 cell lines were obtained through incubating them with 1.75% dimethyl sulfoxide and then were selected as a neutrophil model in this study (18,19).

The cells were maintained in Roswell Park Memorial Institute (RPMI 1640 (Lonza; Verviers, Belgium) medium supplemented with glutamine and 10% FBS, and grown at 37°C in a 5% CO_2 incubator. Cells were grown in six-well tissue culture plates until reaching their desired confluency.

Co-cultures of *H. pylori* strains with THP-1 and neutrophil-differentiated HL-60 cell lines

H. pylori colonies were incubated in 10 mL *Brucella* broth supplemented with 10% FBS and 0.01% vancomycin at 37°C under 5% CO_2 atmosphere for 24 h. After incubation, bacterial motility was verified using a light microscope (40X magnification). *H. pylori* strains were then co-cultured with THP-1 cells and neutrophil-differentiated HL-60 cells in RPMI 1640 medium supplemented with glutamine, 10% *Brucella* broth, 10% FBS, and 0.01% vancomycin. The multiplicity of infection was adjusted to 1:20 and the co-cultures were incubated for 24 h. At the end of this incubation, the supernatants were collected and subjected to cytometric bead array (CBA) analysis. Non-infected THP-1 and neutrophil-differentiated HL-60 cells served as a negative control for the study. All experiments were performed in duplicates.

Cytometric bead array analysis of inflammatory cytokine responses induced in *H. pylori* co-cultured cell lines

Supernatants were obtained from the aforementioned cell lines co-cultured with *H. pylori* strains and they were analyzed for inflammatory cytokines using CBA assay according to manufacturer's instructions (BD Biosciences; New Jersey, United States). The human inflammatory cytokine beads include the following cytokines, IL-1β, IL-6, IL-8, IL-12, TNF-α, and IL-10. After flow cytometer data acquisition (BD Accuri; New Jersey, United States), the results were analyzed using the BD CBA analysis software (BD; New Jersey, United States).

Statistical analysis

The student-t test was used for the statistical analysis of our results by comparative perspective. The Statistical Package for the Social Science version 21.0 (IBM, New York, USA) was used for statistical analyses and $p<0.05$ was accepted as significant value.

RESULTS

Genetic characterization of 21 clinical *H. pylori* strains from Turkish patients

H. pylori strains, isolated from gastric biopsies of 21 patients, were genotyped by PCR. All of them were positive for the *ureC* gene. Approximately 90.4% of *H. pylori* strains had the *cagA* gene. The allelic *vacA* gene variants *vacAs1* (57.1%) and *vacAm2* (90.5%) were more prevalent than *vacAs2* (42.9%) and *vacAm1* (9.5%), respectively. Finally 33.4% of *H. pylori* strains had *babA2*.

All *H. pylori* strains studied were identified by the following genotypes based on their virulence-related genes; *cagA, vacAs1/s2, vacAm1/m2,* and *babA2*: **Genotype 1:** *cagA*-positive and *vacAs1m2*-positive (3/21), **Genotype 2:** *cagA*-positive and *vacAs1m1*-positive (2/21), **Genotype 3:** *cagA*-positive, *vacAs1m2*-positive, and *babA2*- positive (7/21), **Genotype 4:** *cagA*-negative and *vacAs2m2*-positive (2/21), **Genotype 5:** *cagA*-positive and *vacAs2m2*-positive (7/21) (summarized in Table 2).

Cytokine secretion from THP-1 and neutrophil-differentiated HL-60 cells infected with *H. pylori* strains

Histopathological findings of the patients were analyzed according to the Sydney classification: active or chronic gastritis, severity of active or chronic gastritis (mild, moderate, and severe), lymphoid aggregates, lymphoid follicles, and intestinal metaplasia. Only 5 *H. pylori* strains, which have similar histopathological changes in hosts with respect to their genotypes, were selected among the 21 *H. pylori* strains. They were selected prototypically as prospective randomized experimental model for co-culture in THP-1 and neutrophil-differentiated HL-60 cells. Remaning 16 strains were excluded from the study due to the similarities of the genotypical and histopathological features of the prototype strains.

Monocytic THP-1 and neutrophil-differentiated HL-60 cell lines were co-cultured with five *H. pylori* strains for 24 h followed by

Table 2. Presence of *cagA, vacAs1, vacAs2, vacAm1, vacAm2,* and *babA2* genes in *H. pylori* strains with five different genotypes

Genotypes	*cagA**	*vacAs1***	*vacAs2*	*vacAm1*	*vacAm2*	*babA2****
1st	+	+	-	-	+	-
2nd	+	+	-	+	-	-
3rd	+	+	-	-	+	+
4th	-	-	+	-	+	-
5th	+	-	+	-	+	-

*cag: cytotoxin-associated gene
**vac: vacuolating cytotoxin gene
***bab: blood group antigen-binding adhesin

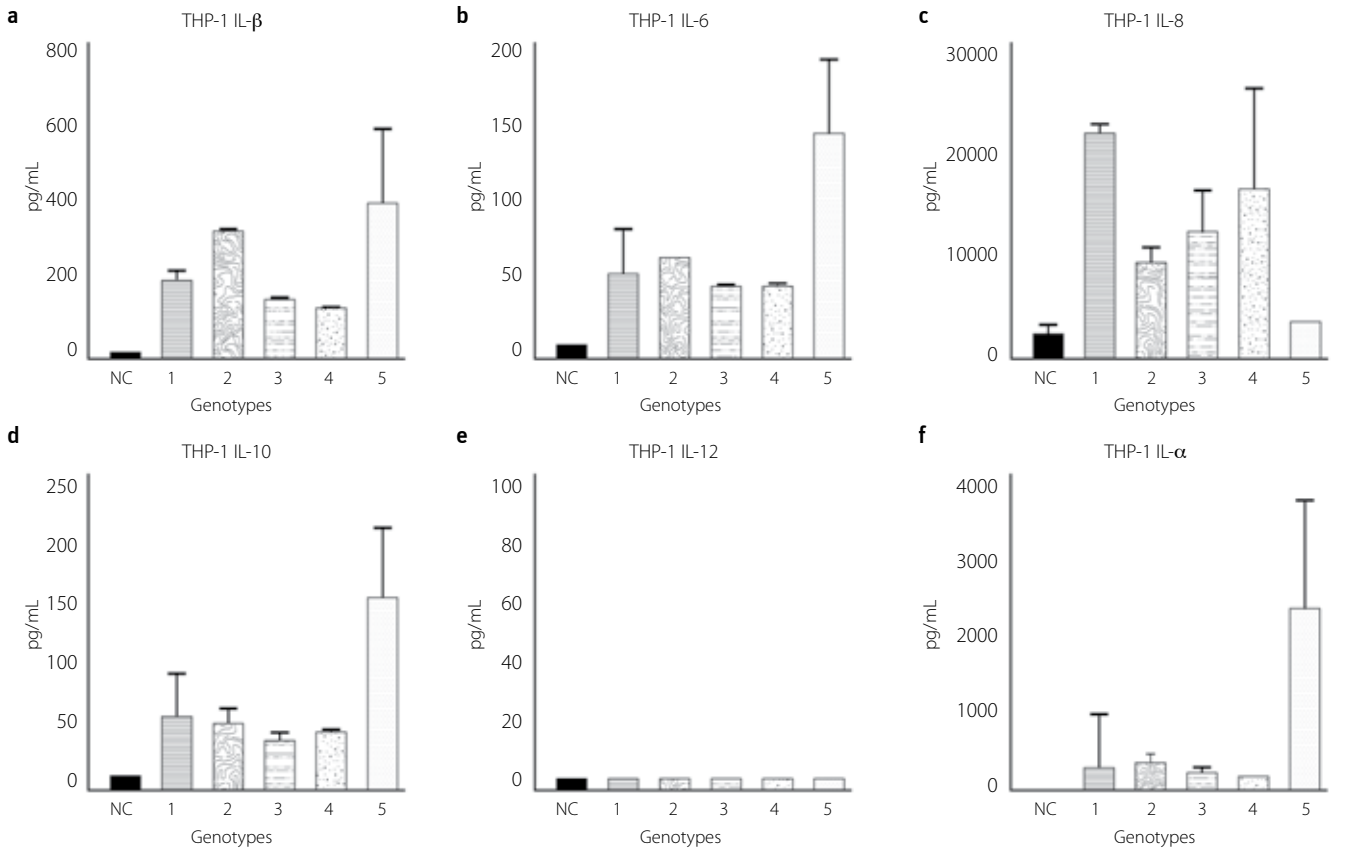

Figure 1. a-f. Various levels of cytokine responses in THP-1 cells after the co-culture of THP-1 cells and *H. pylori* strains with five different genotypes. **(a)** IL-1β secretion level of THP-1 cell line. **(b)** IL-6 secretion level of THP-1 cell line. **(c)** IL-8 secretion level of THP-1 cell line. **(d)** IL-10 secretion level of THP-1 cell line. **(e)** IL-12 secretion level of THP-1 cell line. **(f)** TNF-α secretion level of THP-1 cell line. NC: Negative Control. (All results were averaged from two independent experiments)

analysis of the secreted cytokines. We have analyzed the secretion of IL-1β, IL-6, IL-8, TNF-α, IL-10, and IL-12 from THP-1 and HL-60 cells during infection with five *H. pylori* strains by CBA assay. Our results indicated the significant induction of IL-1β, IL-6, IL-8, TNF-α, and IL-10 in THP-1 cells infected with all five *H. pylori* strains (Figure 1 a,b,c,d,f). However, these *H. pylori* strains did not induce IL-12 in THP-1 cells (Figure 1e). *H. pylori* genotype 5 was responsible for the highest induction of IL-1β, IL-6, TNF-α, and IL-10 ($p<0.001$), and genotype 1 for the secretion of IL-8 ($p<0.001$) in THP-1 cells.

There was a significant increase in IL-6 secretion of neutrophil-differentiated HL-60 cells infected with *H. pylori* genotype 4 ($p<0.001$), but not with the other four genotypes ($p>0.05$) (Figure 2b). Also, IL-8 secretion in neutrophil-differentiated HL-60 cells infected with *H. pylori* genotype 3 and 4 ($p<0.001$) was significantly increased, but the other *H. pylori* genotypes did not induce IL-8 secretion ($p>0.05$) (Figure 2c). In addition, the induction of IL-1β, IL-12, TNF-α, and IL-10 was not statistically significant ($p>0.05$) in neutrophil-differentiated HL-60 cells infected with these *H. pylori* genotypes (Figure 2 a,d,e,f).

DISCUSSION

In this study, we evaluated the cytokine responses of THP-1 (monocyte) and HL-60 (neutrophil-differentiated) cells infected with five *H. pylori* strains having different genotypes. All of these *H. pylori* genotypes caused significant elevations of IL-1β, IL-6, IL-8, TNF-α, and IL-10 levels, but not IL-12 levels in THP-1 cell lines. In neutrophil-differentiated HL-60 cell lines, the secretion of IL-6 was significantly increased only by genotype 4, but not with the other four genotypes. In addition, IL-8 secretion was markedly enhanced by genotypes 3 and 4, whereas no significant change in IL-1β, IL-12, TNF-α, and IL-10 levels was detected.

Development of the gastric *H. pylori* infection is caused by the infiltration of T lymphocytes, plasma cells, mononuclear phagocytes, and neutrophils into the gastric mucosa; the production of various pro-inflammatory cytokines (like IL-1β, IL-6, IL-8, IL-12, and TNF-α); and chemokines from the above immune cells (20-23). IL-8 plays a major role in gastric mucosal damage induced by *H. pylori* with its activator and chemotactic effects (24,25). IL-8 production starts when the *cagPAI* positive *H. pylori* strains interact with the gastric epithelium (26,27). IL-8 was secreted from the gastric epithelial cells infected with *H. pylori* and causes the recruitment of neutrophils to the gastric mucosa and subsequently pro-inflammatory cytokine production like IL-1β. IL-1β have a major role in the initiation of the host immune response against *H. pylori* infections. The mRNA expression of IL-1β was shown to increase in patients

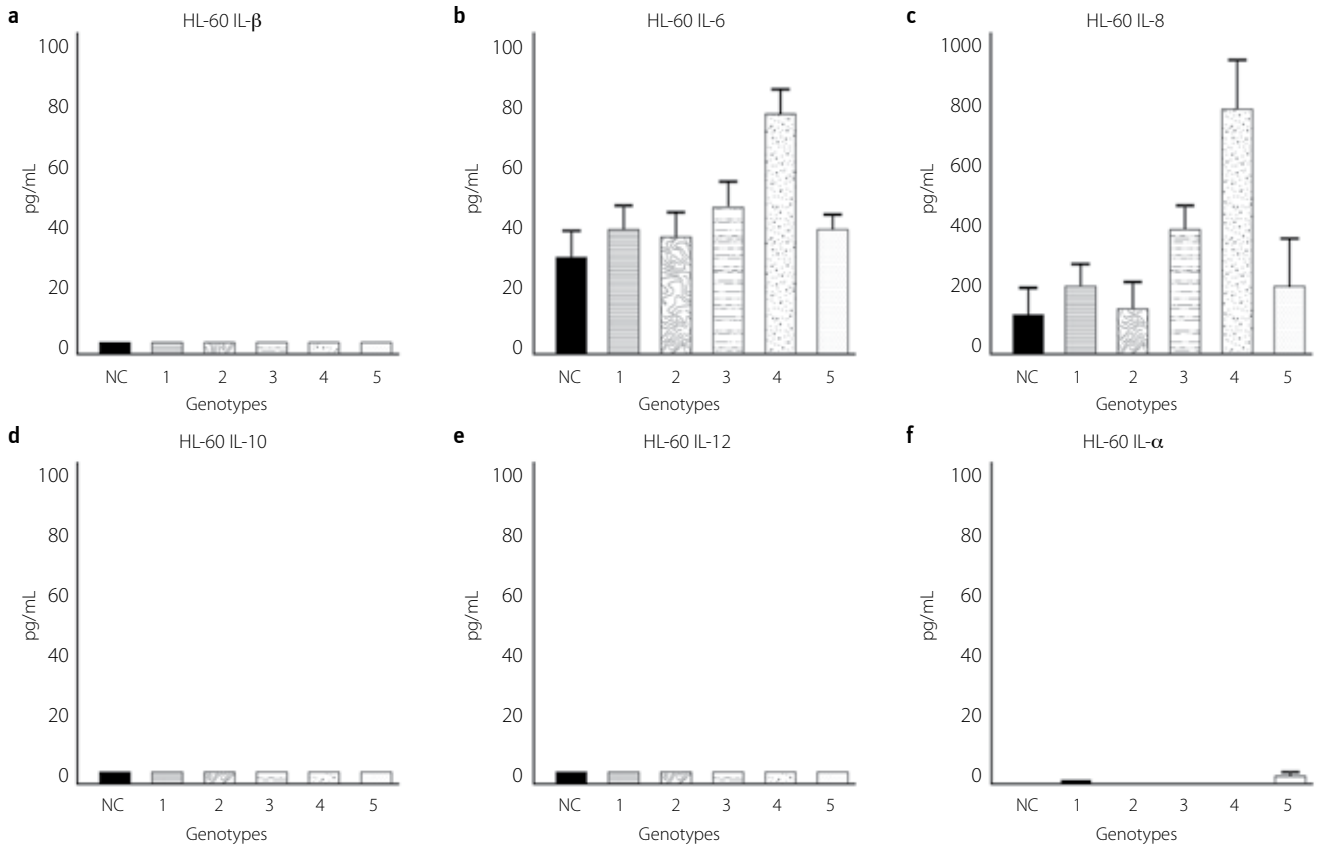

Figure 2. a-f. Various levels of cytokine responses in neutrophil-differentiated HL-60 cells after the co-culture of neutrophil-differentiated HL-60 cells and H. pylori strains with five different genotypes. **(a)** IL-1β secretion level of neutrophil-differentiated HL-60 cell line. **(b)** IL-6 secretion level of neutrophil-differentiated HL-60 cell line. **(c)** IL-8 secretion level of neutrophil-differentiated HL-60 cell line. **(d)** IL-10 secretion level of neutrophil-differentiated HL-60 cell line. **(e)** IL-12 secretion level of neutrophil-differentiated HL-60 cell line. **(f)** TNF-α secretion level of neutrophil-differentiated HL-60 cell line. NC: Negative Control. (All results were averaged from two independent experiments)

with peptic ulcer carrying *cagA* positive *H. pylori* strains (28-30). In addition to IL-1β, the inflammatory cells also secrete TNF-α during *H. pylori*–induced inflammation (31). Furthermore, it has been shown that IL-6 levels are correlated with the severity of atrophic gastritis in *H. pylori* infections (32-34). Also, IL-12 response with IFN-γ plays an important role in the *H. pylori* pathogenesis and development of ulcers (35-37). Furthermore, IL-10, acting as an anti-inflammatory cytokine, suppresses pro-inflammatory cytokines, causes a persistent *H. pylori* infection, and prevents immunopathologies with its inhibitory effects on IL-12-driven Th1 response (25,38). Therefore, inducing immune response related with products of the various gene regions of *H. pylori* strains may cause histopathological changes in the gastric mucosa (6,7).

In this study, we suggested that *H. pylori* genotype 5 (*cagA* and *vacAs2/m2*) may have induced the strongest response for the secretion of IL-1β, IL-6, TNF-α, and IL-10 in THP-1 cells. Kranzer et al. (39) reported that the *cagA* and *vacAs2/m2* positive *H. pylori* strains induced the most prominent elevations of IL-1β, IL-6, IL-8, IL-12, TNF-α, and IL-10 in dendritic cells. Schmidt et al. (10) reported that the *cagA* and *vacAs2/m2* positive *H. pylori* strains induced low levels of IL-8 secretion in AGS cells. The studies performed by Salgado et al. (40) indicated that the *cagA* and *vacAs2/m2* positive *H. pylori* strains caused an increase in TNF-α levels of mice spleen cells. Andres et al. (41) reported that *H. pylori* defines the local immune response in susceptible hosts by interacting with dendritic cells in a strain-specific state, and this interaction can lead to either mild infection or to the disease development. In this study, a case study with *H. pylori* genotype 5 had histopathologically severe gastritis and early stage of *H. pylori* infection could lead to the development of gastritis.

In this study, the most prominent IL-8 secretion was detected in THP-1 cells co-cultured with *H. pylori* genotype 1. Similarly, Schmidt et al. (10) and Andres et al. (41) indicated that *cagA* and *vacAs1/m2* positive *H. pylori* strains led the highest IL-8 secretion in AGS cells. In addition, we determined that the genotypes 1, 2, 3, and 4 had stimulated the secretion of IL-1β, IL-6, TNF-α, and IL-10 with different levels. Thus, our findings were similar with the previous studies (10,14,33,39,41,42).

Notably, not all genotypes cause IL-12 secretion in THP-1 cells in this study. Similar to our results there were reports indicating no increase in IL-12 secretion. Sakai et al. (43) showed that there was no increase in the IL-12 secretion of the gastric mucosa

infected with *H. pylori*. Additionally, IL-12 secretion of dendritic cells inoculated with *H. pylori* was lower than that of dendritic cells inoculated with *Acinetobacter woffii* (44). Obonya et al. (45) indicated that a decrease in the secretion of IL-12 by *H. pylori* inoculated dendritic cells may depend on excessive IL-10 secretion; consequently, they suggested that IL-10 may have a suppressive effect on the secretion of IL-12. We therefore suggested that high levels of IL-10 may partly explain the suppressed levels of IL-12 in this study.

In the present study, *H. pylori* genotype 5 isolated from the patients with gastritis caused the most prominent response in the secretion of IL-1β, IL-6, TNF-α, and IL-10 in THP-1 cells. We suggested that the severity of host immune response may differ due to the different types of *H. pylori* strains. In this study, patients with genotype 2 and 3 have been diagnosed to have intestinal metaplasia, although these genotypes stimulated lower cytokine secretion than that by genotype 5. These findings suggested that host immune (cytokine) responses may show differences regarding different *H. pylori* strains.

Therefore, although we had limited number of strains in this study, it is important to note that this is the first study performed in Turkey. As conclusion, we suggest that the severity of host immune response may differ due to the different types of *H. pylori* strains. Further studies including more strains, different genotypes and pathologies, and examination of the host-specific genetic factors (such as MHC I and MHC II, particularly miRNA) are needed to understand, in detail, the relationship between genotype/cytokine levels and gastric pathologies.

Ethics Committee Approval: The study was approved by the Clinical Research Ethics Board of İstanbul University, Cerrahpaşa Faculty of Medicine No/Year: A-15/2012.

Informed Consent: Written informed consent was obtained from patients who participated in this study.

Peer-review: Externally peer-reviewed.

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