Alterations in Th17 and the Respective Cytokine Levels in Helicobacter pylori-Induced Stomach Diseases

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Keywords
Th17, IL-17A, IL-21, IL-22, IL-23, TGF-β, Helicobacter pylori.

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Abstract

Background: Infections by Helicobacter can cause the stimulation of sophisticated immune response in mucosal immunity. Among the different lymphocytes, Th17 plays an important role in the defense against H. pylori and may cause gastritis and peptic ulcer due to the increased activation of Th17 and cytokine changes.

Aim: To find a relationship between Th17 and IL-17A, IL-21, IL-22, IL-23, TGF-β in the patients with H. pylori infection having signs including gastritis and peptic ulcer.

Methods: A total of 36 samples from the patients [24 Hp+ and 12 Hp− cases] with dyspepsia symptoms were collected. The percentage of Th17 was measured by flow cytometry. The levels of Th17-associated cytokines in the sera and supernatants of peripheral blood mononuclear cells (PBMCs) which were stimulated with the H. pylori antigen, phytohemagglutinin (PHA), or Dynabeads were measured by ELISA.

Results: Patients were divided into two groups of having either H. pylori infected (peptic ulcer, gastritis (mild, moderate)) or being uninfected. The percentage of Th17 in the patients with peptic ulcer and gastritis was significantly higher than their uninfected counterparts (p ≤ .001). The serum levels of IL-17A, IL-23, and TGF-β in the peptic ulcer and gastritis groups were significantly higher compared with the corresponding levels in the uninfected population (p < .05). A significant association of TGF-β, IL-21, and Th17 was observed with low levels of IL-17A in the mild gastritis patients (p < .05). Significantly higher levels of IL-22, IL-17A, IL-23, and higher Th17 frequencies were detected in the moderate gastritis patients, as compared with the uninfected patients (p ≤ .001).

Conclusion: It can be concluded that among the cytokines associated with Th17, the two cytokines of IL-21 and TGF-β play a more critical role in peptic ulcer and gastritis in the individuals infected with H. pylori. Furthermore, inflammation varies depending on the type of the cytokine and its secreted level.

Helicobacter pylori (H. pylori) is a gram-negative bacterium that selectively colonizes the mucus layer of the human stomach, where it resides in the mucus and on the surface of gastric epithelial cells. It is the most common bacterial infection worldwide [1] and is also recognized by the World Health Organization as a risk factor for the development of gastric adenocarcinoma [2]. H. pylori infections have been associated with different types of gastric diseases, including gastritis, gastric adenocarcinoma, peptic ulcer disease, and gastric mucosa-associated lymphoma. Helicobacter pylori infection is common in both developed (30–50% of adults) and developing countries (80% of adults) [3]. However, it is recommended that the patients with lower levels of infection undergo H. pylori eradication therapy [4]. H. pylori induce both humoral and cellular immune
responses. In the course of *H. pylori* infection, a marked specific acquired immune response is generated by the production of antibodies, the differentiation and activation of effector T cells such as helper (Th1) and Th2 [5], and the number of cells generating interferon (IFN)-γ [6].

Th subsets are characterized by their cytokine profiles and their lineage-specific transcription factors. Some evidence indicates that *H. pylori* infection is accompanied by Th1 cell response, which is associated with reduced *H. pylori* colonization density [7]. Th17 cells, a third subset of the effector Th cells, play a serious role in the host defense against extracellular bacteria, fungi, and in the pathogenesis of autoimmune diseases [8]. These cells develop in a pathway independent from Th1 and Th2 differentiation. IL-1, IL-6, IL-21, and IL-23 or IL-1β stimulate the differentiation in humans through the signal transducer and activator of transcription 3 (STAT-3) signaling pathway [9–12]. On the other hand, Th17 cells produce IL-17A, IL-17F, IL-22, and IL-21. The gastric epithelial cells and lamina propria mononuclear cells are capable of giving responses to IL-17 because these cells express IL-17R on their surface. The increased levels of IL-17 during *H. pylori* infection are suggested to be responsible for mucosal damage during gastritis [13–15]. In addition, IL-21 can act in an autocrine manner to induce Th17 development. It also enhances IL-23R expression, which prompts Th17 cells [16]. Th cells and NKT cells produce IL-21 [17], which is also expressed at the highest level by follicular helper T (Tfh) cells and Th17 cells [18]. Its receptor is expressed on T cells, B cells, NK cells, macrophages, and DCs [19]. IL-21 is a requirement for Tfh cell differentiation. It also affects directly GC B cells, plasma cell differentiation and promotes antibody production [20].

As exposure to IL-23 helps Th17 cells to acquire effector functions and become consistent, and Th17 produces IL-17A, IL-17F, and IL-22, it seems that IL-23 has a key feature that determines the pathogenicity of Th17 cells [21]. TGF-β, an anti-inflammatory cytokine, works with IL-6, a pro-inflammatory factor, to prompt Th17 cells [22]. Th17 cells are the link between innate and adaptive immunity and could attract other subsets of Th cells, neutrophils, and macrophages to the sites of infection for the effective clearing of the pathogens [23]. They are also affected by the several cytokines mentioned above.

Therefore, the aim of this study was to investigate the effects of the excessive production of Th17-related cytokines and Th17 cells in the patients with *H. pylori* infection with signs including gastritis and peptic ulcer.

### Materials and Methods

#### Study Design and Population

This study was approved by the ethics committee of Shiraz University of Medical Sciences, Shiraz, Iran. Informed consents were obtained from all participants. In total, 36 adults, consisting of 24 *H. pylori*-infected (11 male, 13 female) and 12 uninfected (four male, eight female) patients, were enrolled in the study. Peripheral blood and gastric biopsy samples were collected from the 36 patients, all of whom underwent endoscopy at Nemaze hospital within 1 year. Signs for endoscopy in these patients were epigastria pain, dyspepsia, and abdominal pain. Excluded were the patients with gastric cancer, gastrointestinal bleeding, autoimmune diseases, acute or chronic renal failure, and those who received anticholinergic, antimicrobial drugs, anti-inflammatory agents, or proton pumps at least 4 weeks prior to endoscopy. For each patient, three biopsy specimens were obtained from the adjacent areas of the gastric antrum. One biopsy sample was sent for histological assessment with hematoxylin and eosin stain, one sample was used for culture, and one for the rapid urease test. If *H. pylori* was recognized in the culture or the rapid urease test and histology test, the case was considered *H. pylori* infected. When the results were negative for all of the tests, the patient was categorized as *H. pylori* uninfected. The *H. pylori* infected were classified into two groups, either having gastritis or peptic ulcer, as determined by a gastroenterologist. The same patients were also classified as having mild or moderate gastritis by a pathologist, based on the intensity of the inflammatory cells within the lamina propria.

#### Culture

The gastric antrum biopsies were cultured on to Columbia agar with sheep blood (10%) nalidixic acid (10 μg/ml), trimethoprim (10 μg/ml), and amphotericin B (2 μg/ml). The plates were micro-aerophilic incubated at 37 °C for 7 to 10 days, using Anoxomat (the Netherlands), and *H. pylori* in the culture was identified by colony formation and gram stain morphology, as well as by the positive urease, catalase, and oxidase tests.

#### Histology

One sample, from the antrum of each patient, was fixed in 10% formalin and was sent to the laboratory. Samples were embedded in paraffin, cut at 3–4 μm...
Isovalurin 190 l phytohemagglutinin (PHA) (Sigma), and 2 wells containing RPMI 1640, containing cell suspension with 10 % RPMI 1640, containing cell suspension together with 10 pg/mL H.pylori antigen, 2 wells containing 100 µL RPMI 1640, containing cell suspension together with 10 pg/mL H.pylori antigen, 2 wells containing 190 µL RPMI 1640, containing cell suspension with 10 µg/mL phytohemagglutinin (PHA) (Sigma), and 2 wells containing 190 µL RPMI 1640, containing cell suspension with 10 µL anti-CD3/CD28 (Dynabead). These samples were kept at 37 °C with CO2 for 4 days duration. The supernatants were collected and stored at −70 °C until examination.

**Intracellular IL-17 staining and the analysis by flow cytometry**

Briefly explained, the PBMCs (2 × 10⁶ cells), isolated from blood samples, were first stained with either anti-CD4-FITC or isotype control antibody (both from BD pharmingen) and incubated at room temperature (RT) for 30 minutes. The PBMCs were stimulated with 1 mg/mL 4α-phorbol 12-myristate 13 acetate (PMA) (Sigma-Aldrich), 1 µg/mL ionomycin (Sigma-Aldrich), and 0.7 µg/mL Golgistop (BD pharmingen) for 5 hours at 37 °C with CO2, then, fixed and permeabilized with the BD cytofix/cytoperm kit (BD pharmingen) and stained intracellularly with anti-IL-17PE (BD pharmingen) or isotype control (BD pharmingen). Incubations were carried out at 4 °C for 30 min. Finally, the cells were washed twice with Perm–wash buffer, re-suspended in staining buffer (PBS, sodium Azide, FBs), and measured by flow cytometry. The T cells were gated with positive CD4 cells; then, the percentage of IL-17+ cells was selected out of the gated CD4+ cells. The data were analyzed, using BD Cell Quest Pro software (BD Biosciences) (Fig. 1).

**Detection of Cytokines**

The levels of IL-17A, IL-21, IL-22, IL-23, and TGF-β in the culture supernatants of PBMCs and sera were evaluated, using ELISA kits (eBioscience), according to the manufacturer’s instructions.

**Statistical Analysis**

The Kruskal–Wallis test was used for the statistical analysis of the data for all of the patient groups. The Mann–Whitney test with the Bonferroni correction was used for the comparison between each pair of the patients in the three groups. Chi-square tests were also used to evaluate the qualitative data (sex, age, etc.), and p < .05 was considered as significant. The Pearson correlation test and the linear regression model were used for the comparison and correlating relationships between the quantitative variables.

**Results**

**Patients’ Characteristics**

Thirty-six patients were divided into three groups, based on their endoscopy results (peptic ulcer N = 12,
gastitis $N = 12$, and uninfected $N = 12$) and pathology results (mild gastritis $N = 16$, moderate gastritis $N = 8$, and uninfected $N = 12$). The number of male and female patients was 15 (41.5%) and 21 (58.3%), respectively. The ages ranged between 30 to 63 years, with a mean age of 46.5 ± 16.5 for infected patients, and 25 to 50 years, with a mean age of 37.5 ± 12.5 for uninfected patients.

No significant difference was observed in the mean age of the $H. pylori$-infected gastritis, the peptic ulcer and the uninfected groups ($p = .626$). There were not any significant differences in the sexes among the three groups of patients, either ($p = .2$). The baseline laboratory medicine and the patients’ characteristics are presented in Tables 1 and 2, respectively.

### Th17 Frequency in Gastritis (Moderate, Mild) and Peptic Ulcer Patients with $H. pylori$

Peptic ulcers and gastritis caused by $H. pylori$ infections are specifically associated with the presence of inflammation. Th17-associated cytokines and Th17 play a

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>Gastritis</th>
<th>Ulcer</th>
<th>Uninfected</th>
<th>$p$</th>
<th>$p1/p2/p3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age/years</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>43 ± 19.5</td>
<td>43.5 ± 17.4</td>
<td>37.5 ± 12.5</td>
<td>.62</td>
<td>p1–p2 = 0.88, p1–p3 = 0.13, p2–p3 = 0.10</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Male</td>
<td>7 (58.3%)</td>
<td>4 (33.3%)</td>
<td>4 (33.3%)</td>
<td>.22</td>
<td>p1–p2 = 0.2, p1–p3 = 0.2, p2–p3 = 0.67</td>
</tr>
<tr>
<td>Female</td>
<td>5 (41.7%)</td>
<td>8 (66.7%)</td>
<td>8 (66.7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urease</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>12 (100%)</td>
<td>12 (100%)</td>
<td>0.0 (0%)</td>
<td>.000</td>
<td>p1–p2 = 0, p1–p3 = 0.000, p2–p3 = 0.000</td>
</tr>
<tr>
<td>Negative</td>
<td>0.0 (0%)</td>
<td>0.0 (0%)</td>
<td>12 (100%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Culture</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>2 (16.7%)</td>
<td>6 (50%)</td>
<td>0.0 (0%)</td>
<td>.08</td>
<td>p1–p2 = 0.09, p1–p3 = 0.24, p2–p3 = 0.007</td>
</tr>
<tr>
<td>Negative</td>
<td>10 (83.3%)</td>
<td>6 (50%)</td>
<td>12 (100%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$H. pylori$ seen in pathology slide</td>
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</tr>
<tr>
<td>Seen</td>
<td>9 (75%)</td>
<td>7 (58.3%)</td>
<td>0.0 (0%)</td>
<td>.000</td>
<td>p1–p2 = 0.33, p1–p3 = 0.000, p2–p3 = 0.002</td>
</tr>
<tr>
<td>Nonseen</td>
<td>3 (25%)</td>
<td>5 (41.7%)</td>
<td>12 (100%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

p1–p2 (The comparison between gastritis and ulcer groups), p1–p3 (The comparison between gastritis and uninfected groups), p2–p3 (The comparison between ulcer and uninfected groups).
key role in these conditions. Th17 frequency was significantly higher in the peptic ulcer and gastritis groups than that in their uninfected counterparts ($p \leq .001$, $p \leq .05$, respectively). It was also significantly higher in the peptic ulcer patients compared with the gastritis patients (Fig. 2A), and its frequency in the moderate and mild gastritis cases was significantly higher than in the uninfected group ($p \leq .001$, $p \leq .05$, respectively). Compared with mild gastritis, Th17 frequency was greater in the moderate gastritis cases (Fig. 2B).

### Changes of IL-17A serum levels in the peptic ulcer and gastritis (moderate, mild) patients

Th17 cells, and their products, are associated with the pathology of many inflammatory diseases. They preferentially produce IL-17A. In this study, increased levels of IL-17A were observed in the peptic ulcer and gastritis patients, compared with in the uninfected group (Fig. 3A). However, there was no significant difference among the patient groups themselves ($p = .12$, $p = .18$, respectively). The concentrations of IL-17A were significantly higher in the mild gastritis group compared with their uninfected counterparts ($p = .04$) (Fig. 3B).

### Serum levels of IL-21, IL-22, IL-23, and TGF-β

IL-21 is a major product of Th17 cells, and compared with Th1/Th2 cells, Th17 cells selectively produce this cytokine [24]. IL-21 can have an influential role in the development of Th17, causing inflammation [25]. In the infected patients, the serum levels of IL-21 were lower in the peptic ulcer and gastritis patients, compared with the uninfected patients. There were no statistical significant differences in IL-21 production between the gastritis patient groups and the uninfected cases ($p = .138$, $p = .24$, respectively) (Fig. 3C and D). IL-22 is predominantly produced by Th17 [26]. This cytokine enhances the integrity and regeneration in the stomach’s epithelial cells. There were no significant differences in the serum levels of IL-22 cytokine when the peptic ulcer, gastritis, and uninfected group were compared ($p = .1$) (Fig. 3E). IL-22 did not differ significantly among the uninfected, and the moderate and mild gastritis groups ($p = .16$) (Fig. 3F).

IL-23 is important in promoting and maintaining Th17 cells [6]. It can promote IL-17 secretion during bacterial infections [27]. Increased levels of IL-23 were observed in the gastritis patients, as compared with the uninfected patients ($p = .04$) (Fig. 3G). IL-23

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**Table 2** The results of the bacterial culture, urease test, histopathology for the patients with mild and moderate gastritis caused by *H. pylori* infection and the uninfected group

<table>
<thead>
<tr>
<th></th>
<th>Mild gastritis</th>
<th>Moderate gastritis</th>
<th>Uninfected</th>
<th>$p$</th>
<th>$p_{1/2/3}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age/years</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>43.3 ± 20.21</td>
<td>43.13 ± 14.08</td>
<td>37.50 ± 12.51</td>
<td>.62</td>
<td>$p_{1/2} = 0.83$</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>$p_{1/3} = 0.04$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$p_{2/3} = 0.24$</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>6 (37.5%)</td>
<td>5 (62.5%)</td>
<td>4 (33.3%)</td>
<td>.22</td>
<td>$p_{1/2} = 0.23$</td>
</tr>
<tr>
<td>Female</td>
<td>10 (62.5%)</td>
<td>3 (37.5%)</td>
<td>8 (66.7%)</td>
<td></td>
<td>$p_{1/3} = 0.57$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$p_{2/3} = 0.2$</td>
</tr>
<tr>
<td>Urease</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>16 (100%)</td>
<td>8 (100%)</td>
<td>0.0 (0%)</td>
<td>.000</td>
<td>$p_{1/2} = 0.00$</td>
</tr>
<tr>
<td>Negative</td>
<td>0.0 (0%)</td>
<td>0.0 (0%)</td>
<td>12 (100%)</td>
<td></td>
<td>$p_{1/3} = 0.00$</td>
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<td></td>
<td>$p_{2/3} = 0.00$</td>
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<tr>
<td>Culture</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Positive</td>
<td>5 (31.3%)</td>
<td>3 (37.5%)</td>
<td>0.0 (0%)</td>
<td>.09</td>
<td>$p_{1/2} = 0.55$</td>
</tr>
<tr>
<td>Negative</td>
<td>11 (68.8%)</td>
<td>5 (62.5%)</td>
<td>12 (100%)</td>
<td></td>
<td>$p_{1/3} = 0.04$</td>
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<td></td>
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<td></td>
<td>$p_{2/3} = 0.049$</td>
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<tr>
<td><em>H. pylori</em> seen in pathology slide</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Seen</td>
<td>8 (50%)</td>
<td>8 (100%)</td>
<td>0.0 (0%)</td>
<td>.000</td>
<td>$p_{1/2} = 0.01$</td>
</tr>
<tr>
<td>Nonseen</td>
<td>8 (50%)</td>
<td>0.0 (0%)</td>
<td>12 (100%)</td>
<td></td>
<td>$p_{1/3} = 0.004$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$p_{2/3} = 0.000$</td>
</tr>
</tbody>
</table>

$p_{1/2}$ (The comparison between mild gastritis and moderate gastritis groups), $p_{1/3}$ (The comparison between mild gastritis and uninfected groups), $p_{2/3}$ (The comparison between moderate gastritis and uninfected groups).
serum levels were also significantly higher in the mild gastritis cases, compared with the uninfected group, \( p = .01 \) (Fig. 3H). Increased levels of IL-23 were associated to the high frequencies of Th17 in \( H. pylori \)-infected patients with peptic ulcers and gastritis \( p = .0001 \). TGF-\( \beta \) is required for the differentiation of both Th17 and Treg cells. Of note, TGF-\( \beta \) plus IL-6 can have important roles in the development of Th17 cells [28]. In the current study, in addition to the increased Th17 frequencies (Fig. 2A), TGF-\( \beta \) levels were significantly higher in the peptic ulcer and gastritis patients compared with the uninfected counterparts \( p = .001, p = .006, \) respectively) (Fig. 3I). Interestingly, the highest level of TGF-\( \beta \) was detected for the mild gastritis, in comparison with the uninfected \( p = .002 \) (Fig. 3I, J).

**Cytokines Relationships and their Correlations with Th17**

Our results demonstrated that, there are significant positive correlations between IL-21 serum levels with the serum levels of IL-17A and IL-22 \( r = .333, r = .945 p < .001, \) respectively), and between TGF-\( \beta \) serum levels with Th17 cell frequencies and IL-17A cytokine levels \( r = .333, r = .655 p < .001, \) respectively) in all of the patients. Figure 4 demonstrates the correlations and regressions between our measured parameters.

A linear regression analysis revealed that IL-21 serum levels were positively correlated with IL-17 and IL-22 serum levels in all of the patients \( r = .111 p = .047, r = .89 p = .000, \) respectively), and the increases in IL-22 levels were significantly greater than those of IL-17. The same positive correlation was observed between TGF-\( \beta \) levels and Th17 cell frequencies and IL-17 cytokine levels \( r = .111 p = .047, r = .43 p = .000, \) respectively).

**The Association of IL-21 and TGF-\( \beta \) with Th17 Frequency**

IL-6, IL-21, and low concentrations of TGF-\( \beta \) induce the differentiation of Th17 cells [28]. We evaluated the effects of a combination of TGF-\( \beta \) and IL-21 in inducing inflammation in \( H. pylori \)-infected patients. The IL-21 concentration, alone, was not significantly different among the patient groups (Fig. 3C, D), but in the combination with TGF-\( \beta \), the level of this cytokine has shown to be significantly higher in the patients infected with \( H. pylori \) compared with the uninfected group \( p = .001, p = .001, \) respectively) (Fig. 5A, B). Higher levels of IL-21 with TGF-\( \beta \) were observed in the moderate and mild gastritis patients compared to the uninfected patients \( p = .007, p = .002, \) respectively) (Fig. 5B). We investigated the relationship of TGF-\( \beta \), IL-21, and Th17 with IL-17A in \( H. pylori \)-infected peptic ulcer and gastritis groups. While the observed correlation between TGF-\( \beta \), IL-21, and Th17 with the level of IL-17A for \( H. pylori \)-infected peptic ulcer and gastritis groups was positive, this correlation was negative for the mild gastritis group. In fact, the lower level of IL-17A was associated with higher levels of TGF-\( \beta \), IL-21, and Th17 in this group \( p = .03 \).

**The Association of IL-22, IL-17A, and IL-23 with Th17 Frequency**

We observed significantly higher levels of IL-22, IL-17A, IL-23 cytokines, associated with Th17 in the peptic ulcer and gastritis patients, compared with the uninfected \( p = .004 \) (Fig. 6A, B). High levels of IL-23 were
Figure 3  (A) The comparison of the serum concentrations of IL-17A, (C)IL-21, (E)IL-22, (G)IL-23, and (I)TGF-β (pg/mL) in the patients with gastritis and peptic ulcer due to H. pylori infection and the uninfected group (B) The comparison of the serum concentrations of IL-17A, (D)IL-21, (F)IL-22, (H)IL-23, and (J)TGF-β (pg/mL) in the patients with moderate gastritis and mild gastritis due to H. pylori infection and the uninfected group. The data were analyzed by Kruskal–Wallis test (p ≤ .05 significant, NS, Nonsignificant).
seen in the *H. pylori*-infected gastritis mucosa. IL-17, alone, induced low inflammation in gastric mucosa, but could occur synergistically with IL-22 to increase inflammation. As shown in Fig. 6B, significantly higher levels of IL-22, IL-17A, and IL-23 were detected in the moderate gastritis patients as compared with the uninfected group (*p* = .03). Increased levels of these three cytokines and Th17 frequencies were also observed in the moderate and mild gastritis patients, compared with the uninfected group (*p* = .003).

**Cytokine analysis in cell cultures of PBMCs stimulated with the *H. pylori* antigen**

The PBMCs were isolated from peptic ulcers, and the gastritis and uninfected groups were stimulated with *H. pylori* antigens. IL-21, IL-22, IL-23, and TGF-β secreted by PBMCs, in response to *H. pylori* antigens, were significantly higher in the gastritis cases (*p* = .02, *p* = .02, *p* = .002, and *p* = .001, respectively) (Fig. 7C, E, G, I), especially the moderate type, compared with those in the *H. pylori*-uninfected cases (*p* = .001, *p* = .0001, *p* = .007, and *p* = .0001, respectively) (Fig. 7D, F, H, J). The levels of IL-23 and TGF-β were also significantly higher in the peptic ulcer patients than in the uninfected cases (*p* = .04, and *p* = .001, respectively) (Fig. 7G, J). Although the levels of IL-17A were slightly higher for the moderate gastritis compared with the uninfected group, the difference was not significant. Furthermore, the differences were not statistically significant among the peptic ulcer, gastritis, and uninfected groups (*p* = .1) (Fig. 7A, B).

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**Figure 4** The correlations and regressions between the measured parameters in this study. The following equations were correlated for the above depicted parameters: (A) Th17 = (0.03 × TGFβ)/1000 + 0.903; (B) IL22 = (0.343 × IL21) + 12.396; (C) IL17A = [0.03 × IL21] + 7.731; (D) IL17A = (0.243 × TGFβ)/1000) + 12.014.
Significantly increased levels of TGF-β with IL-21 in the supernatants of PBMCs, stimulated with H. pylori antigens, were observed in the peptic ulcer and gastritis groups, as compared with the uninfected group (\(p \leq .0001\), \(p \leq .0001\), respectively) (Fig. 8A,B). Among the gastritis groups (both moderate and mild), the levels were significantly higher in the moderate gastritis group than in their uninfected counterparts (\(p \leq .0001\)) (Fig. 8B). The association of TGF-β, IL-21, and IL-17A secretions by PBMCs was significantly higher in the gastritis patients (\(p \leq .0001\)) (Fig. 8A), especially in the moderate type, as compared with the uninfected group (\(p \leq .0001\)) (Fig. 8B).

As seen, the rising levels of IL-22 and IL-17A were observed to be significantly correlative with the increased levels of IL-23, especially in the moderate gastritis group, compared with the uninfected group (\(p = .002\), \(p = .001\), respectively) (Fig. 8C,D). Similarly, the increased levels of IL-22 and IL-17A were significantly associated with the higher frequency of Th17 in the same group, compared with the uninfected group (\(p = .004\), \(p = .004\), respectively).

Cytokine levels in the supernatants of PBMCs stimulated with anti-CD3/CD28 or phytohemagglutinin (PHA)

The levels of IL-17A, IL-22, and IL-23 in response to anti-CD3/CD28, were significantly increased in the peptic ulcer (\(p = .001\), \(p = .002\), \(p = .04\), and \(p = .0001\), respectively) and gastritis groups (\(p = .001\), \(p = .002\), \(p = .007\), and \(p = .0001\), respectively) (Fig. 9 A1, C1, E1, I1), as well as the moderate gastritis patients, compared with the uninfected group (\(p = .0001\), \(p = .002\), \(p = .002\), and \(p = .0001\), respectively) (Fig. 9B1, D1, F1, J1). IL-23 levels in the uninfected group were not significantly different from those of the other groups (\(p = .2\)) (Fig. 9G1, H1).

Notably, when the PBMCs were stimulated with PHA, the levels of IL-17A, IL-21, IL-22, and IL-23 were significantly increased in the moderate gastritis group (\(p = .001\), \(p = .02\), \(p = .03\), and \(p = .005\), respectively) (Fig. 9B2, D2, F2, H2), and the TGF-β level was significantly increased in the peptic ulcer and mild gastritis.
Figure 7 The culture supernatants of PBMCs stimulated by H. pylori - antigen were analyzed by ELISA for measuring the levels of (A) IL-17, (C) IL-21, (E) IL-22, (G) IL-23, and (I) TGF-β (pg/mL) in the H. pylori-infected patients suffering from gastritis and peptic ulcer and the uninfected group. The same analysis was carried out for measuring the levels of (B) IL-17A, (D) IL-21, (F) IL-22, (H) IL-23, and (J) TGF-β (pg/mL) in the H. pylori-infected patients suffering from moderate gastritis and mild gastritis, and the uninfected group. (p ≤ .05 significant. NS, Nonsignificant).
patients, compared with the uninfected group ($p = 0.03$, and $p = 0.02$, respectively) (Fig. 9I2, J2).

**Discussion**

In this study, the activity of Th17 was investigated in the patients infected and uninfected with *H. pylori*. Moreover, the association of Th17-related cytokines, in gastritis and peptic ulcer patients, was evaluated, and significantly higher frequencies of Th17 were observed in the peptic ulcer and gastritis patients compared with their uninfected counterparts. Similar results were found for moderate and mild gastritis in comparison with the uninfected groups. These results indicate the important role of Th17 in the peptic ulcers and gastritis, especially in the moderate gastritis during *H. pylori* infection.

*H. pylori* infection persists throughout respective adult patients’ lives and predisposes them to severe diseases such as peptic ulcers or gastric carcinoma [29]. In the stomach and the peripheral blood of the patients with gastric cancer, it was shown that Th17 cell frequency is higher than that of the normal individuals [30]. A previous study has shown that the progression of tumors is correlated with increased frequencies of Th17 cells [9]. Several studies have also reported that IL-17A is present in the stomachs of experimentally infected mice and *H. pylori*-infected humans, which proposes that a Th17 response may also be exploited [27,31]. Several reports suggest that IL-17 may be more...
Figure 9 The culture supernatants of PBMCs stimulated by anti-CD3/CD28 or PHA were analyzed by ELISA for measuring the levels of (A1,2) IL-17A, (C1,2) IL-21, (E1,2) IL-22, (G1,2) IL-23, and (I1,2) TGF-β (pg/mL) in the H. pylori-infected patients suffering from gastritis and peptic ulcer and the uninfected group. The same analysis was done for measuring the levels of (B1,2) IL-17A, (D1,2) IL-21, (F1,2) IL-22, (H1,2) IL-23, and (J1,2) TGF-β (pg/mL) in the H. pylori-infected patients suffering from moderate gastritis and mild gastritis, and the uninfected group. (p ≤ .05 significant, NS, Non-significant).
Figure 9 (Continued).
The immunological events taking place in the gastric mucosa might be decisive in the immune response and may determine the final outcome in the patients [32]. TGF-β is required for the differentiation of both Th17 and T regulatory (Treg) cells by inducing their key transcription factors, that is, RORγ/RORe and foxp3, respectively [22,25,33,34]. TGF-β plus IL-6 can play important roles in the development of Th17 cells. In addition, a low concentration of TGF-β with IL-6 and IL-21 promotes IL-23R expression, and Th17 cell differentiation. On the other hand, a high concentration of TGF-β inhibits IL-23R expression, favoring Treg cell generation [32]. TGF-β plays a role in preventing inflammation, but it is not effective enough to prevent ulcers caused by H. pylori. In the present study, the concentration of TGF-β was found to be significantly higher in the peptic ulcer and gastritis patients than in the uninfected people. Previous studies showed that IL-23 is a self-determining mediator in the pathogenesis of peptic ulcers [27]. It was shown that IL-23 takes part in the process of inflammation, irrespective of the presence of an ulcer [35]. The evidence indicates that IL-23 is an important factor in the process of H. pylori infection-related gastric cancer [27]. Our results indicated that the levels of IL-23 were higher in the peptic ulcer and gastritis patients, compared with the uninfected, suggesting that IL-23 could be implicated in inflammatory response. In addition, the stability of Th17 cells in sites of infection is implied. During H. pylori infection in gastric mucosa, IL-23 is over-produced. IL-23 enhances Stat3 activation and, then, IL-17 is synthesized by normal gastric lamina propria mononuclear cells (LPMC) [6]. IL-17 may cause local tissue inflammation in such conditions [31]. Fabr rico et al. observed increased levels of IL-17A in the gastric mucosa of H. pylori -positive adult patients [32]. In agreement with the above studies, we also found higher levels of IL-17A in the gastritis and peptic ulcer patients compared with the normal controls.

IL-21 is an autocrine cytokine, which enhances the generation of Th17 cells. It is a T-cell-derived cytokine, which also exerts multiple effects on the inflammatory process. This cytokine is directly implicated in the inflammation-related tissue damage, and architectural subversion observed in the gut of IBD patients, affected by long-standing inflammation [10]. IL-21 is a stimulator for Th17 cells; therefore, it can amplify the differentiation and expression of IL-23R and maturation of Th17. It has also a paracrine role in the chronic maintenance of the T-cell-mediated inflammation in H. pylori -infected patients [36].

In the present research, the level of IL-21 was not significantly different among the investigated groups, consistent with the results of the research by Caruso [37], but in IL-21 association with TGF-β, the levels of these two cytokines were significantly higher for the peptic ulcers and gastritis, especially the moderate gastritis, as compared with the uninfected. These results suggest that the association of IL-21 and TGF-β cytokines induces effector Th17 cells production, leading to inflammation.

IL-22 is a recently discovered cytokine belonging to the IL-10 cytokine family [26]. Previous studies indicate that IL-22 is expressed by Th17 cells and propose a synergistic interaction between IL-22 and IL-17 in tissue inflammation and autoimmune diseases [38,39]. In another study on the human, a relationship between IL-22 and the severity of gastritis inflammation in H. pylori -infected patients was observed [40]. It has been reported that IL-22 has tissue protective, as well as pro-inflammatory properties. The balance between these two activities might be governed by the extent of IL-22 co-expression with IL-17A. In the absence of IL-17A, IL-22 has tissue-protective effects. However, IL-22 and IL-17A synergistically induce inflammation when co-expressed [41]. No significant difference was observed between the IL-22 serum levels of gastritis and peptic ulcer patients, as compared with the corresponding levels in the uninfected patients. We observed that the association of IL-22 and IL-17A levels is higher in the peptic ulcer patients than in the uninfected group. It seems that IL-22 acts in synergy with IL-17A, what causes increased inflammation. IL-22 and IL-17A receptors exist on epithelial and fibroblast cells and act together to adjust local inflammation [42].

A recent research on mice has shown that Th17 cells can become either “classical” or “alternative” Th17 cell [43]. The affluence of TGF-β causes the production of “classical” Th17 cells, while the presence of IL-23 enhances the generation of “alternative” Th17 cells, which produces IL-22 and IL-17 with low levels of IL-10. These cells are less regulated and more pathogenic [43]. In the present study, we have demonstrated significantly higher levels of IL-22, IL-17A, and IL-23 cytokines, associated with Th17, in the moderate gastritis and peptic ulcer patients. Probably, “alternative” Th17 cells contribute to these diseases more than other Th17 cells.

The positive correlation between IL-21 and IL-17 serum and IL-22 levels in all of the patients could be explained by IL-21R expression on Th17 [24,33]. Therefore, IL-21 being an autocrine cytokine influences the production and differentiation of Th17. It can be suggested that the increase in Th17 frequency, and con-
subsequently, the increase in IL-17 and IL-22 levels, are responsible for gastritis and peptic ulcers in the studied patients. As shown, the positive correlation between TGF-β levels and Th17 cell frequencies and IL-17 cytokine levels indicates that TGF-β plays a role in Th17 production, leading to IL-17 cytokine secretion [44,45]. According to the above-mentioned comments, IL-17 is a critical factor in the development of such GI conditions.

In addition to evaluating the serum levels of IL-17A, IL-21, IL-22, IL-23, and TGF-β, and their differences in the above-mentioned patients, we also evaluated the levels of these cytokines in the same groups of patients through stimulating the PBMCs by *H. pylori* antigen, PHA, or anti-CD3/CD28.

IL-23 and IL-18 levels in the supernatants of the samples of gastric mucosa, taken from patients with duodenal ulcers, with gastric ulcers and with chronic gastritis, were higher among the *H. pylori* -positive patients, as compared with the *H. pylori* -negative patients [35]. In a recent study, increased IL-23, IL-6, and TGF-β cytokines were observed in the culture-containing macrophage cells stimulated with *H. pylori* antigen, followed by the activation of Th17, and the secretion of IL-17 [46]. In the present study, we observed the increase in IL-23, IL-22, and IL-21 levels in the supernatants of stimulated PBMCs with *H. pylori* antigen in the moderate gastritis patients. The levels of IL-23 and IL-17A were also significantly higher in the response to PHA in the moderate gastritis patients, as compared with the uninfected group. Bhuiyan also showed increased IL-17A cytokine levels in adults and children who were *H. pylori* -positive [47]. Furthermore, TGF-β secretion, in response to PHA and *H. pylori* antigen, was significantly higher in the peptic ulcer and gastritis patients, as compared with the uninfected cases, consistent with the results of the research of Sebahat.

**Conclusions**

Based on the obtained results, we can conclude that Th17, and its associated cytokines such as IL-21, IL-23, IL-22, IL-17A, and TGF-β, can play a critical role in peptic ulcer and gastritis in individuals infected with *H. pylori*. Serum and supernatant cytokine levels showed an increase in all of the patients with peptic ulcer and gastritis, and in particular, in the moderate gastritis group, in comparison with the uninfected individuals. Also, we found an association of IL-21, TGF-β with Th17, both in vitro and in vivo, in the peptic ulcer and moderate gastritis patients. As the concentrations of the cytokines, namely IL-23, IL-22, IL-17A, were simultaneously increased in the patients with peptic ulcer and gastritis, it can be suggested that the predominant Th17 is the “alternative” Th17 cells in these patients.

**Acknowledgements and Disclosures**

The funding of this project was provided by Prof. Alborzi Clinical Microbiology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran (Grant. No 92-3), and our gratitude goes to Miss. Esmat Kazemi and Dr. Hassan Khajehei for their linguistically edited draft. The authors would also like to thank the Clinical Studies Center of Nemazee Hospital for the statistical assistance.

**Competing interests:** The authors declare no conflict of interests related to this work.

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