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Frequency of γδ T Cells and Invariant Natural Killer T Cells in *Helicobacter Pylori*-infected Patients with Peptic Ulcer and Gastric Cancer

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ABSTRACT

To clarify the effect of γδ T cells and invariant Natural Killer T (iNKT) cells in pathophysiology of dyspeptic disorders, number of these two cells in patients with non-ulcer dyspepsia (NUD), peptic ulcer disease (PUD), and gastric cancer (GC) were compared.

Patients with dyspepsia were divided into three groups of NUD, PUD, and GC according to their endoscopic and histopathological examinations. *Helicobacter pylori* infection was diagnosed by rapid urease test and histopathology. The number of peripheral blood $CD3^+TCR\gamma\delta^+$ T cells and $CD3^+V\alpha24J\alpha18^+$ iNKT cells were determined by flow cytometry. Immunohistochemistry (IHC) was also used for identifying the $TCR\gamma\delta^+$ cells.

Forty two patients with NUD (31.6%), 44 with PUD (33.1%), and 47 with GC (35.3%) were included in the study. The frequency of $CD3^+TCR\gamma\delta^+$ T cells in peripheral blood of patients with GC (2.71±0.25) was significantly lower than that in NUD (3.97±0.32, $p<0.05$) and PUD groups (3.87±0.32, $p<0.05$). However, there was no significant difference in $CD3^+TCR\gamma\delta^+$ T cell percentage between the NUD and PUD groups. The frequency of $TCR\gamma\delta^+$ lymphocytes was significantly lower in tissue samples from patients with GC (4.81±0.53) than in NUD (11.09±1.09, $p<0.0001$) and PUD groups (11.11±1.01, $p<0.0001$). Also, we could not find any significant difference in the percentage of mucosal $TCR\gamma\delta^+$ cells between the NUD and PUD groups. The results showed no significant difference in iNKT cells percentage among the three groups of patients.

The results suggest that decreasing number of γδ T cells may be related to development and progression of gastric cancer.

Keywords: Antigen; Helicobacter pylori; Invariant Natural Killer T Cells; Peptic Ulcer; Receptor; Stomach Neoplasms; T-Cell, gamma-delta

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INTRODUCTION

Helicobacter pylori (H. pylori), a gram negative microaerophilic bacterium, is able to cause a wide range of gastric mucosal pathologies from asymptomatic gastritis to peptic ulcer and gastric cancer.[1,2] Host inflammatory response plays a pivotal role in *H. pylori* colonization and inducing inflammation by disturbing acid homeostasis.[3] The clinical outcome of gastric infection has been reported to be dependent to bacterial virulence factors, host mucosal factors, and environmental factors.[4] *H. pylori* virulence factors, mainly Cytotoxin-associated gene A (Cag A), and Vacuolating Cytotoxin A (Vac A), have been suggested to be important in bacterial entrance to gastric mucosa following the secretion of pro-inflammatory cytokines by epithelial cells. Chronic inflammation is associated with sustained interaction between *H. pylori* and host immune defense leading to the development of peptic ulcer or gastric cancer.[5] Indeed, the alternation of mucosal micro-environment by *H. pylori*[4] increases gastric epithelial cell apoptosis, cell cytotoxicity and inflammatory responses.[6-10] Persistence of *H. pylori* infection in gastric mucosa triggers innate immune responses which might lead to increased bacterial colonization, intensity of inflammation and initiation of adaptive immune responses.[11-14] On the other hand, the innate immune response might play a role in gastric carcinogenesis.[5] It has been shown that eradication therapy for *H. pylori* significantly reduces the risk of gastric cancer in infected individuals without premalignant lesions underlining the role of *H. pylori* in gastric carcinogenesis.[15] Innate immune T-cells, including invariant Natural killer T (iNKT) and γδ T cells, have been suggested to play a role in mucosal immune responses against *H. pylori.*[16]

T cells expressing TCRγδ, so called γδ T cells, seem to react to ligands that are not recognized by conventional T cells expressing TCRαβ. Although the main role of γδ T cells in immune responses against infections and cancers has not been completely understood,[17] they have been shown to link innate and acquired immunity and also associated with immune-regulation and immune-surveillance.[18,19] These cells are capable of recognizing antigens independently of MHC presentation and then kill infected and tumor cells.[18,20-22] The association between γδ T cells and *H. pylori*-mediated gastritis have been investigated in several studies. For example, it has been reported that infiltration of γδ T cells in *H. pylori*-infected gastric mucosa was significantly increased in patients with gastritis while it was decreased after *H. pylori* eradication therapy.[23]

iNKT cells are an unconventional T-cell subset with properties of the innate immune system.[24] They are characterized in human by expressing an invariant T cell receptor (TCR)-α chain (Vα24-Jα18) paired preferentially with Vβ11 chain.[25] These cells can recognize glycolipid antigens presented by non-polymorphic CD1d molecule.[25] Activated iNKT cells can mediate inflammation in several different organs systems.[26] The exact role of iNKT cells in *H. pylori*-mediated inflammation of the gastric mucosa has not been studied, yet; however, it has been reported that cholesteryl α-glucosides, which constitute 25% of total *H. pylori* lipids, can induce an immune response by iNKT cells causing inflammation in gastric mucosa.[27] Moreover, the role of iNKT cells in promoting antitumor immunity has been previously reported.[24]

Despite investigations, little is known about the exact role of iNKT and γδ T cells in the pathogenesis of peptic ulcer and gastric cancer in *H. pylori*-infected individuals. Therefore, this study aimed to determine γδ T iNKT cell and numbers in peripheral blood and gastric mucosa of patients with peptic ulcer and gastric cancer.

MATERIALS AND METHODS

Patients

Patients with dyspepsia who underwent esophago-gastro-duodenoscopy at Imam Hospital or Tooba Outpatient Clinic (Mazandaran University of Medical Sciences, Sari, Iran) between January 2011 and December 2012 were enrolled in the study. The study was approved by the Ethics Committee of Mazandaran University of Medical Sciences. Clinical history, demographic data, and written informed consent were taken from all study subjects.

Two tissue samples from the body or antrum of the stomach were taken from each patient, based on the endoscopic examination. Half of the first biopsy specimen was fixed and processed for routine histological examination. The other half was freshly embedded in optimal cutting temperature compound

(Tissue-Tek; Miles Inc., Elkhart, Ind.), immediately snap-frozen, and stored at -70°C for immunohistochemistry (IHC). The second biopsy specimen was embedded in urease test solution.

Based on the endoscopic and histopathological assessments, samples were divided into three groups of non-ulcer dyspepsia (NUD), peptic ulcer disease (PUD), and gastric cancer (GC). GC samples were included in the study if they were histopathologically diagnosed as adenocarcinoma of body or antrum of the stomach. None of the study subjects had a history of chronic inflammatory, autoimmune disorders, or received non-steroidal anti-inflammatory drugs (NSAIDs) during past two weeks, or had a history of *H. pylori* eradication therapy. Among patients with GC, none of them had received surgery, radiotherapy, chemotherapy, or any other form of medical interventions before the samples were collected.

The histpathological grade of the gastric tumors was determined on the basis of differentiation as described in Table 1.[28] The presence of *H. pylori* infection was determined by histopathological examination (including Giemsa staining) and a positive result for a rapid urease test performed on at least one additional biopsy sample. Patients were considered as *H. pylori*-positive if the results by at least one diagnostic method were positive and *H. pylori*-negative if results by both methods were negative. *H. pylori*-negative samples were then excluded from the study. A five-mL sample of peripheral blood was collected from each subject using ethylenediamine tetraacetic acid (EDTA, Sigma Chemical Co., St. Louis, MO) as the anticoagulant. The whole peripheral blood samples were then analyzed by flow cytometry.

Flow Cytometric Analysis of γδ T and iNKT Cells

White blood cells (WBC) were analyzed as follows: 100 µl of peripheral blood samples which had been collected in Vacutainer tubes containing EDTA were mixed in 12×75 mm test tube with 5 µl monoclonal antibodies specific for cell surface markers. To determine the frequency of γδ T cells, WBCs were labeled with mouse anti-human monoclonal antibodies targeted against CD3-FITC (SK7) and TCRγδ-PE (B1-1) (eBioscience, San Diego, CA, USA). To measure the frequency of iNKT cells, cells were stained with optimized amounts of fluorochrome labeled mouse anti-human monoclonal antibodies (clones) targeted against CD3-FITC (SK7) and Vα11Jα18-PE (6B11) (eBioscience). Mouse IgG1κ-FITC (P3.6.2.8.1) and IgG1κ-PE (P3.6.2.8.1) (eBioscience) were also used as isotype controls. The tubes were incubated in the dark for 30 min at room temperature. Following incubation, erythrocytes were lysed with BD lysing solution (BD Biosciences, San Jose, CA). The remaining cells were then washed twice with 2 ml phosphate-buffered saline (PBS) containing 0.01% sodium azide. Cell preparations were fixed in 500 µl fix solution (2% paraformaldehyde in PBS). Two color flow cytometric analysis was performed on a FACS Calibur Cytometer (BD FACS Calibur, BD) using the CellQuest software.

Immunohistochemical Analysis

TCRγδ staining was conducted using the avidin–biotin–peroxidase complex method. Before cutting sections, the temperature of the block was allowed to equilibrate to temperature of the cryostat (–20°C). For immunostaining, 5-µm cryosections were cut and mounted onto slides precoated with Poly L-Lysin. After fixation in cold acetone, endogenous peroxidase activity was blocked by Peroxidase 1% (Dako) and incubated with primary monoclonal antibody to the TCRγδ protein (clone B1, 1:100; BD Biosciences) for 60 min at room temperature. Samples were then incubated with a biotinylated secondary antibody (1:50) and streptavidin–horseradish peroxidase (both included in Anti-Ig HRP Detection Kit, BD Biosciences) for 30 min at room temperature following by development with diaminobenzidine for 5 min and counterstaining with hematoxylin. Slides were mounted using Entelan, and viewed under a light microscope. The number of $TCR\gamma\delta^+$ cells was counted in three microscopic fields. Negative control staining was performed with cold PBS, instead of the primary antibody. All IHC evaluations were performed in a blinded manner.

Statistical Analysis

Statistical analysis was performed using the SPSS statistical package (SPSS, Chicago, IL, USA). The results were evaluated by independent-samples t-test, Mann-Whitney U test, and Pearson and Spearman correlation tests where appropriate. Findings were considered significant when P-values were <0.05. The results presented in the text and tables represent mean ± standard deviation (SD) or standard error (SE) where appropriate.

Table 1. Characteristics of the study subjects

		NUD (n=42)	PUD (n=44)	GC (n=47)
Age (mean±SD)		48.9±15.7	47.3±18.7	63.2±17.0
Sex	Male	20 (43.5%)	21 (47.5%)	29 (56.9%)
	Female	26 (56.5%)	25 (54.3%)	22 (43.1%)
Tumor Grade	I (Well differentiated)			3 (5.9%)
	II (Moderately differentiated)			10 (16.9%)
	III (Poorly differentiated)			15 (29.4%)
	IV (Undifferentiated)			4 (7.8%)

NUD: Non-ulcer dyspepsia, PUD: Peptic ulcer disease , GC: Gastric Cancer , ND: Not defined

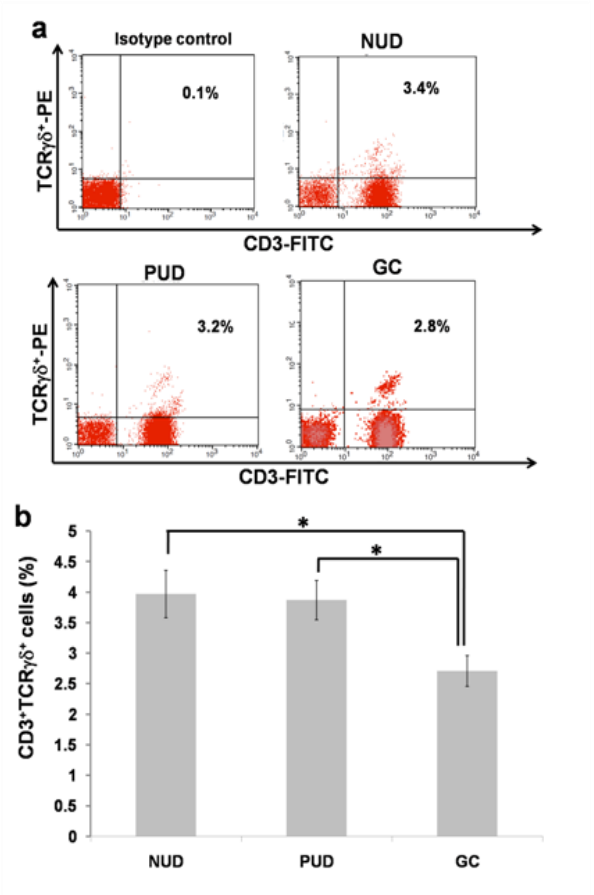

Figure 1. (a) Frequency of γδ T cells in peripheral blood of NUD, PUD, and GC subjects. The dot plots demonstrate typical staining of $CD3^+TCR\gamma\delta^+$ T cells in three patients with NUD and GC, respectively. The populations of $CD3^+TCR\gamma\delta^+$ cells as a percentage of total $CD3^+$ T cells in the peripheral blood are shown. (b) Mean frequencies of γδ T cells in peripheral blood from NUD, PUD, and GC patients.* $p<0.05$

RESULTS

Patients

A total of 133 cases including 42 patients with NUD (31.6%), 44 with PUD (33.1%), and 47 with GC (35.3%) were included in the study based on the endoscopic and histo-pathological assessments. The characteristics of the study subjects are summarized in Table 1. All participants were *H. pylori* positive. The histological grades of the GC samples are also shown in Table 1.

The Frequency of γδ T Cells in NUD, PUD, and GC Subjects

To analyze the frequency of γδ T cells, $CD3^+TCR\gamma\delta^+$ T cells were evaluated by flow cytometry and expressed as a percentage of the total $CD3^+$ cells. Representative flow cytometric data in peripheral blood from patients with NUD, PUD, and GC are shown in Table 2 and Figure 1a. The results showed that the frequency of γδ T cells in peripheral blood of patients with GC (2.71±0.25) were significantly lower than that in NUD (3.97±0.32, $p<0.05$) and PUD groups (3.87±0.32, $p<0.05$) (Figure 1b). However, there was no significant difference in γδ T cell percentage between the NUD and PUD groups ($p>0.05$) (Figure 1b).

To enumerate γδ T cells present in the gastric mucosa, we determined the number of $TCR\gamma\delta^+$ cells by IHC in three consecutive sections. The expression of TCRγδ was observed on the surface of lymphocytes (Table 2 and Figure 2a). Summarized data with quantitative analysis of IHC showed that the frequency of $TCR\gamma\delta^+$ lymphocytes was significantly lower in

tissue samples from patients with GC (4.81±0.53) than in NUD (11.09±1.09, $p<0.0001$) and PUD groups (11.11±1.01, $p<0.0001$) (Figure 2b). However, there was no significant difference in mucosal TCRγδ$^+$ cell number between the NUD and PUD groups ($p>0.05$) (Figure 2b). Further analysis of patients with GC showed that the frequencies of TCRγδ$^+$ cell were not significantly different among the four grades of tumors.

The results of analysis of γδT cells in peripheral blood and in gastric mucosa among the three groups of patients were in agreement with each other, although Pearson's correlation showed no significant correlation between the two variables.

The Frequency of CD3$^+$Vα24Jα18$^+$ iNKT Cells in NUD, PUD, and GC Subjects

To analyze the frequency of CD3$^+$Vα24Jα18$^+$ iNKT cells, peripheral blood samples were evaluated by flow cytometry and expressed as a percentage of the total CD3$^+$ cells. Representative flow cytometric data in peripheral blood from patients with NUD, PUD, and GC are shown in Table 2. The results showed no significant difference in iNKT cells percentage among patients with NUD (0.30±0.05), PUD (0.25±0.05), and GC (0.21±0.05).

Table 2. Frequencies of γδ T and iNKT cells in peripheral blood or gastric mucosa of NUD, PUD, and GC patients.

	NUD		PUD		GC	
	Number of Patients	**% of cells (mean±SE)**	**Number of Patients**	**% of cells (mean±SE)**	**Number of Patients**	**% of cells (mean±SE)**
γδ T cells in peripheral blood	42	3.97±0.32*	44	3.87±0.32*	47	2.71±0.25
γδ T cells in gastric mucosa	22	11.09±1.09***	26	11.11±1.01***	27	4.81±0.53
iNKT cells in peripheral blood	24	0.30±0.05	20	0.25±0.05	24	0.21±0.05

∗A $p<0.05$ difference when compared with those of GC group

∗∗∗A $p<0.0001$ difference when compared with those of GC group

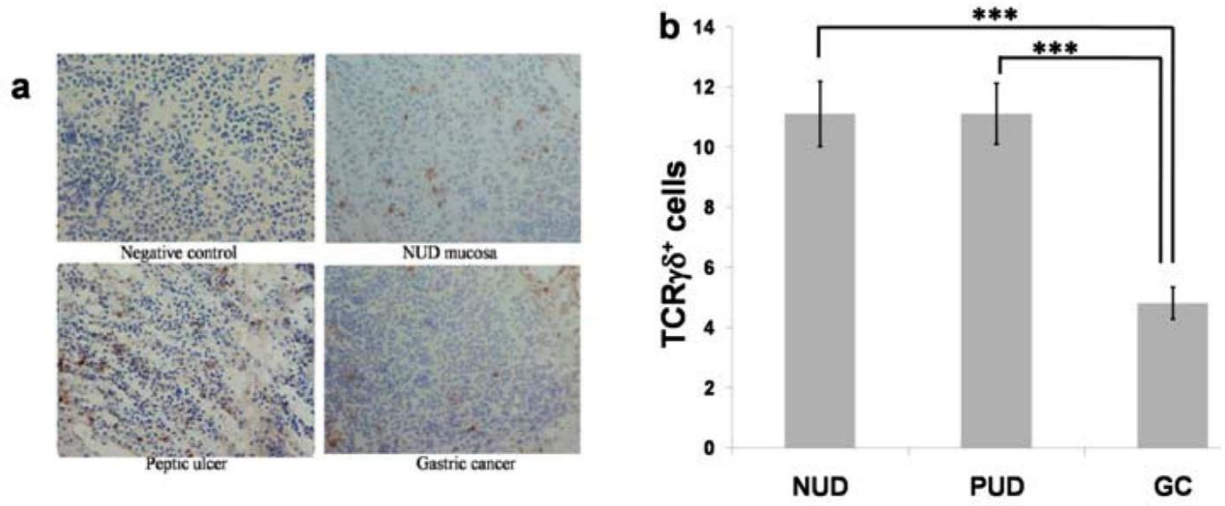

Figure 2. IHC of TCRγδ$^+$ cells in NUD, PUD, and GC subjects. (a) Representative immunostaining for TCRγδ$^+$ is shown for negative control, NUD, PUD, and GC. (b) The number of TCRγδ$^+$ cells in gastric mucosa of patients with NUD, PUD, and GC.*$p<0.0001$**

DISCUSSION

The present study compared the number of two major innate immune T cells, γδ T and iNKT, in patients with NUD, PUD, and GC. Given the role of immune responses against *H. pylori* infection in the development of PUD and GC, only *H. pylori*-positive subjects were enrolled in the study.

The present study showed that the number of γδ T cells in both peripheral blood and gastric mucosa of GC patients was significantly lower than that in NUD or PUD groups. Concurrently, Brant et al. observed only reduced numbers of γδ T cells within renal cell carcinoma (RCC) specimens.[29] Decreased numbers of γδ T cells within RCC tumors were also reported in studies using IHC and flow cytometry,[30,31] where a subset of γδ T cells (Vδ2) in peripheral blood of gastric cancer patients was found to be fewer than that in healthy controls.[17]

Comparing numbers of γδ T cells in peripheral blood of patients with early and advanced gastric cancer, Kim et al. found that patients with early gastric cancer showed significantly higher proportion of γδ T cells than healthy controls.[32] In a study by Ma et al., γδ T cells are found to be a dominant population in the breast cancer suppressive microenvironment during the cancer progression.[33] However, the numbers of γδ T cells in peripheral blood or in tumor tissue seem to be different in different types of cancer. As a result, the physiologic or pathophysiologic functions of γδ T cells are still extremely ambiguous. They may be influenced by organ-, host-, and disease-specific factors.[29]

The present study shows that γδ T cells in both peripheral blood and mucosal tissue of GC patients were reduced simultaneously. Although γδ T cells have been considered as immune effector cells in the early stage of cancer, the tumor development in gastric cancer may be impacted by the number and function of these cells. Considering that we previously found that the number of the two immune-suppressor cells, regulatory T cells (Tregs) and Myeloid-Derived Suppressor Cells (MDSCs), increased in patients with gastric cancer (manuscript in preparation), production of inhibitory cytokines by these cells are probably led to reduce the percentage of γδ cells in cancer microenvironment. Another explanation may be associated with exhaustion of cytotoxic T cells during cancer progression that lead to expression of inhibitory molecules on the surface of these cells and subsequently resulted in cell death reducing cell numbers. In agreement with this, recent observations revealed that *H. pylori* Lipopolysaccharide (LPS) decreases IFN-□-related cellular immunity and cytotoxicity of mononuclear cells through LPS-TLR4 binding which lead to gastric cancer expansion and progression. LPS of *H. pylori* therefore can be considered as a negative regulator for the first line of immune defense such as naturally cytotoxic lymphocytes.[34]

In the present study, the results of analysis of γδ T cells in peripheral blood and in gastric mucosa among the three groups of patients were in agreement with each other, although Pearson's correlation showed no significant correlation between the two variables. In this study, we failed to determine the stage of cancer in GC patients. Nevertheless, it seems that in the early stages of gastric cancer, the number of γδ T cells in mucosal tissue is not necessarily correlated with the γδ T cells in peripheral blood. However, in the late stages of gastric cancer, they are more correlated.

This study also determined the number of $CD3^{+}V\alpha24J\alpha18^{+}$ iNKT cells in peripheral blood by flow cytometry among the three groups of NUD, PUD, and GC. The results showed that the number of iNKT cells in peripheral blood of GC patients was lower than that in NUD and PUD patient; however, this decrease was not statistically significant. Similar results were reported by Molling et al. where no significant difference in the percentage of $CD4^{-}CD8^{-}$ cells or NKT cells observed between breast cancer patients and healthy controls.[24] In an earlier study, also researchers found that the percentage of NKT cells producing IFN-γ (approximately 57%) was similar in primary lung cancer patients and healthy controls.[35]

These findings are in contrast with an earlier study by Molling et al. where a significant decrease in the number of circulating iNKT cell (47%) reported compared with the healthy controls.[24] Similar observation, based on a small-scale study, was also reported by Yanagisawa et al. where the number of circulating $V\alpha24V\beta11^{+}$ iNKT cells in cancer patients was lower in comparison to healthy controls.[36] Tahir et al. revealed that $V\alpha24V\beta11^{+}$ iNKT cells numbers are reduced in patients with advanced prostate cancer.[37] The low number of iNKT cells in blood circulation, however, may be a risk factor for the progression but

not initiation of malignant tumors

In addition, tumor microenvironment may result in impaired proliferation or increased death of iNKT cells which, in turn, lead to reduced number of these cells in peripheral blood of cancer patients.[24] In this regard, some studies found only a small number of $CD56^+CD3^+$ NKT cells or $V\alpha24V\beta11^+$ iNKT cells in the liver tumor [38, 39]. Moreover, iNKT cells may accumulate in tumor tissue which leads to decreased number of these cells in peripheral blood.[24]

On the other hand, Motohashi et al. illustrated an increase in $V\alpha24V\beta11^+$ iNKT cell number in lung tumors.[40] Moreover, immature dendritic cells pulsed with α-GalCer were injected into the advanced cancer patients which resulted in increase in the number of the circulating $V\alpha24V\beta11^+$ iNKT cells. However, this increase was transient and the iNKT cells numbers returned to baseline after 7–14 days [41]. The present study was conducted on the number of $CD3^+V\alpha24J\alpha18^+$ iNKT cells in peripheral blood; however, if the number of these cells would be determined in gastric tissue specimens, the results might be more obvious. Based on the above findings, and considering rather small numbers of GC samples in our study, and the negligible number of iNKT cells in the human peripheral blood, it is not surprising to observe no increase or decrease of iNKT cells in the peripheral blood of GC patients. In the present study, the numbers of γδ T cells or iNKT cells in different histo-pathological grades of GC patients were compared and the results showed no significant differences. This might be related to the few numbers of patients in each tumor grade.

In summary, this study showed that the numbers of γδ T cells in both peripheral blood and gastric mucosa of GC patients were significantly lower than those in NUD or PUD groups.

Since γδ T cells have been previously shown to play a protective role against tumors, it is hypothesized that the tumor microenvironment, including regulatory T cells and their inhibitory cytokines, decrease the number and function of γδ T cells which can finally lead to tumor progression. This study failed to show a significant difference in the number of $V\alpha24J\alpha18^+$ iNKT cells in NUD, PUD, and GC groups. Future investigation on the effect of *H. pylori* on the number and function of γδ T cells might lead to better understanding of the pathogenesis of *H. pylori*-related peptic ulcer and gastric cancer and also changes in the current strategies for cancer treatment.

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