Regulatory T Cells and Myeloid-Derived Suppressor Cells in Patients with Peptic Ulcer and Gastric Cancer

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ABSTRACT

Background: Regulatory T Cells (Tregs) and Myeloid-Derived Suppressor Cells (MDSCs) are two main regulatory cells modulating the immune responses in inflammation and cancer. **Objective:** To investigate and compare Tregs and MDSCs in peptic ulcer and gastric cancer. **Methods:** Patients with dyspepsia were selected and divided into three groups of non-ulcer dyspepsia (NUD, n=22), peptic ulcer disease (PUD, n=25), and gastric cancer (GC, n=27) according to their endoscopic and histopathological examinations. *Helicobacter pylori* infection was diagnosed by rapid urease test and histopathology. The number of peripheral blood $CD4^+CD25^+FoxP3^+$ Tregs and $CD14^+HLA\text{-}DR^-$ MDSCs were determined in all patients, by flow cytometry. The number of $FoxP3^+$ regulatory T cells was also determined by immunohistochemistry (IHC). **Results:** The percentage of peripheral blood Treg cells in both PUD (0.81 ± 0.39, $p<0.001$) and GC groups (0.98 ± 0.65, $p<0.001$) were significantly higher than in NUD group (0.46 ± 0.10). These results were also confirmed by IHC. A significantly higher percentage of MDSCs in patients with PUD (0.73 ± 0.19, $p<0.001$) and GC (0.73 ± 0.16, $p<0.001$) was also observed when compared to NUD group (0.46 ± 0.16). There was no difference in the percentages of these two cell types between the PUD and GC groups. The percentages of Tregs and MDSCs in patients with PUD and GC were not significantly correlated. **Conclusions:** Both Tregs and MDSCs showed higher frequencies in PUD and GC. These results suggest that immune-modulation by the Tregs and MDSCs may play a role in the pathogenesis of PUD and GC.

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Keywords: Gastric Cancer, Myeloid-Derived Suppressor Cell, Peptic Ulcer, Regulatory T Cells

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INTRODUCTION

Peptic ulcer disease (PUD) and gastric cancer (GC) are common diseases worldwide. PUD is usually accompanied with a low health-related quality of life, while GC is the fourth most common cancer in the world and the second leading cause of cancer-related death (1,2). Several genetic and environmental factors have been suggested to cause PUD and/or GC (2). Recent studies have demonstrated the role of host immune responses in mucosal inflammation leading to PUD or GC (2). Furthermore, *Helicobacter pylori* infection is now identified as a major cause of both PUD and GC (3-5). Therefore, it seems that suppression of host immune responses against *H. pylori* could be a risk factor for developing both gastric mucosal inflammation and GC (6,7). Two major subsets of regulatory cells, regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) have the potential to suppress immune responses of T cells via diverse mechanisms (8-10).

Tregs are a small population of T lymphocytes playing a central role in the maintenance of immunological self-tolerance and homeostasis of the immune system, preventing the development of autoimmune diseases (3). Tregs represent between 5–10% of peripheral CD4+ T cells in healthy individuals (11). Naturally occurring Tregs originate from the thymus as CD4+ T cells expressing high levels of CD25 along with the transcription factor forkhead box P3 (FoxP3) (12-14). Foxp3 plays an important role in Treg development and function (14). Treg cells are suggested to actively suppress the host immune response to *H. pylori*, promoting bacterial persistence and possibly long-term pathology as a result of chronic infection (13,15).

MDSCs arise from myeloid progenitor cells that have failed to terminally differentiate into mature granulocytes and macrophages. MDSCs are known as potent immunosuppressive agents. They can upregulate the expression of inducible nitric oxide synthase (iNOS), arginase-1 (ARG 1), and increase the production of nitric oxide (NO) and reactive oxygen species (ROS), all of which can suppress the T cell function (16,17). Human MDSCs are mostly defined as CD14-CD11b+ cells or, more closely, as cells that express CD33, the common myeloid marker, without expressing markers of mature myeloid and lymphoid cells and the MHC-class-II molecule HLA-DR (18-21). A monocytic subset of MDSCs with CD14+HLA-DR- phenotype was also reported in patients with different types of cancer (10,20,22). In several cancers including hepatocellular carcinoma, CD14+HLA-DR- MDSCs have been shown to be involved in the induction of Tregs (18,21,22).

Several studies have reported an increased number of CD4+CD25+T cells in the peripheral blood of patients with different types of cancer; however, the number of CD4+CD25+FoxP3+Treg cells in the peripheral blood of patients with PUD and GC has not been studied, yet (23). Furthermore, the role of MDSCs and Tregs in PUD has not been clearly understood. Therefore, the present study aimed to compare the number of Tregs and MDSCs in patients with PUD and GC to elucidate the correlation of these cells with these diseases.

MATERIALS AND METHODS

Patients and Sample Preparation. 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 biopsies were taken from the body or antrum of the stomach of each patient, during endoscopy. 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. Moreover, a 5-mL sample of peripheral blood was collected from each subject using EDTA as the anticoagulant. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll (Sigma-Aldrich, USA) density gradient according to the manufacturer's instruction. The viability of isolated cells was more than 95% as assessed by Trypan blue viability test.

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

Flowcytometric Analysis of Tregs and MDSCs. To determine the frequency of Tregs, PBMCs were first labeled with mouse anti-human monoclonal antibodies (clones) targeted against CD4-FITC (RPA-T4) and CD25-PECy5 (BC96) (eBioscience, San Diego, CA, USA), along with mouse IgG1κ-FITC (P3.6.2.8.1) and IgG1κ-PE (P3.6.2.8.1) (eBioscience) as isotope controls. Intracellular staining for FoxP3 was performed using mouse anti-human FoxP3 antibody-PE (Clone 236A/E7), mouse IgG1κ-PE as the isotype control, and Fixation/Permeabilization kit (all from eBioscience) following the manufacturer's protocol. To determine the frequency of $CD14^{+}HLA\text{-}DR^{-}$ MDSCs, PBMCs were stained with optimized amounts of fluorochrome labeled mouse anti-human monoclonal antibodies (clones) targeted against: CD14-PE (61D3) and HLA-DR-PE-cy5 (LN3) (eBioscience) in combination with mouse IgG1κ-FITC (P3.6.2.8.1) and IgG1κ-PE (P3.6.2.8.1) (eBioscience), as isotype controls. Cells were then finally analyzed on a FACS Calibur (BD). All data analysis was performed using CellQuest software (BD).

Immunohistochemical Analysis. Foxp3 staining was conducted using the avidin–biotin–peroxidase complex method. Before cutting sections, the temperature of the block was allowed to equilibrate to the 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 monoclonal mouse anti-human

FoxP3 antibody (clone 150D/E4, 1:100; eBioscience) 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) 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 $FoxP3^+$ cells was then counted in three microscopic fields, and the mean value was considered as the number of Tregs in each slide. 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.

RESULTS

Patients. A total of 74 patients, including 22 with NUD (29.72%), 25 with PUD (33.74%), and 27 with GC (36.48%), were enrolled in the study based on the endoscopic and histopathological assessments. The characteristics of the study subjects are summarized in Table 1.

Table 1. Characteristics of NUD, PUD, and GC patients.

	NUD (N=22)	PUD (N=25)	GC (N=27)	P Value
Age (years, mean ± SD)	50.41 ± 14.54	47.20 ± 18.34	63.22 ± 14.54	0.018[a]
Gender				0.311 [a]
Male	7 (31.8%)	9 (36%)	14 (51.9%)	
Female	15 (68.2%)	16 (64%)	13 (48.1%)	
Smoking status	0 (0%)	2 (8.3%)	5 (19.4%)	0.020 [a]

[a] Compared among the three groups

All study subjects were *H. pylori* positive. The histological grades of the GC are shown in Table 2. None of the GC patients had received therapy prior to blood and biopsy donation.

Table 2. Histopathological grade of gastric tumors.

Tumor differentiation	Number (%) of Patients
Grade I (Well differentiated)	4 (14.8%)
Grade II (Moderately differentiated)	6 (25.9%)
Grade III (Poorly differentiated)	13 (44.4%)
Grade IV (Undifferentiated)	4 (14.8%)

The Frequency of $CD4^+CD25^+FoxP3^+$ Tregs in NUD, PUD, and GC Subjects. To analyze the frequency of Tregs, $CD4^+CD25^+FoxP3^+$ cells were enumerated by flowcytometry and expressed as a percentage of the total $CD4^+$ cells. Representative flowcytometric data in PBMCs from patients with NUD, PUD, and GC are shown in Figure 1. The results showed that the frequency of Tregs in PBMCs from patients with PUD (0.81 ± 0.39, $p<0.001$) and GC (0.98 ± 0.65, $p<0.001$) were significantly higher than that in NUD (0.46 ± 0.10) (Figure1). However, there was no significant difference in Treg percentage between the PUD and GC groups ($p>0.05$) (Figure1).

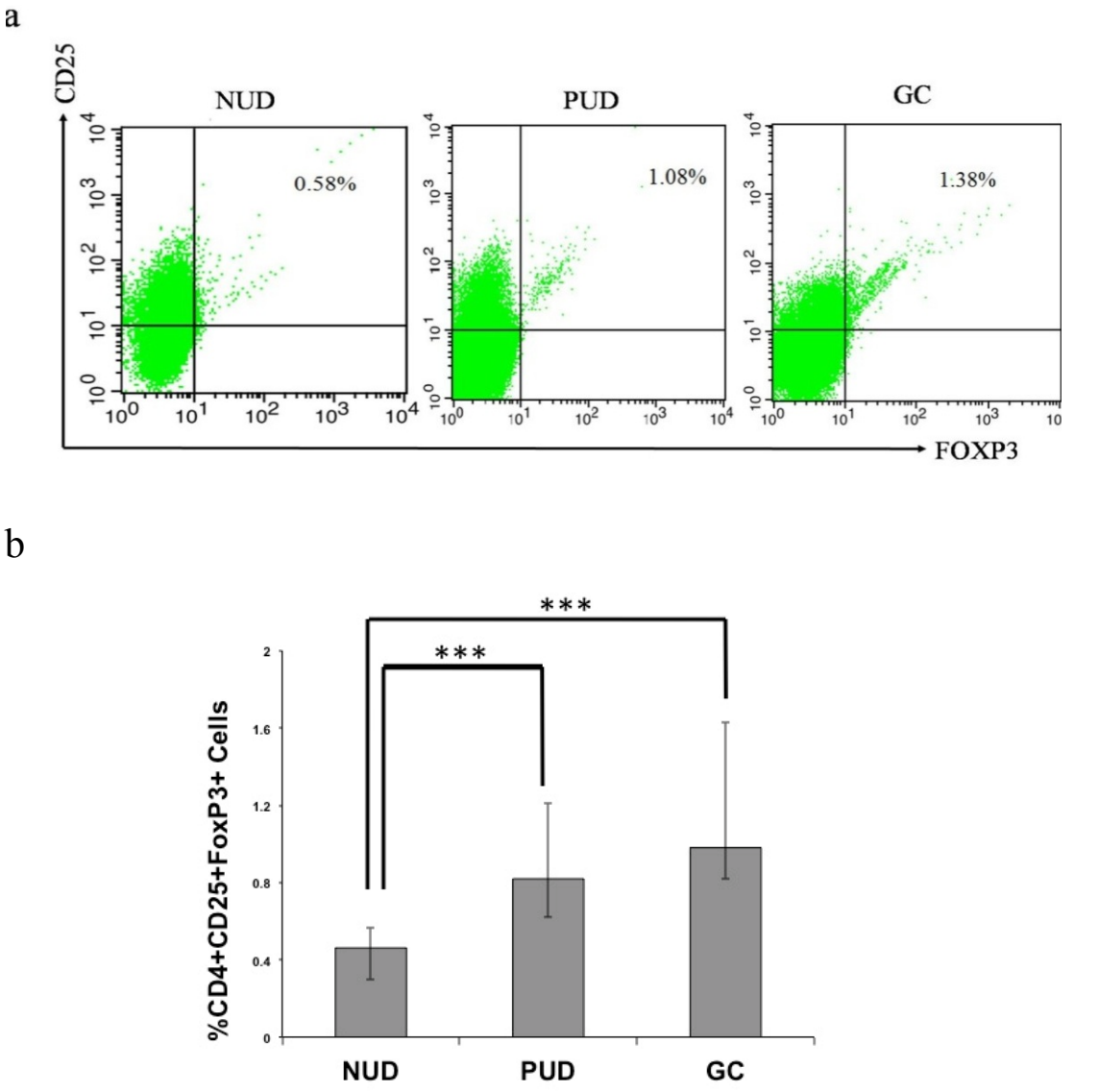

Figure 1. (**a)** Frequency of $CD4^+CD25^+FoxP3^+$ regulatory T cells in PBMCs from three representative patients with NUD, PUD, and GC, respectively. (**b)** Mean frequencies of $CD4^+CD25^+FoxP3^+$ regulatory T cells in PBMCs from patients with NUD, PUD, and GC.*** $p<0.001$.

To enumerate Tregs present in the gastric mucosa, we determined the number of $Foxp3^+$ cells by IHC in three consecutive sections. The expression of FoxP3 was seen in the nucleus of lymphocytes (Figure 2). Summarized data with quantitative analysis of IHC showed that the frequency of FoxP3 positive lymphocytes was significantly higher in tissue samples from PUD and GC patients than those of NUD patients (16.00 ± 1.42 and 20.11 ± 1.36 vs. 2.36 ± 0.53, respectively, $p<0.001$) (Figure2). However, there was no significant difference in mucosal Treg percentage between the PUD and GC groups ($p<0.05$) (Figure 2). Further analysis of samples from patients with GC showed that the frequencies of $CD4^+CD25^+FoxP3^+$ Tregs were not significantly different among the four grades of tumors.

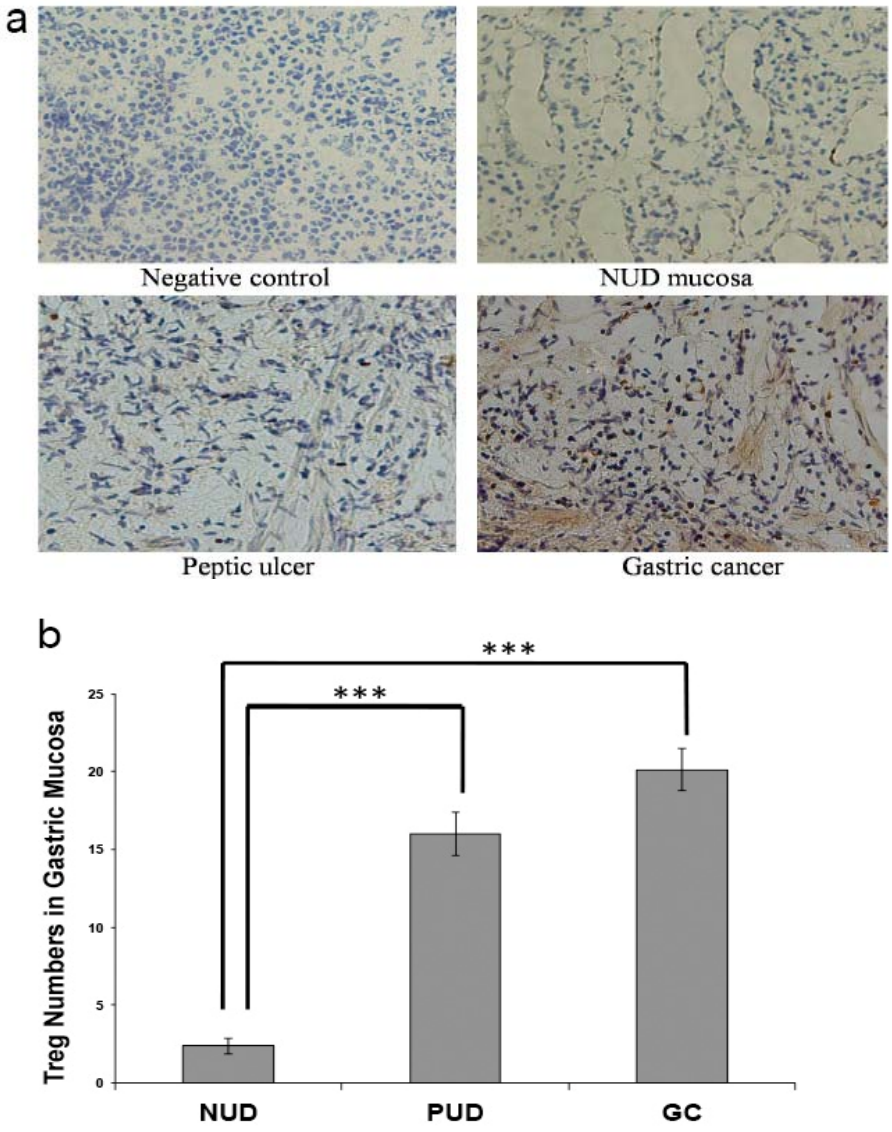

Figure 2. IHC of FoxP3+ cells in NUD, PUD, and GC subjects. **(a)** Representative immunostaining for FoxP3 is shown for negative control, NUD, PUD, and GC. **(b)** The number of $FoxP3^+$ cells in gastric mucosa from patients with NUD, PUD, and GC. ****p*<0.001.

The frequency of $CD14^+HLA\text{-}DR^-$ MDSCs in NUD, PUD, and GC subjects. The frequency of $CD14^+HLA\text{-}DR^-$ cells in PBMCs of patients NUD, PUD, and GC were evaluated using flowcytometry and shown in Figure 3. The percentages of $CD14^+HLA\text{-}DR^-$ MDSCs were significantly higher in PUD (0.73 ± 0.19, $p<0.001$) and GC (0.73 ± 0.16, $p<0.001$) groups compared with NUDs (0.46 ± 0.16) (Figure 3). However, there was no difference in the percentage of these cells between the PUD and GC patients ($p<0.05$) (Figure 3). We next investigated whether the percentage of $CD14^+HLA\text{-}DR^-$ cells in patients is associated with that of $CD4^+CD25^+Foxp3^+$ Treg cells; however, the Pearson's correlation test showed no significant correlation between the percentage of $CD14^+HLA\text{-}DR^-$ cells and Treg ($rs=0.161$, $p>0.05$). Further analysis of patients with GC showed that the frequencies of $CD14^+HLA\text{-}DR^-$ MDSCs were not significantly different among the four grades of tumors.

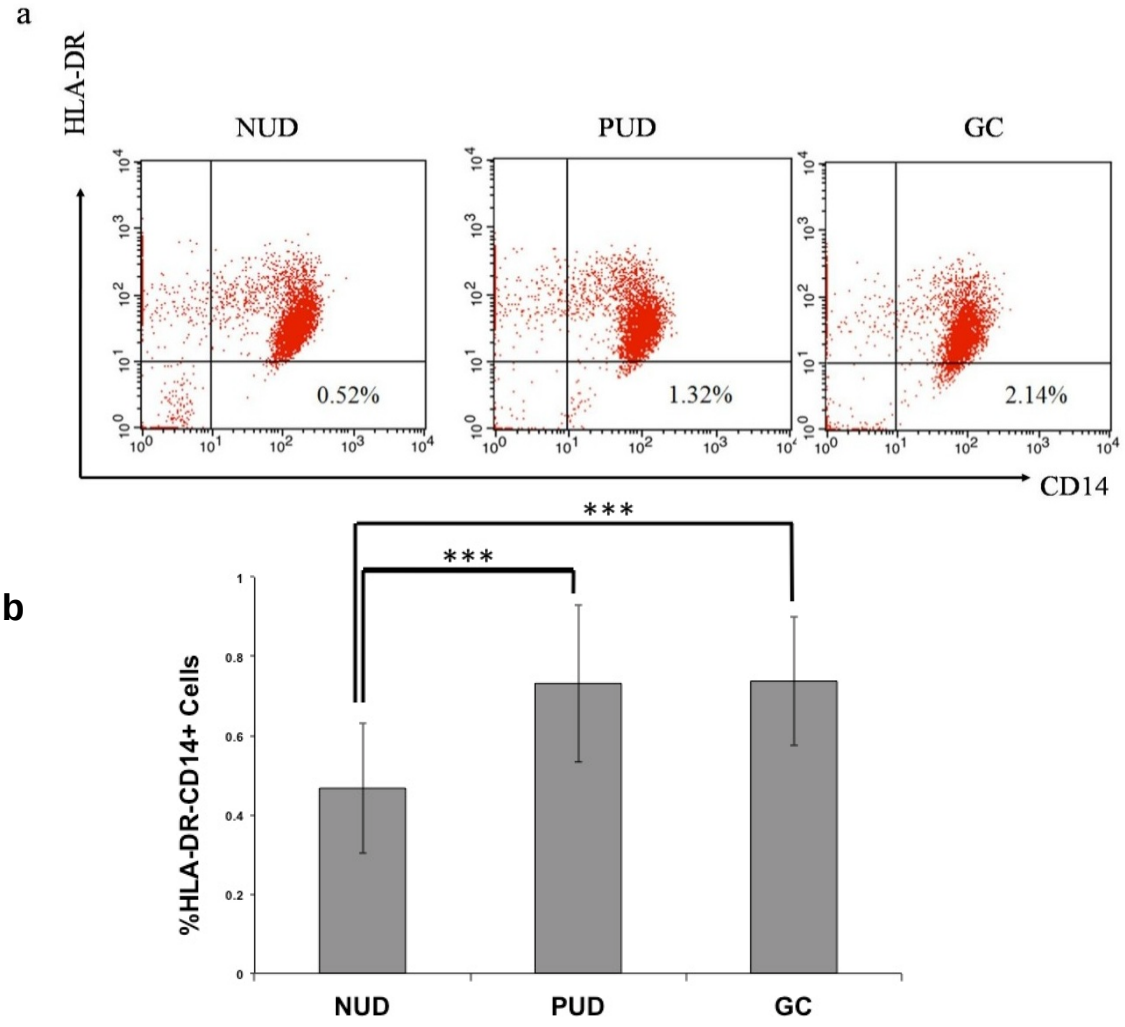

Figure 3. (**a)** Frequency of $CD14^+HLA\text{-}DR^-$ cells in PBMCs from three representative patients with NUD, PUD, and GC, respectively. **(b)** Mean frequencies of $CD14^+HLA\text{-}DR^-$ cells in PBMCs from patients with NUD, PUD, and GC. ****p*<0.001.

DISCUSSION

The present study compared the number of the two major immunosuppressive cells, CD4+CD25+FoxP3+ Treg cells and CD14+HLA-DR- MDSCs among *H. pylori* positive patients with non-ulcer dyspepsia (NUD), peptic ulcer disease (PUD), and gastric cancer (GC).

First, it was shown that the number of CD4+CD25+FoxP3+ Tregs in both peripheral blood and gastric mucosa was higher in PUD and GC groups compared to that of NUD group. Concurrently Cheng *et al.*, using IHC, showed an increased number of CD25+ and FoxP3+ cells in gastric mucosa of both PUD and GC groups compared to that of NUD group (3). Other studies have also shown that infection of the gastric mucosa with *H. pylori* may cause an increase in the number of Treg cells (6,13,24,25). Enarsson *et al.* also revealed that Tregs from patients with gastric adenocarcinoma could suppress *H. pylori*-induced T cell responses *in vitro*. They concluded that Tregs might help bacterial persistence and possibly development of gastric adenocarcinoma (26). The exact role of Tregs in gastric cancer has not been identified, yet. Tregs have been shown to suppress the host immune response against different tumors such as ovarian carcinoma and head and neck squamous cell carcinoma (27,28). More interestingly, FoxP3, as a key molecule and specific marker for the development and function of Tregs, was shown to be upregulated in gastric tumor cells, although its tumor suppressor role could be reduced due to the inflammation (29). Concurrently, Ma *et al.* showed the FoxP3 upregulation in gastric tumor cells and that FoxP3 could increase cell apoptosis (30). Taken together, it can be suggested both Tregs and FoxP3 alone play an important role in gastric cancer; however, finding the exact role of Treg cells in the proliferation or apoptosis of the cancer cells needs further investigations.

The present study also determined the number of CD14+HLA-DR- MDSCs in peripheral blood among the three groups of NUD, PUD, and GC, and showed increased numbers of these cells in PUD and GC groups compared to that of NUD group. Several studies have previously reported increased numbers of CD14+HLA-DR- MDSCs in patients with different types of cancers (8,10,20,31); however, few studies uncovered the number of these cells in inflammatory conditions. It has been reported that proinflammatory cytokines, such as Interleukin-1β (IL-1β) and IL-6, as well as the bioactive lipid, PGE2, can induce the accumulation of MDSCs, which supports the role of inflammation in MDSC expansion (16). Other reports also indicated that S100A8 and S100A9, two calcium-binding proteins released by neutrophils, cause inflammation and subsequent induction of MDSCs, since it has been shown that MDSCs also express receptors for these proteins on their cell surface (32). Cheng *et al.* demonstrated that S100A9 prevents the differentiation of myeloid precursors into functional dendritic cells or macrophages through a STAT3-dependent pathway. They also reported decreased number of MDSCs in mice lacking this protein (33). Taken together, it can be suggested that *H. pylori*-induced inflammation of the gastric mucosa may cause the increased number of MDSCs which, in turn, leads to development to PUD or GC.

Although Tregs and MDSCs showed significantly higher numbers in PUD and GC compared to NUD samples, Pearson's correlation test showed no significant correlation between the two variables. Concurrently, Yuan *et al.* (20) examined the numbers of CD4+CD25+CD127- Tregs and CD14+HLA-DR- MDSCs in patients with bladder cancer which showed no significant correlation between them. In contrast, Gabitass *et al.* showed a positive correlation between HLA-DR-L in Low/-CD33+CD11b+ MDSCs

and CD4+CD25+CD127-FoxP3+ Tregs in patients with esophageal and gastric cancer (9). Finally, Chikamatsu *et al.* observed a significant but reverse correlation between the percentages of CD14+HLA-DR- MDSCs and CD4+CD25+CD127– Tregs in patients with squamous cell carcinoma of the head and neck (10). The discrepancies among the results of different studies can be explained by the differences in precursors of Tregs and MDSCs which may result in separate proliferation of these two cells. Regarding the role of MDSCs in the production of Treg cells, it has been revealed that MDSCs may contribute to induction of Tregs in cancer through TGF-β, IL-10 and/or arginase metabolism (34,35). Recently, Hoechst *et al.* demonstrated that CD14+HLA-DR- cells from patients with hepatocellular carcinoma induced a CD4+CD25+FoxP3+ Treg population when co-cultured with autologous T cells (8).

The numbers of CD4+CD25+FoxP3+ Tregs and CD14+HLA-DR- MDSCs in different grades of GC patients were also compared in this study which showed no significant difference. This may be due tothe few patients in each tumor grade group.

In summary, this study showed the higher number ofCD4+CD25+FoxP3+ Tregs and CD14+HLA-DR-MDSCs in *H. pylori* positive patients with PUD and GC compared to those with NUD. However, no significant difference was found in the number of each of these cells between PUD and GC groups. It can thus be concluded that *H. pylori*-induced inflammation of the gastric mucosa results in the increased production of Tregs and MDSCs, both can modulate the immune responses against *H. pylori*. The subsequent chronic *H. pylori* infection may finally lead to the development of peptic ulcer or gastric adenocarcinoma.

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