


Site-specific differences in T lymphocyte composition of the gastric mucosa after *Helicobacter pylori* eradication

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Abstract

In our earlier work, we revealed that inflammation of the lesser curvature of the gastric body and antrum could constitute independent risk factors for gastric cancer development, while inflammation of the greater curvature was not. The aims of this study were as follows: first, to reveal the differences between T lymphocyte populations of the gastric antrum and the greater and lesser curvatures of the gastric body in patients after *Helicobacter pylori* eradication; second, to analyze the correlation between the composition of the stomach-resident T lymphocytes and time from *H. pylori* eradication; and third, to evaluate the sex differences in T lymphocyte subsets after *H. pylori* eradication. To investigate site-specific differences in stomach-resident T lymphocytes after *H. pylori* eradication, we performed flow cytometry analysis on samples taken from the gastric antrum, greater curvature of the gastric body, and lesser curvature of the gastric body of 20 patients. We also analyzed the correlation between the composition of the stomach-resident T lymphocytes and the time from *H. pylori* eradication. The lymphocyte subsets of the antrum and lesser curvature of the body were similar. In contrast, compared to those in the greater curvature of the gastric body, CD4⁺/CD3⁺ lymphocyte subsets (43.8 ± 19.4% vs 31.7 ± 14.6%) were elevated in the lesser curvature of the body, whereas CD8⁺/CD3⁺ (67.1 ± 21.3% vs 80.4 ± 12.0%), CD7⁺/CD3⁺ (91.2 ± 4.6% vs 93.7 ± 3.8%), CCR4⁺/CD3⁺ (7.7 ± 8.1% vs 10.4 ± 7.0%), CD45RA⁺/CD3⁺CD4⁺ (27.2 ± 24.8% vs 39.5 ± 20.8%), and CD45RA⁺/CD3⁺CD4⁻ (14.2 ± 11.1% vs 18.7 ± 11.5) were lower. Linear regression analysis showed a negative correlation between the time after *H. pylori* eradication and CD4⁺/CD3⁺ ($P < .05$, $R^2 = 0.198$). There were no significant differences between men and women with respect to the lymphocyte populations. These results indicate that there are site-specific differences in lymphocyte composition in the stomach after *H. pylori* eradication.

Keywords: eradication, flow cytometry, *Helicobacter pylori*, T lymphocytes

1. Introduction

Helicobacter pylori infection is believed to be a major risk factor for the development of gastric cancer. Although eradication of *H. pylori* reduces inflammation in the gastric mucosa and decreases the risk of gastric cancer development, patients infected with *H. pylori* have a higher risk for the occurrence of gastric cancer even after eradication.^[1–3] In our earlier work, we conducted a propensity score-matching analysis between patients with gastric cancer that developed after successful

H. pylori eradication, and those who did not develop gastric neoplasm >3 years after successful *H. pylori* eradication.^[4] We showed that inflammatory cell infiltration was more prominent in the background gastric mucosa of patients with gastric cancer than that of those without gastric cancer. Multivariate analysis further revealed that inflammation of the lesser curvature of the gastric body and antrum were independent risk factors for gastric cancer development, while inflammation of the greater curvature was not. Our study results suggested that continuous inflammation of the background gastric mucosa

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The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

The patients were prospectively registered and analyzed in this study. Written informed consent was obtained from all the participants. This study adhered to the principles of the Declaration of Helsinki and was approved by the ethics committee of Okayama University Hospital. The study protocol was registered in the UMIN Clinical Trials Registry (UMIN000040673).

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is a risk factor for gastric cancer onset after *H. pylori* eradication. Furthermore, there may be site-specific differences in lymphocyte phenotypes in the gastric mucosa after *H. pylori* eradication.

The aims of this study were: first, to reveal the differences between T lymphocyte populations of the gastric antrum and the greater and lesser curvatures of the gastric body in patients after *H. pylori* eradication; second, to analyze the correlation between the composition of the stomach-resident T lymphocytes and time from *H. pylori* eradication; and third, to evaluate the sex differences in T lymphocyte subsets after *H. pylori* eradication.

2. Patients and Methods

2.1. Patients

Flow cytometry was prospectively performed between June 2020 and December 2020 at the Okayama University Hospital (Okayama, Japan) on endoscopic biopsy specimens obtained from the gastric mucosa of 20 patients who had undergone *H. pylori* eradication. All patients had a known history of *H. pylori* eradication, and completion of eradication was previously confirmed using urea breath tests. The inclusion criteria were as follows: patients who had never undergone surgical resection of the stomach or gastric cancer treatment, patients who were not taking immunosuppressive or anticancer drugs, and patients without known inflammatory bowel disease or autoimmune gastritis.

In the 20 patients who underwent *H. pylori* eradication, during endoscopy, 3 specimens (one specimen from each area) were obtained using disposable biopsy forceps from the antrum and the lesser and greater curvatures of the body of the stomach. Lymphocytes were isolated from each biopsied specimen using the 1-step lymphocyte isolation procedure, which was reported in our earlier work.^{15,61}

2.2. Flow cytometry

Lymphocytes isolated from the gastric mucosa were used for flow cytometry. Monoclonal antibodies against CD45 (clone J33; Beckman Coulter, Pasadena, CA), CD3 (UCHT1; Beckman Coulter), CD4 (13B8.2; Beckman Coulter), CD8 (B9.11; Beckman Coulter), CD7 (8H8.1; Beckman Coulter), CD25 (B1.49.9; Beckman Coulter), CD30 (HRS4; Beckman Coulter), CD45RA (2H4; Beckman Coulter), CD56 (N901; Beckman Coulter), CD62L (DREG56; Beckman Coulter), CD127 (R34.34; Beckman Coulter), CCR4 (i.e., CD194; L291H4; BioLegend, San Diego, CA), HLADR (Immu-357; Beckman Coulter), and PD-1 (i.e., CD279; PD1.3; Beckman Coulter) were used. The immunostained cells were analyzed using FACScan (Navios flow cytometer, Beckman Coulter) and Kaluza analysis software (version 1.3; Beckman Coulter). Lymphocytes were separated by flow cytometry based on high CD45 antigen expression and forward and side scatter properties. Subsequently, the flow cytometry data were analyzed according to the percentage of cell populations detected in each quadrant on 2-dimensional scatterplots. We calculated the percentages of CD4⁺, CD8⁺, CD56⁺, CD7⁺, PD1⁺, CCR4⁺, CD30⁺, and HLADR⁺ cells among

CD3⁺ cells. We also assessed the percentages of regulatory T, CD45RA⁺, and CD62L⁺ cells among CD3⁺CD4⁺ cells and percentages of CD45RA⁺ and CD62L⁺ cells among CD3⁺CD4⁺ cells. In this study, we defined CD3⁺CD4⁺CD25⁺CD127^{low/} cells as regulatory T cells.¹⁷¹

2.3. Analysis

The primary purpose of this study was to determine the differences in T lymphocyte composition between the sites of the gastric mucosa. To assess the differences, we compared flow cytometric results between the lesser curvature of the body and the greater curvature of the body or the antrum. Second, to evaluate the association between the changes in T lymphocyte subsets and the time after *H. pylori* eradication, we performed a linear regression analysis of the scatterplots. Third, to reveal the differences in T lymphocyte population between sexes, we compared the flow cytometric results of the lesser curvature of the body between men and women.

Statistical analyses were performed using JMP 14.0.0 software (SAS Institute Inc., Cary, NC). Significant differences between samples were determined using Mann–Whitney *U* or Wilcoxon signed-rank tests, where indicated. Differences were considered significant if the *P* value was <.05.

2.4. Ethics approval

The patients were prospectively registered and analyzed in this study. Written informed consent was obtained from all the participants. This study adhered to the principles of the Declaration of Helsinki and was approved by the ethics committee of Okayama University Hospital. The study protocol was registered in the UMIN Clinical Trials Registry (UMIN000040673).

3. Results

3.1. Patients' characteristics

The clinical characteristics of the enrolled patients are shown in Table 1. The study included ten men and ten women. The mean age was 68.5 years (range, 44–82 years). The mean period between completion of *H. pylori* eradication and flow cytometry analysis was 4.1 years, ranging from 0.7 to 7.4 years. The period between *H. pylori* eradication and flow cytometric analysis did not differ between men (mean ± SD, 3.8 ± 2.1 years) and women (4.49 ± 1.85). However, male patients (62.3 ± 10.8 years) were younger than the female patients (74.7 ± 5.8 years).

The numbers of lymphocytes analyzed on flow cytometry, which were isolated from the single biopsied specimen and separated based on high CD45 antigen expression and forward and side scatter properties, were 264.2 ± 229.1, 696.6 ± 382.4, and 715.4 ± 1030.8 in the gastric antrum, greater curvature of the gastric body, and lesser curvature of the gastric body, respectively. The number of lymphocytes separated from the antrum was significantly smaller than that of the lesser curvature of the body (Wilcoxon signed-rank test, *P* = .008).

Table 1

Clinical characteristics of the study patients.

	All patients	Men	Women	<i>P</i> value,* men vs women
N	20	10	10	
Age, yr (mean ± SD)	68.5 ± 10.6	62.3 ± 10.8	74.7 ± 5.8	<.01
Years after HP eradication (mean ± SD)	4.1 ± 2.0	3.8 ± 2.1	4.5 ± 1.9	.54

HP = *Helicobacter pylori*, SD = standard deviation.

*Mann–Whitney *U* test.

3.2. Comparison of lymphocyte composition between the sites of the stomach

The flow cytometric results for all samples are shown in Figure 1. Comparison between the lymphocyte composition of the antrum and that of the lesser curvature of the body revealed that the level of CD62L⁺/CD3⁺CD4⁻ was lower in

the antrum ($0.5 \pm 1.3\%$) than that of the lesser curvature of the body ($1.9 \pm 2.7\%$) (Fig. 2). No differences were observed in the expression of other antigens. Meanwhile, CD8⁺/CD3⁺ ($67.1 \pm 21.3\%$ vs $80.4 \pm 12.0\%$), CD7⁺/CD3⁺ ($91.2 \pm 4.6\%$ vs $93.7 \pm 3.8\%$), CCR4⁺/CD3⁺ ($7.7 \pm 8.1\%$ vs $10.4 \pm 7.0\%$), CD45RA⁺/CD3⁺CD4⁺ ($27.2 \pm 24.8\%$ vs $39.5 \pm 20.8\%$), and CD45RA⁺/CD3⁺CD4⁻ ($14.2 \pm 11.1\%$ vs $18.7 \pm 11.5\%$) levels were

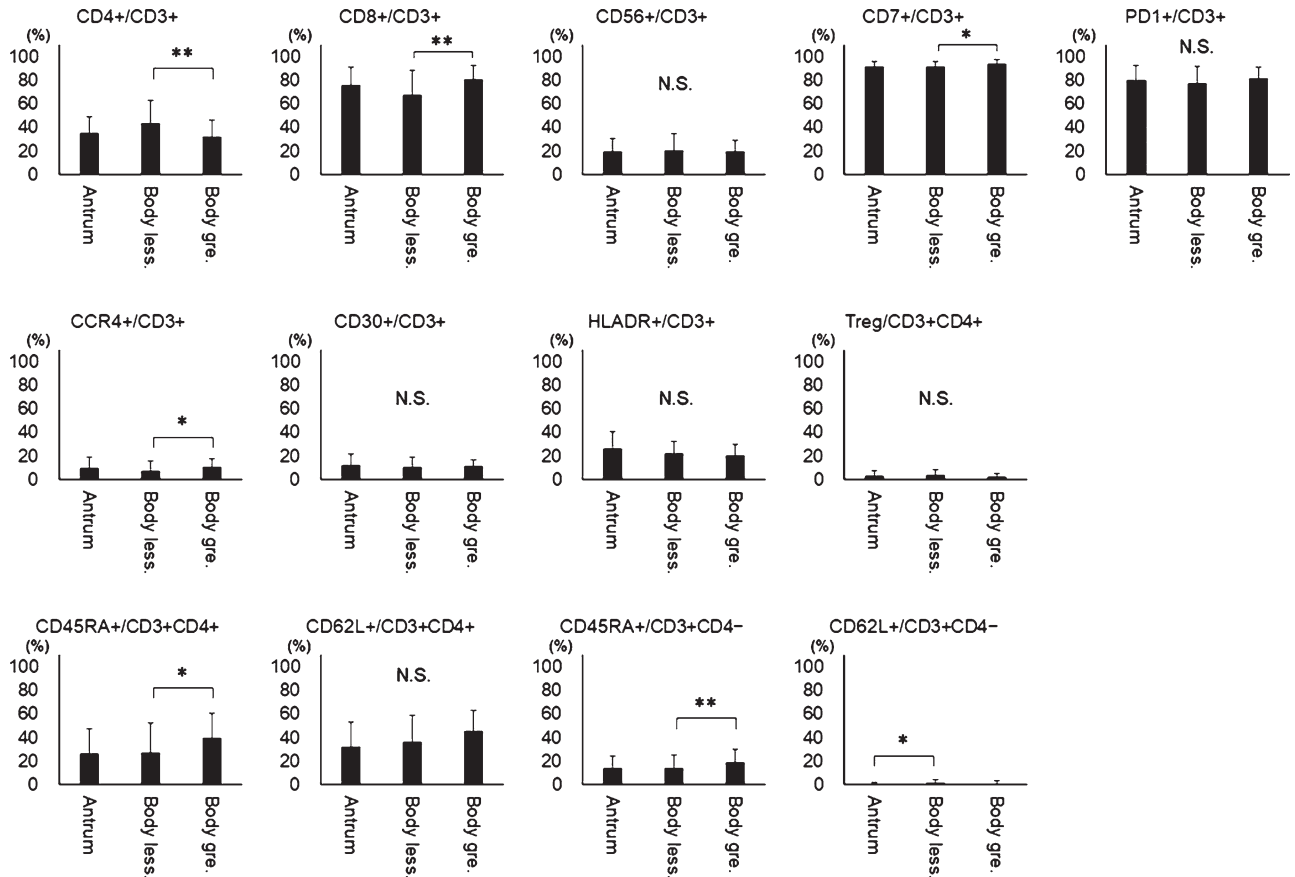


Figure 1. Results of flow cytometric analysis. We comparatively analyzed lymphocyte composition in the gastric antrum, lesser curvature of the stomach (Body less.), and greater curvature of the stomach (Body gre.). Treg cells were defined as CD3⁺CD4⁺CD25⁺CD127^{low/-} cells. **P* < .05; ***P* < .01 (Wilcoxon signed-rank test). N.S. = not significant, Treg = regulatory T.

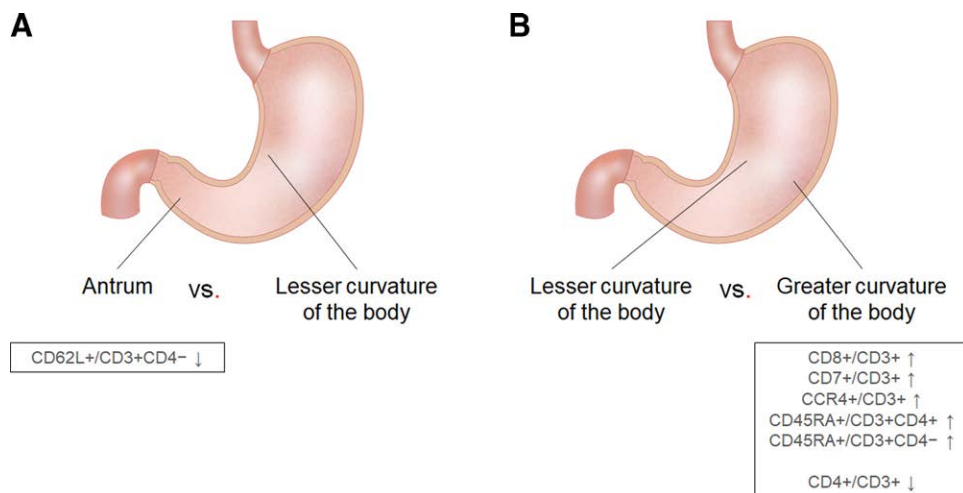


Figure 2. Images depicting differences in lymphocyte composition. Comparison between the lymphocyte subsets in the antrum and the lesser curvature of the body revealed that the level of CD62L⁺/CD3⁺CD4⁻ was lower in the antrum (A). Comparison between the lymphocyte subsets in the lesser and the greater curvatures of the body revealed that CD8⁺/CD3⁺, CD7⁺/CD3⁺, CCR4⁺/CD3⁺, CD45RA⁺/CD3⁺CD4⁺, and CD45RA⁺/CD3⁺CD4⁻ were elevated and CD4⁺/CD3⁺ was lower in the greater curvature of the body (B).

lower and CD4⁺/CD3⁺ ($43.8 \pm 19.4\%$ vs $31.7 \pm 14.6\%$) were higher in the lesser curvature of the gastric body than those in the greater curvature of the body.

3.3. Association between the gastric mucosal lymphocyte composition and the time elapsed after *H. pylori* eradication

On linear regression analysis of the scatterplots, a negative correlation existed between the time elapsed after *H. pylori* eradication and CD4⁺/CD3⁺ ($R^2 = 0.198$, Fig. 3). No significant correlation was found between the expression of other antibodies and the years passed since the completion of *H. pylori* eradication.

3.4. Analysis of sex differences in gastric mucosal lymphocytes

Comparison of the flow cytometry results of the lesser curvature of the gastric body between men ($n = 10$) and women ($n = 10$) revealed that there were no significant differences between the 2 groups with respect to the lymphocyte populations (Fig. 4).

4. Discussion

In this study, we performed flow cytometric analysis of gastric mucosal lymphocytes, primarily to reveal site-specific differences in T lymphocyte composition in the stomach. Statistical differences were observed only in the level of CD62L⁺/CD3⁺CD4⁻ between samples of the antrum and those of the lesser curvature of the stomach. However, we consider that the difference is clinically insignificant as the CD62L⁺/CD3⁺CD4⁻ fractions accounted for the small percentages both in the antrum ($0.5\% \pm 1.3\%$) and the lesser curvature of the body ($1.9\% \pm 2.7\%$). In contrast, we showed that the levels of CD8⁺/CD3⁺, CD7⁺/CD3⁺, CCR4⁺/CD3⁺, CD45RA⁺/CD3⁺CD4⁺, and CD45RA⁺/CD3⁺CD4⁻ were elevated and that of CD4⁺/CD3⁺ were lower in the greater curvature than those in the lesser curvature of the gastric body. Overall, these results indicate that in

the stomach, after *H. pylori* eradication, the lymphocyte subsets of the lesser curvature of the gastric body are quite similar to those of the antrum, while they are different from those of the greater curvature of the body. These results support the findings of our previous study that inflammation of the lesser curvature of the gastric body and that of the antrum were independent risk factors for gastric cancer development, while that of the greater curvature was not.^[4]

Infiltration of CD4⁺ T lymphocytes in *H. pylori*-infected gastric mucosa has been reported previously.^[8-10] With respect to the relationship between the stomach area and *H. pylori*-associated gastritis, it is well known that endoscopic and histopathological alterations, such as inflammation, atrophy, and metaplasia initially occur in the antrum, then involve the lesser curvature of the body, and finally extend to the greater curvature of the body.^[11,12] Therefore, it is likely that CD4⁺ T lymphocytes, which had increased in number in response to *H. pylori* infection, reside in the antrum and lesser curvature of the body even after *H. pylori* eradication. The result of linear regression analysis between the time after *H. pylori* eradication and CD4⁺/CD3⁺ cells indicated that the level of CD4⁺ T lymphocytes in the lesser curvature of the body gradually decreased after *H. pylori* eradication (Fig. 3). This result further supports our hypothesis.

Another noteworthy difference between the lymphocyte subsets of the lesser and greater curvatures of the gastric body was CD45RA⁺/CD3⁺CD4⁺. A CD4⁺CD45RA⁺ T lymphocyte subset represents naïve T helper cells, which are considered immature and have not encountered antigens within the periphery.^[13] In the present study, the level of CD45RA⁺/CD3⁺CD4⁺ cells was lower in the lesser curvature of the body, suggesting that antigen-stimulated CD4⁺ T lymphocytes are more predominant in the lesser curvature of the body than in the greater curvature of the body. Meanwhile, a previous basic study using mouse models of *H. pylori* infection revealed that more severe inflammation with CD8⁺ T lymphocytes occurred in the stomach of CD4⁺ T lymphocyte-deficient mice, while gastric inflammation was mild in mice deficient in both CD4⁺ and CD8⁺ T lymphocytes.^[9] These results suggest the role of CD4⁺ T lymphocytes as a regulator or suppressor of local inflammation in the stomach. Further studies are required to reveal the detailed phenotypes and functions of CD4⁺ T lymphocytes in the stomach mucosa.

Male predominance among patients with gastric cancer with current *H. pylori* infection is well known.^[14] In addition, a 9-year prospective follow-up study of 1299 men and 488 women after successful *H. pylori* eradication revealed the development of gastric cancer in 20 patients, comprising 17 men (85.0%) and 3 women.^[15] In an open-label, randomized controlled trial, 195 men and 60 women were followed up after *H. pylori* eradication and endoscopic resection of early gastric cancer.^[16] Metachronous gastric cancer was identified in 7 men (77.8%) and 2 women. Before conducting the present study, we hypothesized that such male predominance is, at least partly, imparted by the difference in gastric lymphocyte populations between sexes. However, there were no differences between the lymphocyte subsets in the stomach of men and women. Further investigation is required to reveal the mechanisms underlying male-predominant carcinogenesis in the stomach.

Our study has several limitations. The first limitation was the small number of participants. Second, although lifestyle habits, particularly daily eating habits, may affect the lymphocyte composition in the stomach, information about each patient's eating habits is missing. However, despite the small sample size and lack of lifestyle habits, we found several significant differences in lymphocyte subsets. Third, as most of the enrolled patients were elderly, with a mean age of 68.5 years, gastric lymphocyte subsets in younger population must be investigated hereafter. Further investigations with a greater number of patients, including younger individuals, will reveal the true nature of lymphocytes in the stomach after *H. pylori* eradication. Fourth,

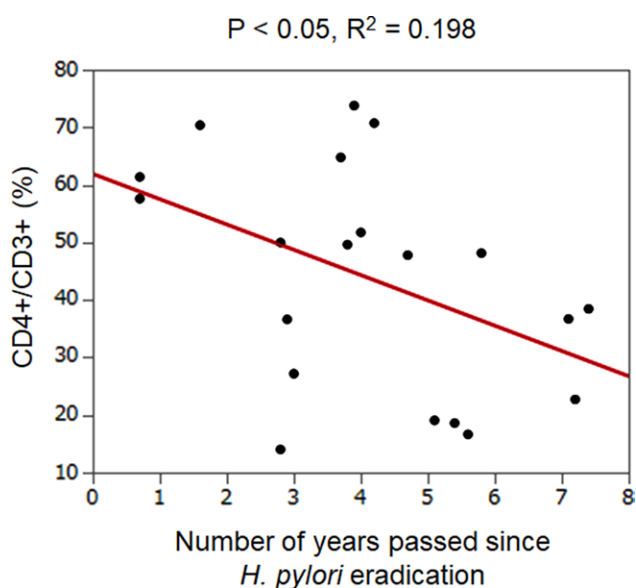


Figure 3. Linear regression analysis between the time after *Helicobacter pylori* eradication and lymphocyte subsets. A negative correlation existed between the time after *H. pylori* eradication and CD4⁺/CD3⁺. No significant correlation was found in other antibody expressions.

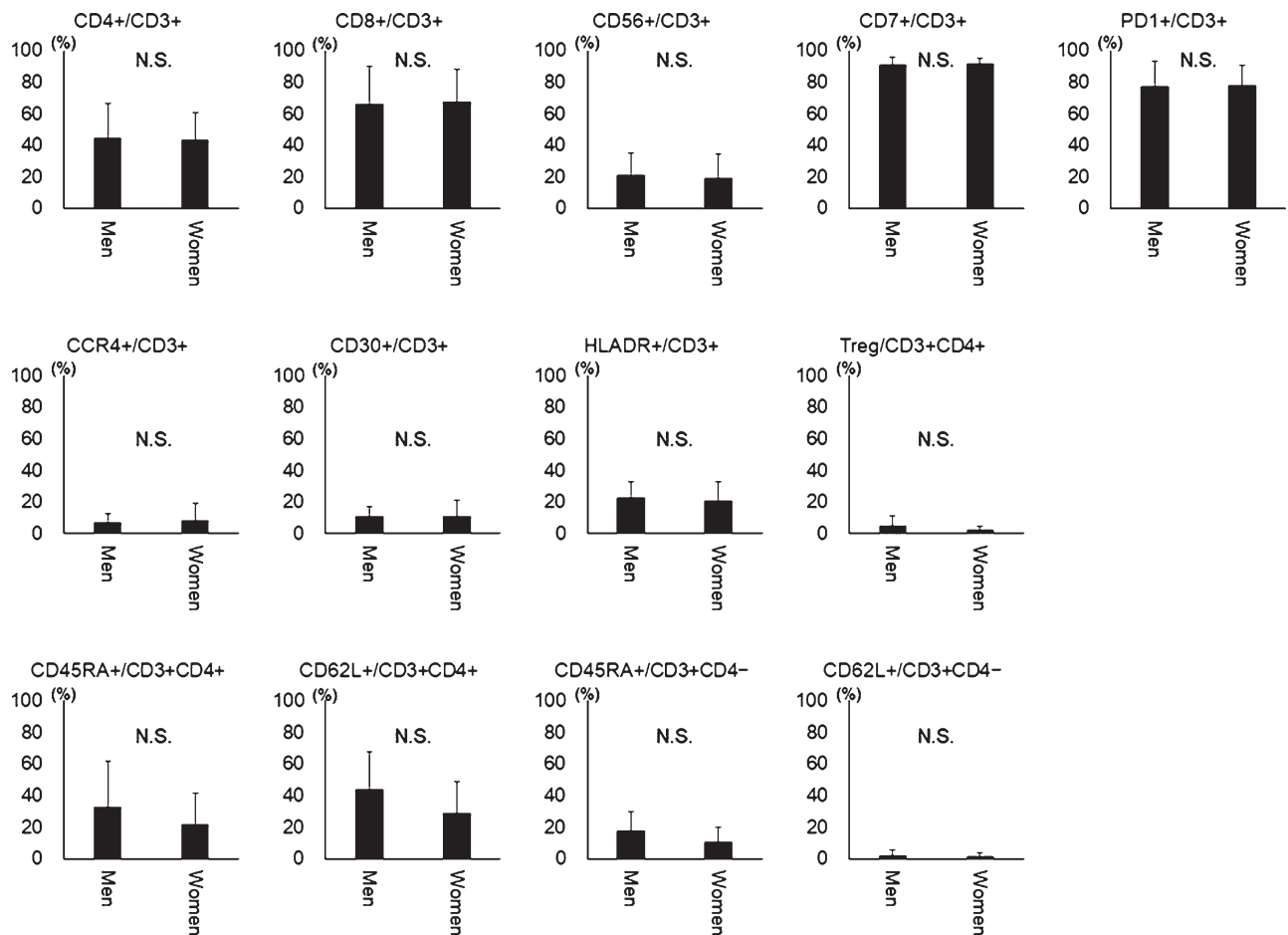


Figure 4. Comparison of the flow cytometry results of the lesser curvature of the gastric body between men ($n = 10$) and women ($n = 10$). There were no significant differences between the 2 groups. N.S. = not significant (Mann–Whitney U test).

the relationship between the site-specific differences in gastric lymphocytes and disease pathogenesis remains to be elucidated.

In conclusion, we performed flow cytometric analysis of stomach-resident lymphocytes in patients after *H. pylori* eradication. We revealed that the lymphocyte compositions were quite similar between the gastric antrum and the lesser curvature of the gastric body, while they were different from the greater curvature of the gastric body. The level of CD4⁺/CD3⁺ cells was elevated in the lesser curvature of the gastric body than in the greater curvature, and it gradually decreased as the years progressed since *H. pylori* eradication. These results indicate that there are site-specific differences in lymphocyte subsets in the stomach after *H. pylori* eradication.

Author contributions

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Supervision: Hiroyuki Okada.

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