In a previous study in mice, mRNA expression of TSLP could be detected throughout the small and large intestines (20, 21). In patients with eosinophilic esophagitis, mRNA expression of TSLP in the inflamed esophageal tissues was significantly overexpressed (18, 19). TSLP expression was also found in gastric mucosal lesions from patients with *Helicobacter pylori*-induced chronic gastritis and *H. pylori* colonization-induced TSLP production in gastric epithelial cells (27). In the intestine, mRNA expression of TSLP was overexpressed in patients with ulcerative colitis, and proinflammatory cytokines induced overexpression of TSLP in colonic epithelial cells (32, 33). Therefore, TSLP can be expressed in the intestinal tract throughout the esophagus, stomach, and small and large intestines, both in humans and mice, suggesting that TSLP is involved in various immune responses in the gastrointestinal tract.

We also demonstrated in this study that TSLPR deficiency induced early onset of AIG and that the increased susceptibility was associated with exacerbated histopathology accompanied by enhanced Th1 responses. In addition, in AIG-bearing mice, TSLP negatively regulated production of IL-12/23p40 by DCs, as described (21). Therefore, these data suggested that TSLP expression in inflamed mucosa negatively modulates DC activation to promote Th1-type autoimmunity in the stomach. In mice, TSLP is involved in allergic diarrhea and Th2-type intestinal immunity against helminth infection, and it regulates Th1-type inflammation in colitis induced by dextran sodium sulfate (17, 21). In humans, TSLP is a candidate gene critically involved in susceptibility to eosinophilic esophagitis. TSLP overexpression is thought to be involved in *H. pylori*-induced chronic gastritis with formation of B cell follicles (18, 27). In addition, mRNA expression of TSLP was significantly reduced in patients with Crohn's disease, whereas TSLP was overexpressed in patients with ulcerative colitis (32, 33). Taking these results together, although TSLP is deeply involved in the pathophysiology of some allergic or Th2-dependent inflammatory conditions, its primary role in physiological conditions of the gastrointestinal tract may be to maintain intestinal immune homeostasis, including regulation of autoimmunity.

Although inflammatory Th1 responses induced by autoreactive $CD4^+$ T cells have the potential to develop AIG in any mice, various regulatory mechanisms in normal mice suppress the development of AIG. NTx-BALB/c mice possess disease-relevant Tregs, but these cannot prevent AIG development (7–9), suggesting that Tregs are critically involved in negatively regulating the development of AIG. In addition, programmed cell death 1 (PD-1) provides negative costimulation to lymphocytes. PD-1–deficient BALB/c mice spontaneously develop AIG (25, 34), suggesting that PD-1–mediated signaling is critical for the negative regulation of AIG development. These regulatory mechanisms are primarily involved in suppressing the development of AIG, whereas although TSLP may be secondarily induced after triggering inflammation of AIG, induced TSLP did not regress the inflammation of AIG. However, it is not clear whether TSLP might have a regulatory function if there were more of it. Further studies are required to ascertain whether TSLP might be used as a therapeutic target for treating patients with AIG.

A previous report showed that adding recombinant TSLP enhanced the proliferation capacity of TCR-stimulated $CD4^+$ T cells in vitro and that $CD4^+$ T cells from TSLPR-deficient mice expanded less efficiently than did $CD4^+$ T cells from wild-type mice in irradiated γc/Rag2-deficient hosts (35). However, we found that infiltrating mononuclear cells in the gastric mucosa of NTx-$TSLPR^{-/-}$ mice were also mainly $CD4^+$ T cells and that TSLPR deficiency increased the cell numbers of $CD4^+$ T cells in the gastric mucosa (Fig. 5). These data suggested that the enhanced proliferative capacity of $CD4^+$ T cells in a Th1-dominant autoimmune setting may overcome a deficiency of direct action of TSLP on $CD4^+$ T cells.

In adult NTx mice, $CD4^+Foxp3^+$ Tregs were observed in the periphery, as described (7–9). We also found that 12-wk-old NTx-$TSLPR^{-/-}$ mice had a number of $CD4^+Foxp3^+$ Tregs in the periphery, comparable to those found in NTx-$TSLPR^{+/+}$ mice (data not shown). These findings suggested that it is not likely that impaired generation and expansion of induced Tregs enhance $CD4^+$ T cell infiltration in the gastric mucosa of NTx-$TSLPR^{-/-}$ mice.

In conclusion, we demonstrated that TSLP was expressed in the inflamed stomach of AIG-bearing mice and that TSLPR deficiency both increased susceptibility to AIG and exacerbated its severity. These data suggested that TSLPR-mediated signaling negatively regulates organ-specific Th1-dependent autoimmunity in the gastric mucosa.

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Disclosures

The authors have no financial conflicts of interest.

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Long-term clinical outcome of gastric MALT lymphoma after eradication of *Helicobacter pylori*: a multicentre cohort follow-up study of 420 patients in Japan

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ABSTRACT

Objective A multicentre cohort follow-up study of a large number of patients with gastric mucosa-associated lymphoid tissue (MALT) lymphoma was conducted to elucidate the long-term outcome of the disease after *Helicobacter pylori* eradication.

Methods 420 patients with gastric low-grade MALT lymphoma who had undergone successful *H pylori* eradication and been followed up for at least 3 years were registered from 21 participating institutes. Responders to treatment were defined as patients whose post-treatment biopsies showed complete histological response (ChR) or probable minimal residual disease (pMRD). Treatment failure was defined as the status of progressive disease or lymphoma relapse after ChR/pMRD.

Results 323 patients (77%) responded to *H pylori* eradication. A logistic regression analysis showed that absence of *H pylori*, submucosal invasion determined by endoscopic ultrasonography and t(11;18)/*API2-MALT1* were independent predictors of resistance to *H pylori* eradication. During the follow-up periods ranging from 3.0 to 14.6 years (mean 6.5 years, median 6.04 years), the disease relapsed in 10 of 323 responders (3.1%) while progressive disease was found in 27 of 97 non-responders (27%). Thus, 37 of 420 patients (8.8%) were regarded as treatment failures. Of these 37 patients, transformation into diffuse large B cell lymphoma occurred in nine patients. Among the non-responders and relapsed patients, 17 patients were subjected to a 'watch and wait' strategy while 90 patients underwent second-line treatments including radiotherapy (n=49), chemotherapy (n=26), surgical resection (n=6), chemoradiotherapy (n=5), antibiotic treatment (n=2), rituximab monotherapy (n=1) or endoscopic resection (n=1). Probabilities of freedom from treatment failure, overall survival and event-free survival after 10 years were 90%, 95% and 86%, respectively. Cox multivariate analysis revealed endoscopic non-superficial type to be an independent prognostic factor for adverse freedom from treatment failure, overall survival and event-free survival.

Conclusions The excellent long-term outcome of gastric MALT lymphoma after *H pylori* eradication was confirmed by this large-scale follow-up study.

Significance of this study

What is already known about this subject?

- *Helicobacter pylori* plays a causative role in the development of gastric mucosa-associated lymphoid tissue (MALT) lymphoma.
- The eradication of *H pylori* leads to a complete remission (CR) of the disease in 50–90% of cases of gastric MALT lymphoma.

What are the new findings?

- The excellent long-term clinical outcome of gastric MALT lymphoma after *H pylori* eradication was confirmed by the follow-up study ranging from 3.0 to 14.6 years (mean 6.5 years, median 6.04 years); the probabilities of freedom from treatment failure, overall survival and event-free survival after 10 years were 90%, 95% and 86%, respectively.

How might it impact on clinical practice in the foreseeable future?

- Patients with localised gastric MALT lymphoma who achieved CR after *H pylori* eradication can be managed only by follow-up endoscopy with multiple biopsies every 1 or 2 years.
- Even in non-responders to *H pylori* eradication, a 'watch and wait' strategy may be recommended for more than 2 years unless progression or relapse of lymphoma can be demonstrated in patients with localised gastric MALT lymphoma.

INTRODUCTION

Extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma) is a distinct clinicopathological entity in the recent classification of malignant lymphomas.[1 2] Gastric MALT lymphoma accounts for 40–50% of primary gastric lymphomas, 20–40% of extranodal lymphomas, 4–9% of all malignant lymphomas and 1–6% of all gastric malignancies.[1–3] *H pylori* plays a causative role in the development of gastric MALT lymphoma, and the eradication of *H pylori* leads to a complete remission (CR) of lymphoma in

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50–90% of cases.[4–32] A systematic review of the data from 32 publications revealed that CR was achieved in 78% of 1408 patients with low-grade gastric MALT lymphoma in stage I/II_1 treated by *H pylori* eradication.[33] Thus, *H pylori* eradication is now regarded as the first-line treatment for gastric MALT lymphoma.[33 34] However, large-scale long-term follow-up outcome after the eradication therapy is still limited, particularly with regard to relapse or progression and survival.

We therefore conducted a multicentre cohort follow-up study of a larger number of patients with gastric MALT lymphoma in order to elucidate the long-term clinical outcome of *H pylori* eradication therapy including predictive factors for resistance to the therapy, probabilities of freedom from treatment failure (FFTF) and survival, as well as the prognostic factors for the disease.

METHODS

Study design

This was a Japanese multicentre cohort follow-up study. Invitations to join the study were sent to all members of the JAPAN GAST Study Group (JGSG) and 21 institutes across Japan participated. The method used was a questionnaire-based inquiry on clinical and pathological data in each institute. Data for all consecutive patients with primary gastric MALT lymphoma diagnosed and treated by *H pylori* eradication between March 1994 and March 2007 were collected. The inclusion criteria were: (1) presence of gastric extranodal marginal zone lymphoma of MALT (MALT lymphoma) according to the WHO classification[2] and compatible with Wotherspoon's histological score of 4 or 5 in the pretreatment gastric biopsy specimens[4]; (2) having undergone *H pylori* eradication therapy using a proton pump inhibitor (omeprazole, lansoprazole or rabeprazole) plus a combination of two antibiotics (amoxicillin, clarithromycin or metronidazole) as the first-line treatment for MALT lymphoma; and (3) follow-up period of at least 3 years after successful eradication therapy. Exclusion criteria were: (1) primary MALT lymphoma arising in the extragastric organs; (2) presence of diffuse large B cell lymphoma (DLBCL) prior to the *H pylori* eradication; and (3) positive *H pylori* infection after the final eradication therapy. Study protocols were approved by the institutional review board at each institute and complied with provisions of the Declaration of Helsinki.

Subjects

A total of 420 patients with gastric MALT lymphoma were registered from the following 21 institutes: Kyushu University (n=87), Tohoku University (n=71), Hokkaido University (n=41), Aichi Cancer Center Hospital (n=39), Hiroshima Red Cross Hospital and Atomic-Bomb Survivors Hospital (n=33), Hiroshima University (n=30), Kawasaki Medical School (n=26), Shinshu University (n=25), Hitachi General Hospital (n=12), Kyoto University (n=10), Iwate Medical School (n=9), Nihon University (n=7), Fukuoka University (n=5), Hyogo College of Medicine (n=5), Social Insurance Shiga Hospital (n=4), Hamamatsu University (n=4), Oita University (n=4), Toyama University (n=3), National Hospital Organisation Kyoto Medical Center (n=3), Tsukuba Memorial Hospital (n=1), and Hirosaki University (n=1). A certain proportion of the study population had been included in previous studies.[18 23 25 27 31 32]

The *H pylori* status was determined by histology, culture, rapid urease test, ^{13}C urea breath test and/or serology. *H pylori* infection was judged to be positive if one or more of the tests showed a positive result and to be negative when all tests were negative. The endoscopic type of lymphoma was classified as either superficial, ulcerative, polypoid or others, based on the classification reported previously.[3] The clinical stage was determined on the basis of the Lugano staging system, a modification of the Ann-Arbor classification.[35] Endoscopic ultrasonography (EUS) was also performed to evaluate the depth of tumour invasion and the degree of perigastric lymphadenopathy.[36 37] t(11;18)(q21;q21)/*API2-MALT1* was investigated by reverse-transcription PCR[38] and/or interphase fluorescence in situ hybridisation.[39]

Assessment after *H pylori* eradication therapy

The biopsy specimens after *H pylori* eradication and other non-surgical treatments was assessed according to the Groupe d'Etude des Lymphomes de l'Adulte (GELA) histological grading system, being classified as either complete histological response (ChR), probable minimal residual disease (pMRD), responding residual disease (rRD) or no change (NC).[34 40] Responders were defined as patients whose post-treatment biopsies showed ChR or pMRD. Those patients were regarded as showing clinical CR.[34] The remaining patients (rRD or NC) were considered as non-responders. rRD was regarded as clinical partial remission (PR).[34] Follow-up endoscopic examinations with biopsies were performed every 3–6 months until confirmation of ChR or pMRD, and repeated every 6–12 months after ChR/pMRD. Treatment failure was defined as the relapse after ChR/pMRD or progressive disease (PD), including transformation into DLBCL. All the biopsy specimens were reviewed by the senior pathologist in each institute.

Second-line treatments for non-responders

Patients with treatment failure or persistence of lymphoma following *H pylori* eradication were subjected to either a 'watch and wait' strategy (no treatment) or various antineoplastic treatments including radiotherapy, chemotherapy with or without rituximab, chemoradiotherapy, rituximab alone, surgical resection or others.

Prognostic factors and statistical analysis

A logistic regression analysis was performed to identify the predictive factors for response to *H pylori* eradication. FFTF was measured from the date of start of the final *H pylori* eradication to the treatment failure, which was censored at the date when patients underwent additional therapy without PD or when patients died of causes unrelated to lymphoma. Overall survival (OS) was measured from the date of the eradication to death from any cause, and event-free survival (EFS) was measured from the date of the eradication to treatment failure or death from any cause. Probabilities of FFTF, OS and EFS were calculated by the Kaplan–Meier method and the values were compared using the log-rank test and generalised Wilcoxon test. All variables with $p<0.1$ by either of the tests were included in multivariate analyses using the Cox proportional hazards model. Other statistical differences were evaluated by the Fisher exact probability test, the χ^2 test or the Mann–Whitney U test. p Values <0.05 were regarded as statistically significant. For multiple comparisons, however, p values were interpreted after the Bonferroni correction.

RESULTS

Patient characteristics at baseline

The baseline characteristics of 420 patients are summarised in table A in the online supplement. The median age was 61 years

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(range 16–87) and 56% of the patients were women. *H pylori* infection was negative in 10%. The most frequently involved site was the middle third of the stomach (45%), followed by the proximal third (22%). Endoscopically, 67% of tumours were classified as superficial type, 16% as ulcerative type, 12% as polypoid type and 4% as others; 90% of patients had clinical stage I disease. Among 341 patients who underwent EUS, the depth of lymphoma infiltration in the gastric wall was: mucosa in 52%, submucosa in 38% and muscularis propria or beyond in 10% of cases. t(11;18)/*API2-MALT1* was positive in 30 of 206 patients (15%) examined.

Response to *H pylori* eradication

The clinical course after the successful eradication of *H pylori* is summarised in figure 1. After *H pylori* eradication, ChR was achieved in 284 patients (68%) and pMRD in 39 (9%). Thus, 323 patients (77%) were responders. There were 97 non-responders (23%), including rRD in 13 (3%) and NC in 84 (20%). The median time from *H pylori* eradication to ChR or pMRD was 4 months (range 1–94). As shown in table 1, non-responders were significantly associated with male sex, absence of *H pylori*, proximal or multiple locations, endoscopically non-superficial type, advanced stage ($\geq II_1$), deep submucosal invasion and t(11;18)/*API2-MALT1*. Multivariate logistic regression analysis indicated that male sex, absence of *H pylori*, the location in the proximal or multiple areas, non-superficial type and advanced stage were independent predictors of resistance to *H pylori* eradication therapy (table 2). In 175 patients who underwent both EUS and investigation for t(11;18), absence of *H pylori*, submucosal invasion under EUS and t(11;18)/*API2-MALT1* were independent predictors of resistance to *H pylori* eradication.

Relapse in responders

The median follow-up period of 323 responders after ChR/pMRD was 5.48 years (range 1.0–14.3). During the period, lymphoma relapse was observed in 10 patients (3.1%). The time duration from ChR/pMRD was 1–131 months (median 21.5). Only one patient showed transformation into DLBCL, which was treated by surgical resection. Six patients were treated by either chemotherapy (n=4) or radiotherapy (n=2). CR was achieved in those seven patients. Other one patient developed macroscopic local relapse of MALT lymphoma 10.9 years after ChR which was associated with reinfection of *H pylori*. Re-eradication of *H pylori* resulted in rRD (PR) in this case. The remaining two patients who showed histological relapse 12 and 13 months after the initial response spontaneously achieved ChR again without any additional treatment.

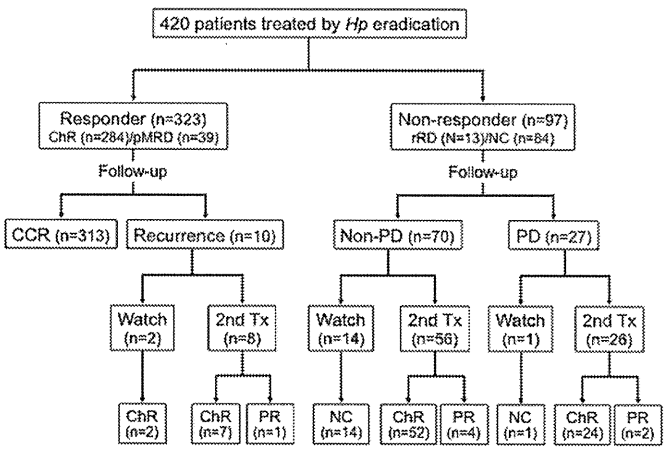

Figure 1 Clinical course of the 420 study patients. *Hp*, *Helicobacter pylori*; ChR, complete histological response; pMRD, probable minimal residual disease; rRD, responding residual disease; NC, no change; CCR, continuous complete remission; PD, progressive disease; Tx, treatment; PR, partial remission.

Table 1 Association between clinicopathological factors and response to the *Helicobacter pylori* eradication

Factors	No. of response/ all patients	Response rate (%)	p Value*
Age			
≤60 years	164/206	80	0.2048
≥61 years	159/214	74	
Sex			
Male	128/184	70	0.0023
Female	195/236	83	
H pylori status			
Positive	317/376	84	<0.0001
Negative	6/44	14	
Dominant site of lesion			
Proximal/multiple	113/172	66	<0.0001
Distal two-thirds	210/248	85	
Endoscopic type			
Superficial	241/282	85	<0.0001
Others	82/138	59	
Clinical stage			
I	304/378	80	<0.0001
II_1 or more	19/42	45	
Depth of invasion by EUS (n=341)			
Mucosa	156/179	87	<0.0001
Submucosa or beyond	98/162	60	
t(11;18)/API2-MALT1 (n=206)			
Positive	3/30	10	<0.0001
Negative	130/176	74	

*Fisher exact probability test; p<0.0062 is significant using the Bonferroni correction.
EUS, endoscopic ultrasonography.

Treatments for non-responders

As shown in figure 1, PD was observed in 27 of 97 non-responders. Eight patients showed transformation into DLBCL. One PD patient, who was positive for t(11;18) and had been subjected to a 'watch and wait' strategy, developed lung metastasis of MALT lymphoma 12 years later.[25] The other 26 patients were treated by radiotherapy (n=11), chemotherapy (n=10), chemoradiotherapy (n=3) or surgical resection (n=2). Twenty-four patients achieved CR and two patients treated with radiotherapy or chemotherapy showed PR.

Fourteen of 70 non-responders without PD were subjected to a 'watch and wait' strategy while 56 patients underwent second-line treatments. One patient showing rRD (clinically PR) at 20 months was treated with 'third-line' antibiotic therapy with gatifloxacin, amoxicillin and rabeprazole which resulted in ChR 15 months later. The remaining 55 patients underwent radiotherapy (n=36), chemotherapy (n=12), surgery (n=3), chemoradiotherapy (n=2), rituximab monotherapy (n=1) or endoscopic resection (n=1). Fifty-two of these 55 patients achieved CR while three patients treated with radiotherapy, chemotherapy or endoscopic resection showed PR.

Figure 1 summarises the clinical course after second-line treatments applied to 97 non-responders and 10 responders with relapse. The second-line treatments resulted in CR in 85 patients, PR in seven patients and NC in 15 patients. The rate of

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Table 2 Predictive factors for resistance to *Helicobacter pylori* eradication therapy as determined by logistic regression analysis

	All patients (n=420)		Selected patients (n=175*)	
Factors	**OR (95% CI)**	**p Value**	**OR (95% CI)**	**p Value**
Age (each incremental year)	2.39 (0.43 to 13.83)	0.323	4.63 (0.23 to 111.91)	0.329
Sex (male)	2.04 (1.15 to 3.65)	0.015	2.27 (0.89 to 5.93)	0.087
H pylori status (negative)	31.73 (12.89 to 91.49)	<0.0001	24.29 (5.12 to 189.15)	0.0003
Location (proximal or multiple)	2.63 (1.48 to 4.73)	0.001	1.18 (0.44 to 3.11)	0.742
Endoscopic type (non-superficial)	2.32 (1.28 to 4.18)	0.005	2.33 (0.73 to 7.47)	0.152
Clinical stage (II_1 or more)	2.91 (1.28 to 6.53)	0.0097	2.69 (0.69 to 10.89)	0.156
Depth of invasion by EUS (≥SM)			6.02 (2.12 to 19.13)	0.0012
t(11;18)/*API2-MALT1* (positive)			44.03 (7.89 to 388.27)	<0.0001

*Patients who underwent both EUS and investigation for t(11;18).
EUS, endoscopic ultrasonography; ≥SM, submucosa or beyond.

the CR induction by each treatment was 94% by radiotherapy, 88% by chemotherapy, 100% by surgical resection, 100% by chemoradiotherapy, 50% by antibiotics, 100% by rituximab monotherapy and 0% by endoscopic resection.

Treatment failure, long-term outcome and prognostic factors

Overall follow-up periods of 420 patients after *H pylori* eradication ranged from 3.0 to 14.6 years (mean 6.5, median 6.04). PD and disease relapse occurred in 27 patients and 10 patients, respectively. Thus, 37 of 420 patients (8.8%) showed treatment failure. Probabilities of FFTF after 5, 10 and 12 years were 92%, 90% and 80%, respectively (figure 2A). Univariate and multivariate analyses for FFTF are summarised in table B in the online supplement. Univariate analysis showed absence of *H pylori*, non-superficial type, advanced stage and submucosal invasion under EUS to be significantly associated with an adverse prognosis. Cox multivariate analysis excluding EUS assessment showed non-superficial type to be the single independent predictor for adverse FFTF.

During the follow-up period two patients died from transformed DLBCL and 11 from causes unrelated to lymphoma. Probabilities of OS after 5, 10 and 12 years were 99%, 95% and 91%, respectively (figure 2B). Univariate analysis for OS showed that older age, endoscopic non-superficial type, advanced stage and submucosal invasion determined by EUS were significant factors (see table C in online supplement). Cox multivariate analysis showed that older age and non-superficial type were independent predictors for poor OS.

Probabilities of EFS after 5, 10 and 12 years were 92%, 86% and 76%, respectively (figure 2C). As shown in table D in the online supplement, univariate analysis for EFS revealed location in the proximal/multiple areas, non-superficial type, advanced stage and submucosal invasion under EUS to be significantly associated with poor EFS. Cox multivariate analyses excluding or including EUS assessment showed that either non-superficial type or submucosal invasion was the single independent predictor for worse EFS (figure 2D).

Other malignant neoplasms

Other malignancies were found in 35 patients (8.3%), 17 of whom (4.0%) had metachronous gastric cancer. Among these 17 patients, five (29%) had undergone either radiotherapy (n=3) or chemotherapy (n=2). The other malignancies included colorectal cancer (n=3), hepatocellular carcinoma, head and neck cancer, prostatic cancer, uterine cancer (n=2 in each), NK/T cell lymphoma of nasal type, chronic myelomonocytic leukaemia, oesophageal cancer, lung cancer, breast cancer, pancreatic cancer, cholangiocarcinoma, renal cell carcinoma and metastatic bone

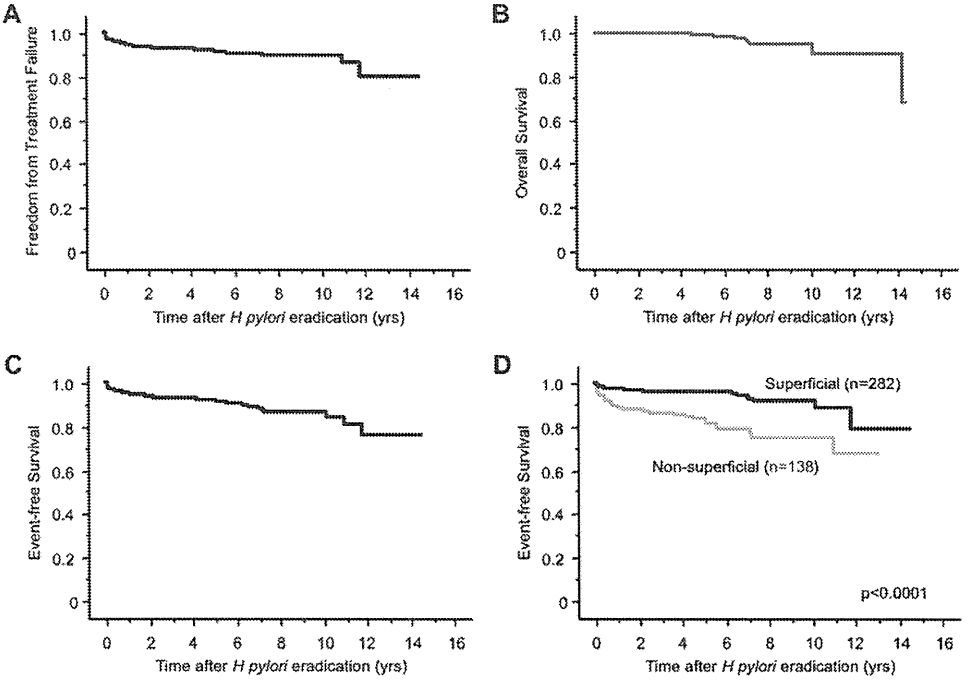

Figure 2 Kaplan–Meier curves of 420 patients with gastric MALT lymphoma after *Helicobacter pylori* eradication. (A) Freedom from treatment failure. (B) Overall survival. (C) Event-free survival. (D) Event-free survival as stratified by endoscopic types (p<0.0001).

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marrow carcinoma of unknown primary site (each in one patient). Two patients with metachronous gastric cancer also had oesophageal cancer or hepatocellular carcinoma.

DISCUSSION

While many publications have evaluated the efficacy of *H pylori* eradication for gastric MALT lymphoma, the number of patients in each study is small and the follow-up periods are relatively short. Table E in the online supplement summarises 28 studies that included more than 20 patients treated by successful *H pylori* eradication.[5–32] The number of patients ranged from 21 to 199 and CR was achieved in 1361 of 1877 patients (73%). During the follow-up period (median 0.8–6.3 years), PD was observed in 17 of 1576 patients (1.1%), relapse was recorded in 60 of 1203 patients (4.9%) with CR and treatment failure (PD or relapse) was observed in 118 of all 1877 patients (6.3%). Only five studies had a median follow-up period exceeding 5 years.[19 20 29 30 32] Compared with these studies, our investigation is large and the median follow-up period of 6.04 years is the third longest. Based on the cancer incidence data reported from the National Cancer Center, Japan (http://ganjoho.jp/professional/statistics/statistics.html), our study population is presumed to comprise approximately 7% of all patients with gastric MALT lymphoma diagnosed during the period 1994–2006 in Japan. As a result, the rates of ChR/pMRD (77%), relapse (3.1%) and treatment failure (8.8%) in our study were similar to those in the previous studies. However, our PD rate (27 of 420 patients, 6.4%) was higher than previous data (1.1%, table E in online supplement).

Various predictive factors for resistance to *H pylori* eradiation in gastric MALT lymphoma have been reported, such as absence of *H pylori* infection,[7 13 16 23 31] advanced stage,[13 31] DLBCL component,[18 19 25 37] proximal location,[7 18 24 33] endoscopic non-superficial type,[16 18] deep invasion of lymphoma in the gastric wall[13 18 19 31 36 37] and t(11;18)/*API2-MALT1* translocation.[15 31 33 38 39] In our subjects, absence of *H pylori* was an independent predictor both in a whole analysis (n=420) and in a stratified analysis of 175 patients who underwent EUS and t(11;18) investigation (table 2). In the latter analysis, deep submucosal invasion by EUS and t(11;18) were also determined to be independent predictors. We therefore consider that, in addition to evaluation for *H pylori* status, the depth of tumour invasion and t(11;18) should also be considered prior to the eradication therapy.[34 37] It has also become clear in this investigation that non-antral (middle and proximal thirds) involvement of MALT lymphoma in Japanese subjects, which is presumably associated with the high prevalence of pangastritis in the population, should be taken into consideration before eradication therapy.

To date, prognostic factors for FFTF have not been reported in gastric MALT lymphoma. We found that non-superficial type was the most significant factor for adverse FFTF (see table B in online supplement). Among patients with treatment failure, the number of PD (n=27) was greater than that of relapse (n=10). It has been reported that relapse of MALT lymphoma is occasionally associated with reinfection with *H pylori*.[15 19 21 22 29] In our study, however, only one of 10 patients with relapse had reinfection. Interestingly, the patient developed macroscopic local relapse 10.9 years after the initial ChR. These observations suggest that the clonal memory B cells may exist for a long time even after ChR was achieved by removing stimuli from *H pylori*-specific T cells.[1 41]

The probability of 5-year OS after *H pylori* eradication has been reported to be 82–96%.[5 18 20 22 29 30] The probabilities of OS after 5 and 10 years in the present investigation were 99% and 95%, respectively (figure 2B), which was much better than those reported previously. By contrast, the prognostic factors for OS in gastric MALT lymphoma have rarely been analysed. In a report by Stathis *et al*,[30] age, previous hepatitis C virus infection and performance status according to the Eastern Cooperative Oncology Group scale were associated with OS in the univariate analysis, and only age remained statistically significant in multivariate analysis. In our study, age and also non-superficial type were found to be independent prognostic factors for adverse OS by Cox multivariate analysis (see table C in online supplement).

Similarly, EFS in patients with gastric MALT lymphoma treated by *H pylori* eradication has been described in only a few publications,[5 18 32] and a probability of 5-year EFS ranging from 67% to 85% has been reported. In our patients the probability of 5-year EFS was 92%, which was higher than that in previous reports. Pinotti *et al* reported that performance status and secondary malignancy were significantly associated with EFS.[5] Another study reported that DLBCL component, deep submucosal invasion and age showed a significant association with EFS in univariate analysis, and that only deep submucosal invasion by EUS was significantly associated with EFS in multivariate analysis.[18] In our current multivariate analysis, either non-superficial type or submucosal invasion was an independent prognostic factor for poor EFS (see table D in online supplement).

The development of synchronous or metachronous other malignant neoplasms has been observed in 1–20% of patients with gastric MALT lymphoma. Our analysis of pooled data from 32 studies found synchronous or metachronous cancers in 136 of 2300 patients (5.9%).[5–32 42–45] Approximately 1–5% of patients with gastric MALT lymphoma developed metachronous gastric cancer after successful eradication of *H pylori*.[18 20 23 25 30 32] We also confirmed an equivalent incidence of metachronous malignancies (8.3%) and gastric cancer (4.9%) in our study population. While the incidence of second malignancy in patients with gastric MALT lymphoma is not high compared with the general population,[43 44] a study from Netherlands reported that the risk of gastric cancer in patients with gastric MALT lymphoma was significantly higher than in the general population.[46] Longer endoscopic follow-up would be warranted even after CR by *H pylori* eradication to detect metachronous gastric cancer as well as relapse of lymphoma.

It is beyond doubt that patients with PD or clinically evident relapse should undergo oncological treatment. Radiotherapy and chemotherapy have a curative potential in localised gastric MALT lymphoma.[34] We confirmed the high response rate of radiotherapy (94%) and chemotherapy (88%) as second-line treatments. On the other hand, the strategy for non-responders without PD or patients with histological relapse without DLBCL remains controversial. In our 10 responders it took more than 2 years from the eradication until confirmation of ChR/pMRD (data not shown). In addition, two other patients who showed histological relapse returned to ChR without any treatment. This presumably transient relapse or histological residual disease has also been reported in previous studies.[8 15 18 20 27] A recent European consensus report recommends that patients with rRD or NC can be followed for up to 2 years by a 'watch and wait' strategy.[34] We consider that 'watch and wait' follow-up can be prolonged for more than 2 years unless PD or relapsed endoscopic lesions can be demonstrated. The decision to continue a 'watch and wait' strategy or to start oncological treatment should be made based on

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multidisciplinary factors including *H pylori* status, clinical stage, presence of t(11;18) or DLBCL component.

There are some limitations in the present study. The retrospective nature seems to have been a source of selection biases. The OS and EFS in our patients might have been overestimated because patients who died within 3 years were excluded. The lack of the central review of all biopsy specimens might have induced some heterogeneity in the histological assessment. Patients in whom assessment of t(11;18) and EUS were not applied might have influenced the results of multivariate analyses for predictive factors. Nevertheless, these shortfalls did not hinder the main conclusions of the study.

In conclusion, this large-scale follow-up study shows that the long-term clinical outcome of gastric MALT lymphoma after *H pylori* eradication is excellent. An endoscopic appearance of non-superficial type is predictive of adverse FFTF, OS and EFS after *H pylori* eradication for patients with gastric MALT lymphoma. Based on these results, we propose that patients with stage I disease who achieved CR after *H pylori* eradication can be managed only by follow-up endoscopy with multiple biopsies every 1 or 2 years. Other staging procedures such as CT or PET are not required frequently. The metachronous development of gastric cancer should also be kept in mind, especially for patients who underwent radiotherapy or chemotherapy.

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Contributors SN, TC and TS conceived and designed this study with the JAPANGAST Study Group. SN, KI, SO, MT, AT, YK, HM, TN, TK, NW, TC and TM collected the data of patients. SN and HO analysed and interpreted the results. SN and TM drafted the manuscript. MA and TS were responsible for the overall planning and conduct of the study and critically reviewed the manuscript. All authors have read and approved the final version of the manuscript.

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INTERNAL MEDICINE

□ CASE REPORT □

Acute Airway Obstruction in a Patient with Achalasia

Shin'ichi Miyamoto, Yoshitaka Konda, Masashi Matsui, Kazuya Sawada, Kazuki Ikeda, Norihiko Watanabe, Chiharu Kawanami and Tsutomu Chiba

Abstract

Megaesophagus resulting from achalasia is a rare but serious cause of acute airway obstruction. We treated achalasia in a 52-year-old woman with acute respiratory distress and stridor. Chest X-ray and endoscopy showed a marked dilatation of the cervical esophagus with a large amount of undigested food. Emergency suction of the food through a nasogastric tube led to decompression of the esophagus and the immediate relief of respiratory symptoms. These findings suggest a dysfunction of the upper esophageal sphincter as a possible mechanism. As this exceptional complication of achalasia is fatal, a wider appreciation is required.

Key words: esophageal achalasia, acute airway obstruction, upper esophageal sphincter

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Introduction

Achalasia is a motility disorder caused by denervation of the esophagus leading to failure of peristalsis, raised pressure in the lower esophageal sphincter (LES), and incomplete relaxation of the LES during swallowing (1-3). Dysphagia is the classic and most common symptom. Other forms of presentation include weight loss, heartburn, nocturnal regurgitation, pneumonia, asthma, and chest pain. Respiratory obstruction due to tracheal compression caused by a massively dilated esophagus is a very rare but fatal complication. The pathophysiology of achalasia leading to acute airway obstruction has not been clearly defined, but the inability to swallow and the failure of the upper esophageal sphincter (UES) to relax are thought to contribute to esophageal distension. Swallowed foods can trap air in the esophagus, which can lead to rapidly worsening respiratory obstruction. In this paper we discuss the probable mechanism for this rare complication of esophageal achalasia and its management.

Case Report

A 52-year-old woman was transported from a nearby sushi restaurant to the emergency ward in acute respiratory distress. Her symptoms began with the acute onset of stridor and increased difficulty breathing immediately after eating sushi. She had a 12-year history of untreated esophageal achalasia and had noticed difficulty in belching for a long time. She had no previous history of respiratory or cardiac disease. On physical examination she was afebrile but diaphoretic and her neck bulged at the level of the larynx bilaterally. No thyroid masses were detectable. In addition to loud stridor, chest auscultation revealed decreased breath sounds bilaterally with an audible inspiratory and expiratory wheeze. Laboratory investigations, including blood gas data, revealed no abnormal findings. The electrocardiogram was normal. An asthma attack was suspected initially, and she was given 500 mg of methylprednisolone intravenously and 0.3 mg of epinephrine subcutaneously, but her symptoms did not change. Chest radiography showed pronounced dilatation of the esophagus in the upper thorax extending up to the neck, with a widened mediastinum (Fig. 1). Both lung fields were clear. Emergency esophagogastroduodenoscopy (EGD) showed marked dilatation of the cervical esophagus (Fig. 2), but she could not tolerate further examination because of severe dyspnea. The supraglottic airway and motility of the vocal cords were normal. A large amount of undigested food and saliva were sucked from the esophagus through the nasogastric tube, resulting in decompression of the esophagus and immediate relief of her stridor and dyspnea. She was admitted to our hospital without tracheal intubation. The same symptom occurred twice during her first

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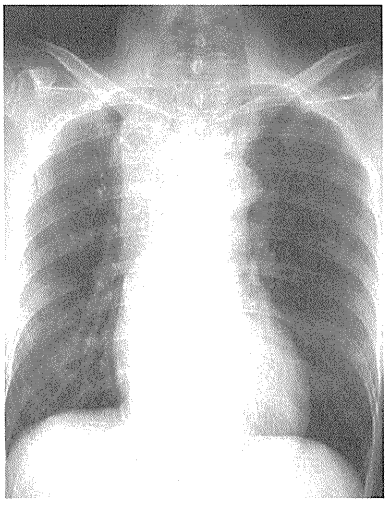

Figure 1. Chest X-ray on admission showing striking dilatation of the upper esophagus with a widened mediastinum.

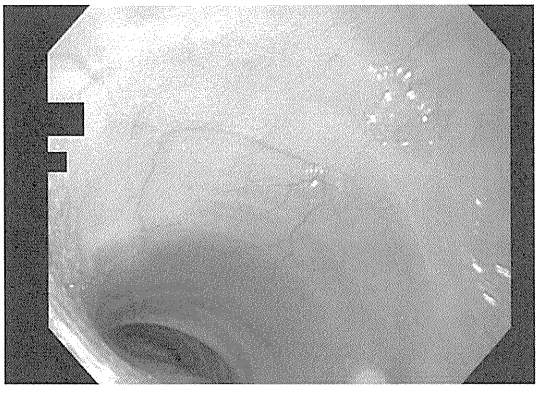

Figure 2. Upper gastrointestinal endoscopy in the emergency room demonstrating the massively dilated lumen of the cervical esophagus.

night of hospitalization, but it was improved immediately by nasogastric tube aspiration of the air and saliva in the esophagus. On the third day of hospitalization, a gastrografin contrast examination showed a large elongated esophagus with a typical sigmoid-shaped appearance (Fig. 3). Re-examination of the EGD was performed subsequently. A large amount of undigested food was observed in the grossly dilated esophagus. The endoscope (9.8 mm in diameter) was able to pass through the esophagogastric junction with no apparent stenosis of the LES. There was no evidence of esophageal or gastric malignancy. After removing the food residue, her symptoms did not recur during hospitalization. Manometric studies were performed using a multilumen probe with a pneumohydraulic water-perfused pump, which was connected to a multichannel polygraph (Esophageal Manometry Systems, Synectics Medical, Stockholm, Sweden). Manometric findings were characterized by aperistalsis of the esophagus, but LES pressure had not increased. The patient underwent a balloon dilatation of the LES twice (Rigiflex Achalasia Dilator; Microvasive, Boston, MA, USA), after which her respiratory symptoms apparently

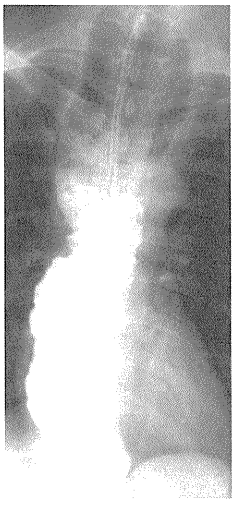

Figure 3. Gastrografin swallow showed a tortuous sigmoid-shaped esophagus with a large amount of food residue, which is typical for achalasia.

improved. Computed tomography of the chest also showed the extremely dilated esophagus located adjacent to the membranous part of the trachea (Fig. 4). From these findings we postulated that dysfunction of the UES played a crucial role in the development of her respiratory symptoms. She was discharged on the 28th day of hospitalization and is currently being followed up in the outpatient clinic at our hospital. Upper airway obstruction has not recurred in the 10 years since her presentation, although she still has mild dysphagia.

Discussion

Achalasia presenting as an acute upper airway obstruction was first reported by Bello et al (4) in 1950, and nearly 40 cases have been described in the literature since then. The clinical features of these cases have been strikingly similar. Most of the patients have been elderly women, with the onset of symptoms often occurring after a meal. Chest radiographs are easy to do and important for the diagnosis. The dilated esophagus is presented as a widened mediastinum, often with a characteristic mottled appearance or air fluid level on posterior-anterior view, which compresses and displaces the trachea forward on lateral view.

Prompt diagnosis and emergency treatment are necessary for this fatal condition. Air and saliva must be evacuated to decompress the megaesophagus; immediate insertion of a nasogastric tube into the esophagus is the most convenient and effective method (5-8). Transcutaneous needle aspiration of the distended esophagus (presented as a soft mass of the neck) is another possible method of emergency evacuation (9). Pharmacological management of this condition has been universally disappointing. However, because administration of sublingual glyceryl trinitrate is simple and familiar, it should be tried once (10).

The cricopharyngeal muscle, of approximately 1 cm in

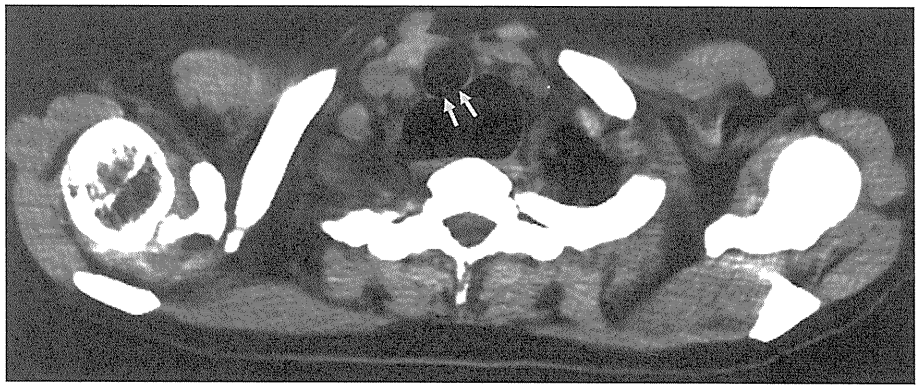

Figure 4. Computed tomography of the chest after balloon dilatation of the lower esophageal sphincter. The dilated esophagus was located adjacent to the membranous part of the trachea (arrows).

length, is a dominant part of the UES, which measures between 2 and 4 cm. Swallowing depends on fine coordination between UES relaxation and pharyngeal contractions, which forces food through the sphincter into the esophagus. A number of abnormalities in UES function have also been described in achalasia, including: (a) elevated UES residual pressure (11, 12), that is, the difference between the pressure recorded at the nadir of UES relaxation and baseline pressure, (b) decreased duration of UES relaxation (13, 14), (c) repetitive UES contractions (15), and (d) a loss of belch reflex with normal deglutitive UES relaxation (16, 17). The present patient had experienced difficulty in belching for a long time and her symptoms were consistent with failure of the belch reflex caused by insufficient UES relaxation, which led to progressive esophageal distension. Interestingly, Yoneyama et al (12) reported that the increased UES residual pressure in achalasia patients decreased significantly after successful LES dilatation. In fact, pneumatic dilatation of the LES was effective in relieving the present patient's respiratory symptoms. Unfortunately, it is impossible to accurately measure the pressure of UES with our pneumohydraulic pull-through manometer. Another method, such as the multichannel intraluminal impedance technique, may be used to analyze the detailed function of the UES (18, 19). Previous reports suggest that cricopharyngeal myotomy is a logical, well-tolerated procedure that permanently relieves symptoms for this complication (20, 21). If respiratory failure is recurrent, this procedure should be the treatment of choice. Tracheal stenting is an alternative palliative treatment in patients who are unfit for cricopharyngeal myotomy (22).

In conclusion, acute upper airway obstruction resulting from tracheal compression by dilatation of the esophagus may occur in achalasia cases because of UES dysfunction. As any delay in diagnosing this exceptional condition is critical, a wider appreciation of this complication is required. Immediate insertion of a nasogastric tube into the esophagus can lead to prompt decompression of the megaesophagus and marked improvement of respiratory symptoms.

The authors state that they have no Conflict of Interest (COI).

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Dual roles of CagA protein in *Helicobacter pylori*-induced chronic gastritis in mice

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ABSTRACT

Cytotoxin-associated gene A (CagA) acts directly on gastric epithelial cells. However, the roles of CagA in host adaptive immunity against *Helicobacter pylori* (*H. pylori*) infection are not fully understood. In this study, to investigate the roles of CagA in the development of *H. pylori*-induced chronic gastritis, we used an adoptive-transfer model in which spleen cells from C57BL/6 mice with or without *H. pylori* infection were transferred into RAG2$^{-/-}$ mice, with gastric colonization of either CagA^{+} *H. pylori* or CagA^{-} *H. pylori*. Colonization of CagA^{+} *H. pylori* but not CagA^{-} *H. pylori* in the host gastric mucosa induced severe chronic gastritis in RAG2$^{-/-}$ mice transferred with spleen cells from *H. pylori*-uninfected mice. In addition, when CagA^{+} *H. pylori*-primed spleen cells were transferred into RAG2$^{-/-}$ mice, CD4^{+} T cell infiltration in the host gastric mucosa were observed only in RAG2$^{-/-}$ mice infected with CagA^{+} *H. pylori* but not CagA^{-} *H. pylori*, suggesting that colonization of CagA^{+} *H. pylori* in the host gastric mucosa is essential for the migration of *H. pylori*-primed CD4^{+} T cells. On the other hand, transfer of CagA^{-} *H. pylori*-primed spleen cells into CagA^{+} *H. pylori*-infected RAG2$^{-/-}$ mice induced more severe chronic gastritis with less Foxp3^{+} regulatory T-cell infiltration as compared to transfer of CagA^{+} *H. pylori*-primed spleen cells. In conclusion, CagA in the stomach plays an important role in the migration of *H. pylori*-primed CD4^{+} T cells in the gastric mucosa, whereas CagA-dependent T-cell priming induces regulatory T-cell differentiation, suggesting dual roles for CagA in the pathophysiology of *H. pylori*-induced chronic gastritis.

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1. Introduction

Helicobacter pylori (*H. pylori*) infection induces chronic active gastritis, triggering the development of gastric adenocarcinoma [1–3]. Cytotoxin-associated gene A (CagA) protein is a major *H. pylori*-associated virulence factor and CagA^{+} *H. pylori* strains are more potent in developing chronic gastritis than CagA^{-} *H. pylori* strains [4,5]. CagA is encoded within the *cag* pathogenicity island (*cag*PAI). *H. pylori-cag*PAI also encodes a type IV secretion apparatus that delivers bacterial agents, including CagA, into the epithelial cells [6]. Delivered CagA mediates many of the direct actions of *H. pylori* on gastric epithelial cells. In these cells, tyrosine residues at the EPIYA motifs in the C-terminal region of CagA are phosphorylated by Src family kinase, and the tyrosine-phosphorylated CagA then forms a complex with the Src homology 2 domain-containing protein tyrosine phosphatase SHP2 [6,7]. The CagA-deregulated SHP2 exerts various functions, such as activation of extracellular regulated kinase and of the mitogen activated protein kinase cascade, as well as modulation of the focal adhesion kinase with resulting induction of the hummingbird phenomenon [7–9]. In contrast to such direct effects of CagA on gastric epithelial cells, however, the roles of CagA in host-adaptive immunity are not fully understood.

The adaptive immunity mediated by infiltrating T cells has a major role in the chronic phase of gastritis in *H. pylori* infection [2,3]. *Helicobacter*-induced chronic gastritis is characterized by a marked infiltration of CD4^{+} T cells, which produce large amounts of interferon (IFN)-γ [10,11]. Indeed, development of *H. pylori*-induced chronic gastritis is impaired in mice lacking CD4^{+} T cells or IFN-γ production [12,13]. On the other hand, forkhead box P3 (Foxp3) expressing CD4^{+}CD25^{+} regulatory T (Treg) cells, which are critical in maintaining immunologic self-tolerance and regulating a variety of pathological and physiological immune responses, also accumulate in the *H. pylori*-infected gastric mucosa [14,15]. Mice lacking Treg cells develop more severe gastritis while possessing reduced *H. pylori* bacterial loads in the gastric mucosa, suggesting that Treg cells are involved in suppressing the host

Abbreviations: *H. pylori*, *Helicobacter pylori*; CagA, cytotoxin-associated gene A; B6, C57BL/6; IFN, interferon; Foxp3, forkhead box P3; Treg, CD4^{+}CD25^{+} regulatory T; RAG2, recombination activating gene 2; PPs, Peyer's patches.

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immune response to *H. pylori* and hence the persistence of *H. pylori*-induced gastritis [16,17].

Recently, we and others reported that Peyer's patches (PPs) in the gut play an essential role in the development of *Helicobacter*-induced chronic gastritis [18,19]. In *Helicobacter* infection, PPs have a role in T-cell differentiation and the recruitment of the *Helicobacter*-specific Th1 cells into the *Helicobacter*-colonized gastric mucosa [18,19]. However, it is unclear whether CagA is indispensable for T-cell differentiation and migration in adaptive immunity against *Helicobacter* infection.

In this study, to investigate the roles of CagA in the priming and migration of T cells for the development of *H. pylori*-induced chronic gastritis, we used an adoptive-transfer model in which spleen cells of C57BL/6 (B6) mice with or without *H. pylori* infection were transferred to recombination activating gene 2 $(RAG2)^{-/-}$ mice with gastric colonization of either $CagA^+$ *H. pylori* or $CagA^-$ *H. pylori*. We found that colonization of $CagA^+$ *H. pylori* in the host gastric mucosa is essential for the migration of *H. pylori*-primed $CD4^+$ T cells to the gastric mucosa, whereas CagA-dependent T-cell priming induces Treg-cell differentiation, suggesting dual roles for CagA in the pathophysiology of *H. pylori*-induced chronic gastritis.

2. Materials and methods

2.1. Mice

B6 were purchased from Japan SLC (Shizuoka, Japan), and $RAG2^{-/-}$ mice on a B6 background were generated as described previously [18]. Before *H. pylori* infection, all of these mice were bred and housed under specific pathogen-free conditions. All mouse protocols were approved by the Institute of Laboratory Animals at the Kyoto University Graduate School of Medicine.

2.2. H. pylori and infection

H. pylori TN2GF4, isolated from a Japanese patient with a duodenal ulcer, was donated by M. Nakao (Pharmaceutical Research Division, Takeda Chemical Industries, Ltd., Osaka, Japan). TN2GF4 was CagA and VacA positive, as described previously [20]. *H. pylori* CagA-positive strain (TN2GF4) and CagA-negative strain (ΔCagA, [21]) were maintained as described previously [22]. The bacteria were grown in brucella broth at a titer of 1×10^8 organisms/ml. The bacterial suspension was stored at −80 °C until use. Six-week-old normal B6 and $RAG2^{-/-}$ mice were inoculated with 0.5 ml of bacterial suspension into the stomach using a steel catheter.

2.3. Adoptive transfer

A total of 3.0×10^7 spleen cells from *H. pylori*-infected or uninfected normal B6 were injected intravenously into *H. pylori*-infected $RAG2^{-/-}$ recipient mice. At 4 weeks after the completion of transfer, the mice were killed, and gastric tissues were analyzed by histologic and immunohistologic stainings and by real-time quantitative RT-PCR.

2.4. Histological and immunohistological analysis

Gastric tissues were fixed in neutral buffered formalin and embedded in paraffin wax. Sections were stained with hematoxylin and eosin for histopathology. Immunohistology was performed on frozen sections and examined under a microscope as described previously [22]. The following antibodies and reagents were used: FITC-conjugated anti-mouse CD4 (BD Biosciences, San Jose, CA), biotin-conjugated anti-Foxp3 (eBioscience, San Diego, CA), avidin–biotin peroxidase complex (ABC Elite Kit, Vector Laboratories, Burlingame, CA). The degree of gastritis was determined according to the semiquantitative scoring system, as described previously [23]. The infiltration of mononuclear cells was graded from 0 to 3, where 0 = no increase in the number of inflammatory cells, 1 = slight infiltration of the lamina propria by lymphocytes and plasma cells, 2 = moderately dense infiltration of the lamina propria by lymphocytes and plasma cells, and 3 = very dense lymphoplasma-cell infiltration in the lamina propria. Atrophic changes and hyperplasia were graded from 0 to 3 according to the loss of specialized cells, chief and parietal cells (0 = no loss, 1 = mild loss of specialized cells, 2 = moderate loss of specialized cells, and 3 = severe loss of specialized cells), and to the hyperplastic change of mucus neck cells (0 = no change, 1 = mild hyperplastic change of mucus neck cells, 2 = moderate hyperplastic change of mucus neck cells, and 3 = severe hyperplastic change of mucus neck cells).

2.5. Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR)

Real-time quantitative RT-PCR was performed as described previously [18,22]. Gastric tissues were frozen and stored at −80 °C until use. Values are expressed as arbitrary units relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The following primers were used: *GAPDH*: 5′-CAACTTTGTCAAGCTCATTTCC-3′ and 5′-GGTCCAGGGTTTCTTACTCC-3′; *IFN-γ*: 5′-GGATGCATTCATG AGTATTGC-3′ and 5′-CCTTTTCCGCTTCCTGAGG-3′; *IL-17A*: 5′-CTCC AGAAGGCCCTCAGACTAC-3′ and 5′-AGCTTTCCCTCCGCATTGACAC AG-3′; *Foxp3*: 5′-TCAGGAGCCCACCAGTACA-3′ and 5′-TCTGAAGG CAGAGTCAGGAGA-3′.

2.6. Statistical analysis

Statistical analysis was performed by the Student's *t*-test or Mann–Whitney *U* test for pair-wise comparisons. *P*-values below .05 were considered significant.

3. Results

3.1. CagA plays an important role in the development of H. pylori-induced chronic gastritis in the adoptive-transfer model

In this study, we used the cell-transfer model to investigate the influence of CagA in the priming and migration of T cells for the development of *H. pylori*-induced chronic gastritis. Six-week-old T- and B-cell-deficient $RAG2^{-/-}$ mice were infected with $CagA^+$ or $CagA^-$ *H. pylori*. We found that $RAG2^{-/-}$ mice infected with either $CagA^+$ or $CagA^-$ *H. pylori* did not show any inflammation of the stomach (data not shown). These data are in agreement with previous studies using $RAG2^{-/-}$ mice [18,19], suggesting that $CD4^+$ T cells have critical roles in the development of *H. pylori*-induced gastritis. Spleen cells prepared from B6 mice without *H. pylori* infection were intravenously injected into *H. pylori*-infected $RAG2^{-/-}$ recipient mice at 8 weeks after infection. Four weeks after the transfer, the recipient mice infected with $CagA^+$ *H. pylori* showed chronic gastritis with severe lymphocyte infiltration containing a large number of $CD4^+$ T cells, loss of parietal and chief cells, and hyperplasia of the mucus neck cells (Fig. 1A). These findings mimic the pathologic features observed in *H. pylori*-induced chronic gastritis in humans. In contrast, the recipient mice infected with $CagA^-$ *H. pylori* mice showed limited inflammation of the gastric mucosa without any glandular atrophy or foveolar hyperplasia by the spleen-cell transfer (Fig. 1A). These findings were further confirmed by the gastritis scoring system that evaluates (1) chronic inflammation, characterized by the infiltration of mononuclear

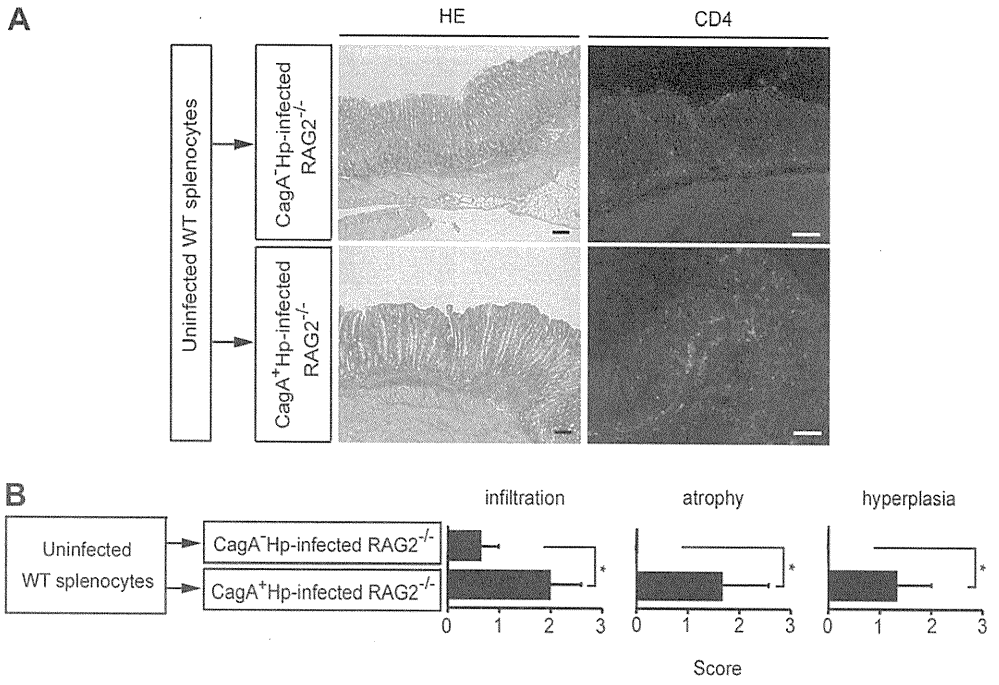

Fig. 1. Histologic and immunohistologic findings of the gastric mucosa in an adoptive-transfer model of *H. pylori*-induced chronic gastritis. Six-week-old RAG2$^{-/-}$ mice were infected with CagA^{+} or CagA^{-} *H. pylori*. Eight weeks after infection, a total of 3.0 x 10^{7} spleen cells prepared from uninfected C57BL/6 (WT) mice were intravenously injected into *H. pylori*-infected RAG2$^{-/-}$ recipient mice. (A) Four weeks after the transfer, the recipient mice were sacrificed and the stomach was analyzed by H&E and immunohistologic staining with FITC-conjugated anti-CD4. All scale bars, 100 μm. (B) The degree of gastritis in H&E staining was determined according to a semiquantitative scoring system for infiltration, atrophy, and hyperplasia. Data are shown as the mean of at least three mice in each group. Error bars represent SD. *Asterisks* indicate $P < .05$.

cells; (2) atrophic changes based on the loss of parietal and chief cells; and (3) hyperplastic changes of foveolar mucus neck cells (Fig. 1B). These data suggest that CagA is important in development of *H. pylori*-induced chronic gastritis in this mouse model.

3.2. Colonization of CagA^{+} H. pylori but not CagA^{-} H. pylori in the host gastric mucosa is critical for the migration of primed CD4^{+} T cells

In *Helicobacter* infection, PPs have important roles in T-cell differentiation and the recruitment of the primed CD4^{+} T cells into the *Helicobacter*-colonized gastric mucosa [18,19]. In *Helicobacter* infection in B6 mice, splenic CD4^{+} T cells contained *Helicobacter*-specific IFN-γ–producing cells [18,19]. When these spleen cells from B6 mice were transferred into RAG2$^{-/-}$ mice, *Helicobacter*-colonized gastric mucosa of the recipient RAG2$^{-/-}$ mice allowed the infiltration of CD4^{+} T cells of *Helicobacter*-infected B6 donor mice [18,19].

Here, we examined whether colonization of CagA^{+} *H. pylori* in the host gastric mucosa is critical for the migration of primed CD4^{+} T cells. Six-week-old normal B6 mice were inoculated with CagA^{+} *H. pylori* or CagA^{-} *H. pylori* suspension into the stomach. Eight weeks after infection, from these B6 mice, CagA^{+} *H. pylori*- or CagA^{-} *H. pylori*-primed spleen cells were prepared and transferred into either CagA^{+} *H. pylori*- or CagA^{-} *H. pylori*-infected RAG2$^{-/-}$ mice (Fig. 2). When CagA^{+} *H. pylori*-primed spleen cells were transferred into *H. pylori*-infected RAG2$^{-/-}$ mice, colonization of CagA^{+} *H. pylori* in the host gastric mucosa induced massive CD4^{+} T cell infiltration in the gastric mucosa (Fig. 3A, 2nd panels). In contrast, colonization of CagA^{-} *H. pylori* in the host gastric mucosa did not allow infiltration of a large number of CD4^{+} T cells derived from

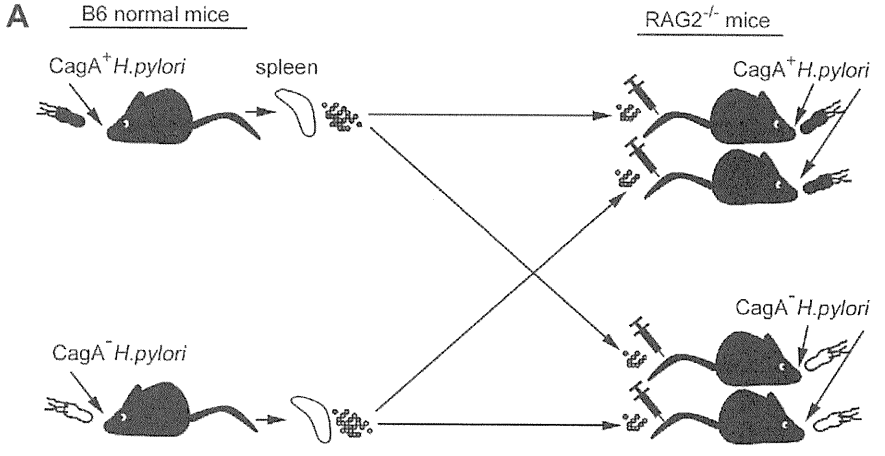

Fig. 2. Adoptive-transfer model in which *H. pylori*-primed spleen cells were transferred into *H. pylori*-infected RAG2$^{-/-}$ recipient mice. The stomachs of six-week-old normal B6 mice were inoculated with either CagA^{+} *H. pylori* or CagA^{-} *H. pylori* suspension. Eight weeks after infection, a total of 3.0 × 10^{7} CagA^{+} *H. pylori*- or CagA^{-} *H. pylori*-primed spleen cells from these B6 mice were prepared and intravenously injected into either CagA^{+} *H. pylori*- or CagA^{-} *H. pylori*-infected RAG2$^{-/-}$ recipient mice.

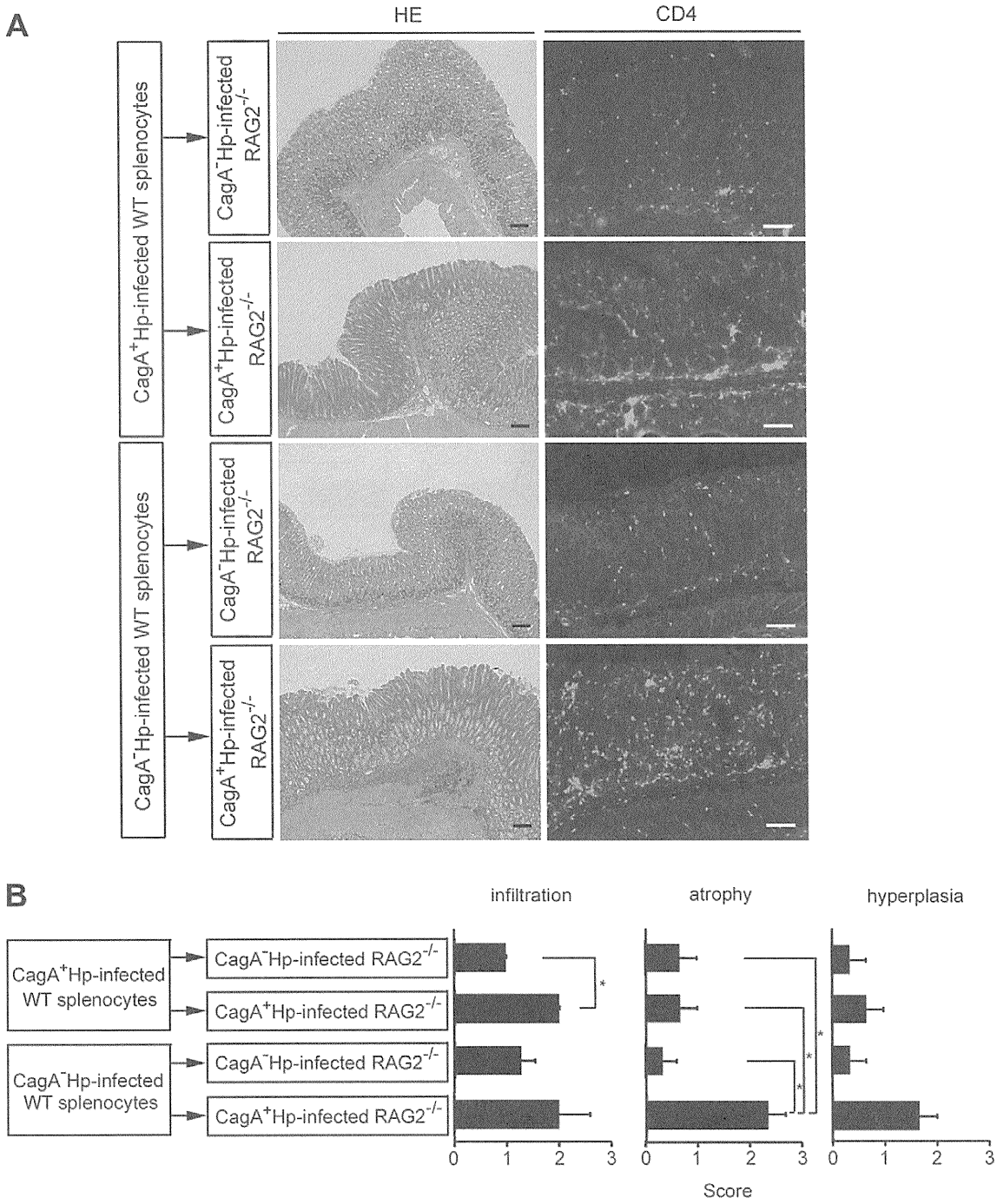

Fig. 3. Histologic and immunohistologic findings of the gastric mucosa in *H. pylori*-infected RAG2$^{-/-}$ recipient mice transferred with *H. pylori*-primed spleen cells. (A) Four weeks after the transfer of *H. pylori*-primed spleen cells into *H. pylori*-infected RAG2$^{-/-}$ recipient mice as described in Fig. 2, the recipient mice were sacrificed and their stomachs were analyzed by H&E and immunohistologic staining with FITC-conjugated anti-CD4. All scale bars, 100 μm. (B) The degree of gastritis in H&E staining was determined according to a semiquantitative scoring system for infiltration, atrophy, and hyperplasia. Data are shown as the mean of at least three mice in each group. Error bars represent SD. *Asterisks* indicate $P < .05$.

CagA$^+$ *H. pylori*-primed spleen cells (Fig. 3A, top panels). These data suggest that colonization of CagA$^+$ *H. pylori* but not CagA$^-$ *H. pylori* in the host gastric mucosa is critical for the recruitment of *H. pylori*-primed CD4$^+$ T cells into the gastric mucosa.

3.3. *CagA$^-$ H. pylori-primed spleen cells induce more severe chronic gastritis than CagA$^+$ H. pylori-primed spleen cells*

Notably, when CagA$^-$ *H. pylori*-primed spleen cells were transferred into *H. pylori*-infected RAG2$^{-/-}$ recipient mice, massive CD4$^+$ T cell infiltration was observed in the CagA$^+$ *H. pylori*-colonized host gastric mucosa but not in the CagA$^-$ *H. pylori*-colonized gastric mucosa (Fig. 3A, lower four panels). The number of infiltrating CD4$^+$ T cells in the gastric mucosa colonized with CagA$^+$ *H. pylori* is similar between recipient mice transferred with CagA$^+$ *H. pylori*- and CagA$^-$ *H. pylori*-primed spleen cells (Fig. 3A and B). Interestingly, however, compared with recipient mice transferred with CagA$^+$ *H. pylori*-primed spleen cells, recipient mice transferred with CagA$^-$ *H. pylori*-primed spleen cells showed more severe loss of parietal and chief cells in the CagA$^+$ *H. pylori*-colonized host gastric mucosa

(Fig. 3A and B). These data suggest that CagA$^+$ *H. pylor i*– but not CagA$^-$ *H. pylori*-primed CD4$^+$ T cells may contain cells with regulatory function to suppress the inflammation inducing obvious destruction of gastric mucosa.

3.4. CagA$^+$ H. pylori priming induces accumulation of regulatory CD4$^+$ T cells in the gastric mucosa

Foxp3 expressing Treg cells accumulate in the *H. pylori*-infected gastric mucosa, and Treg cells are involved in active suppression of the host immune response to *H. pylori* and persistence of *H. pylori*-induced gastritis [15–17]. To examine whether infiltrating CD4$^+$ T cells in CagA$^+$ *H. pylori*-colonized gastric mucosa by the transfer of CagA$^+$ *H. pylori*-primed CD4$^+$ T cells contains the Treg fraction, we performed RT-PCR analysis to measure the expression levels of mRNA encoding cytokines related to T cell lineage, such as IFN-γ and IL-17, and transcription factor Foxp3 in the gastric tissues. Slightly lower expression levels of mRNA encoding IFN-γ and IL-17 were observed in recipient mice transferred with CagA$^+$ *H. pylori*-primed spleen cells than in mice transferred with CagA$^-$ *H. pylori*-primed spleen cells (Fig. 4A). In contrast, gastric expression levels of mRNA encoding Foxp3 were significantly higher in recipient mice transferred with CagA$^+$ *H. pylori*-primed spleen cells than in mice transferred with CagA$^-$ *H. pylori*-primed spleen cells (Fig. 4A). These findings were further confirmed by the Foxp3 immunostaining of inflamed gastric mucosa. When CagA$^+$ *H. pylori*-primed spleen cells were transferred into CagA$^+$ *H. pylori*-infected RAG2$^{-/-}$ mice, the host gastric mucosa contained a certain number of cells whose nucleus were stained by anti-Foxp3, while the staining of nucleus was rarely seen in the gastric mucosa of the recipient mice transferred with CagA$^-$ *H. pylori*-primed spleen cells (Fig. 4B). Taken together, CagA$^+$ *H. pylori*-primed spleen cells induced less severe chronic gastritis accompanied by Foxp3$^+$ regulatory T-cell infiltration, suggesting that CagA plays a role in induction of Treg cells suppress the destruction of gastric mucosa.

4. Discussion

In this study, by using the adoptive-transfer model in which *H. pylori* infection induced severe chronic gastritis in RAG2$^{-/-}$ mice, we demonstrated that CagA is important in the development of *H. pylori*-induced chronic gastritis. We showed that colonization of CagA$^+$ *H. pylori* in the host gastric mucosa is critical for the migration of *H. pylori*-primed CD4$^+$ T cells to the gastric mucosa. In addition, CagA-dependent T-cell priming evokes differentiation of Treg cells. These data suggest that CagA has dual roles in the pathophysiology of *H. pylori*-induced chronic gastritis.

In humans, it is well established that CagA$^+$ *H. pylori* is more potent than CagA$^-$ *H. pylori* in developing chronic gastritis with higher risks for ulcer diseases and gastric cancer [2–4]. In this

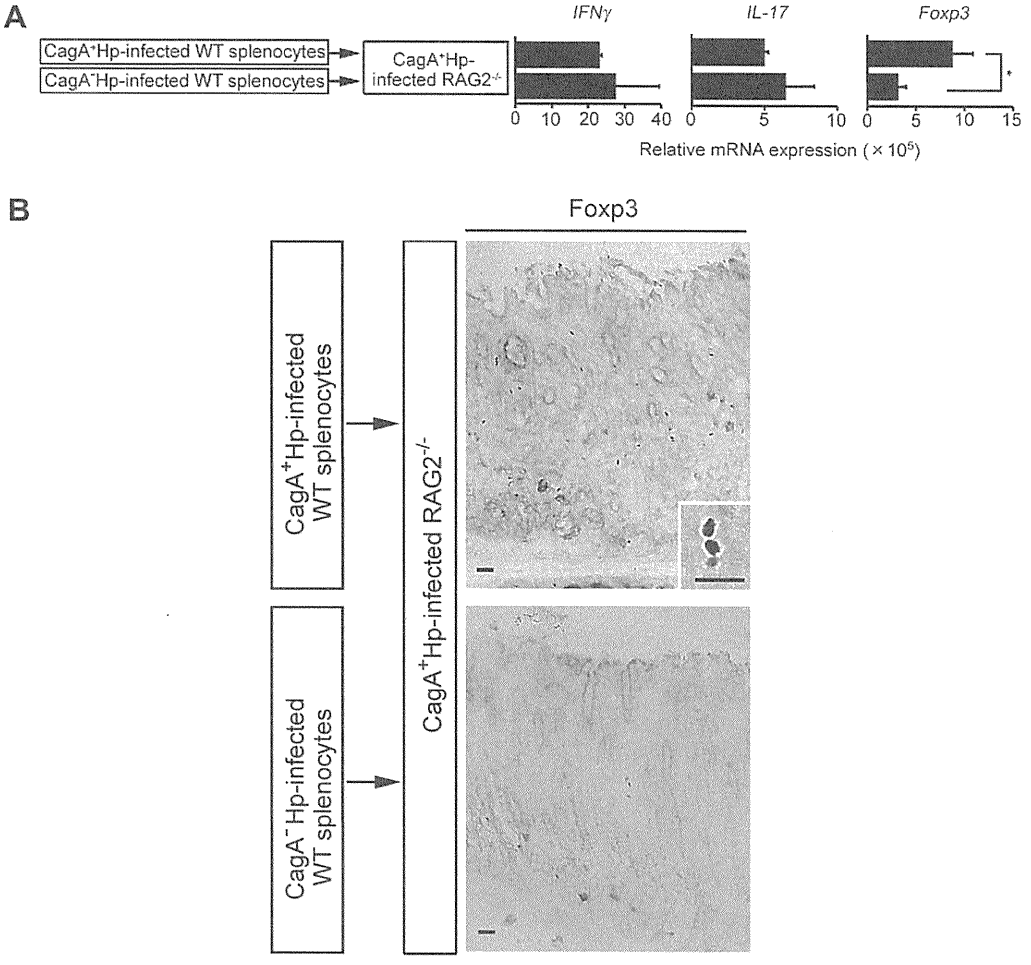

Fig. 4. Expressions of *IFN-γ*, *IL-17*, and *Foxp3* mRNA and Foxp3 immunostaining of the stomach in CagA$^+$ *H. pylori*-infected RAG2$^{-/-}$ recipient mice transferred with CagA$^+$ or CagA$^-$ *H. pylori*-primed spleen cells. (A) For indicated mice under the adoptive-transfer model as described in Fig. 2, gastric tissues were analyzed by RT-PCR. Data are shown as the mean of at least three mice. Error bars represent SD. *Asterisks* indicate $P < .05$. (B) Gastric tissues were analyzed by immunohistologic staining with biotin-conjugated anti-Foxp3, avidin–biotin peroxidase complex, and DAB substrate. All scale bars, 10 μm.

study, we observed that infection of CagA$^+$ *H. pylori* but not CagA$^-$ *H. pylori* resulted in chronic gastritis with loss of parietal and chief cells in RAG2$^{-/-}$ mice in which spleen cells from B6 mice were transferred. The mucosal findings in CagA$^+$ *H. pylori*-infected stomach of our transfer model mimic the pathologic features found in *H. pylori*-induced chronic gastritis in humans [24]. Thus, our data confirm the importance of CagA in the development of chronic gastritis induced by *H. pylori* infection.

Since RAG2$^{-/-}$ mice did not develop gastritis even by infection with CagA$^+$ *H. pylori* when spleen cells were not transferred, it is evident that CagA is not capable of inducing gastritis without T and/or B cells. In this respect, an interesting finding in our study is that when *H. pylori*-primed spleen cells were transferred to *H. pylori*-infected RAG2$^{-/-}$ mice, massive infiltration of CD4$^+$ T cells occurred only in the gastric mucosa with colonization of CagA$^+$ *H. pylori* but not in the mucosa with CagA$^-$ *H. pylori* colonization. These data strongly suggest that CagA is essential for the recruitment of *H. pylori*-primed CD4$^+$ T cells into the gastric mucosa for development of gastritis. We previously reported that direct contact of *H. pylori* with gastric epithelial cells induced epithelial production of CCL20, which can recruit CCR6-expressing T cells into the gastric mucosa [22]. In addition, we found that production of thymic stromal lymphopoietin, a cytokine derived from epithelial cells, was also triggered by direct contact with CagA$^+$ *H. pylori* but not with CagA$^-$ *H. pylori* from epithelial cells ([22] and unpublished data). Furthermore, CagA translocation into host gastric epithelial cells is involved in the induction of IL-8 [24,25]. Taken together, the migration of *H. pylori*-primed T cells into the gastric mucosa with CagA$^+$ *H. pylori* colonization observed in this study may be mediated at least in part by the production of chemokines and cytokines in epithelial cells induced by CagA.

In this study, transfer of not only CagA$^+$ but also CagA$^-$ *H. pylori*-primed spleen cells induced massive CD4$^+$ T cell infiltration in the gastric mucosa of CagA$^+$ *H. pylori*-infected RAG2$^{-/-}$ recipient mice. The data suggest that CagA might be dispensable for T-cell priming in *H. pylori*-induced gastritis, although CagA in the stomach is critical in the migration of primed T cells into the gastric mucosa, as described above. Interestingly, we further observed that transfer of CagA$^-$ *H. pylori*-primed spleen cells induced more severe gastritis, characterized by loss of parietal and chief cells than did the transfer of CagA$^+$ H. *pylori*-primed spleen cells. These data indicate that CagA may have an inhibitory role in priming *H. pylori*-specific T cells that induce gastritis. Interestingly, we further found that the inflamed gastric mucosa of the recipient mice that received CagA$^+$ *H. pylori*-primed spleen cells showed a significantly higher level of Foxp3 mRNA expression than the mice that received CagA$^-$ *H. pylori*-primed spleen cells. In addition, we observed infiltration of Foxp3-positive cells in the gastric mucosa of recipient mice transferred with CagA$^+$ *H. pylori*-primed spleen cells, while those cells were barely seen in mice transferred with CagA$^-$ *H. pylori*-primed spleen cells. These data strongly suggested that CagA protein has an important role in induction of Treg cells that can cause suppression of gastric inflammation leading to destruction of gastric mucosa. We and others previously demonstrated that dendritic cells in PPs mediate *Helicobacter*-specific Th1-cell differentiation and migration into the *Helicobacter*-colonized gastric mucosa [18,19]. Thus, it is tempting to speculate that CagA regulates the function of PPs-dendritic cells to induce Treg differentiation.

In conclusion, we demonstrated in this study that colonization of CagA$^+$ *H. pylori* in the host gastric mucosa is critical for the migration of *H. pylori*-primed CD4$^+$ T cells into the gastric mucosa and development of gastritis. Moreover, CagA-dependent T-cell priming elicits Treg-cell differentiation. These data imply dual roles for CagA in migration of primed T cells into the gastric mucosa and persistence of *H. pylori*-induced gastritis.

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