Cancer Immunology, Immunotherapy (2021) 70:443–452
https://doi.org/10.1007/s00262-020-02696-4

ORIGINAL ARTICLE

The impact of ICOS$^+$ regulatory T cells and *Helicobacter pylori* infection on the prognosis of patients with gastric and colorectal cancer: potential prognostic benefit of pre-operative eradication therapy

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Received: 4 March 2020 / Accepted: 11 August 2020 / Published online: 16 August 2020

Abstract

It remains unclear whether *Helicobacter pylori* (*H. pylori*), a major cause of gastric cancer (GC), is involved in other intestinal cancers. In our previous study, ICOS$^+$ Foxp3$^+$ CD4$^+$ T cells (ICOS$^+$ Tregs) in GC tumors were identified as effector Tregs and associated with *H. pylori*. In the present study, the impact of ICOS$^+$ Tregs on not only GC, but also colorectal cancer (CRC) and their prognosis was investigated in association with *H. pylori*. Tissue-infiltrating lymphocytes (TILs) purified from fresh tumor and sera were obtained from GC and CRC patients prospectively. % ICOS$^+$ Tregs were analyzed by flow cytometry and their production of anti-*H. pylori* antibody (Hp-Ab) in sera was detected by ELISA. % ICOS$^+$ Tregs were higher in GC and CRC patients with Hp-Ab than in those without Hp-Ab, including eradicated patients. ICOS$^+$ Tregs purified had higher potential to produce IL-10 than ICOS$^-$ Tregs. For prognostic analysis, immunohistochemical analysis and ELISA were performed using archival fixed specimens and frozen sera, respectively, obtained from GC and CRC patients. Overall survival was longer in patients with low % ICOS$^+$ Tregs than in those with high % ICOS$^+$ Tregs, and patients with Hp-Ab showed shorter recurrence-free survival than those without Hp-Ab. These results suggested that ICOS$^+$ Tregs in GC and CRC patients were closely associated with *H. pylori* in gastric epithelium and their prognosis, and that pre-operative *H. pylori* eradication has potential as a novel immunotherapy for GC and CRC patients.

Keywords ICOS$^+$ treg · Gastric cancer · *Helicobacter pylori* · Eradication · Colorectal cancer

Abbreviations

CI	Confidence interval
CRC	Colorectal cancer
CT	Cancer/testis
eTregs	Effector Tregs
EC	Esophageal cancer
GC	Gastric cancer
HR	Hazard ratio
H. pylori, Hp	*Helicobacter pylori*
Hp-Ab	*H. pylori* Antibody
IHC	Immunohistochemistry
OC	Ovarian cancer
OS	Overall survival
PBMCs	Peripheral blood mononuclear cells
pDC	Plasmacytoid dendritic cell
RCC	Renal cell carcinoma
RFS	Recurrence-free survival
TILs	Tumor tissue-infiltrating lymphocytes
TLRs	Toll-like receptors
Tregs	Regulatory T cell

ICOS$^+$ regulatory T cells in gastric and colorectal tumors were closely associated with *H. pylori* infection and the prognosis of these patients. Pre-operative *H. pylori* eradication has potential as a novel cancer immune therapy.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00262-020-02696-4) contains supplementary material, which is available to authorized users.

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Introduction

Helicobacter pylori (*H. pylori*), the most common bacteria in the human intestinal flora, selectively colonizes in the gastric epithelial layer, competing against host defense mechanisms, e.g., gastric acidity and the mucosal barrier [1–3]. *H. pylori* also has to act against host immune defenses. Regarding host innate immunity, dendritic cells with abundant toll-like receptor-4 (TLR4) is impaired since *H. pylori* lipopolysaccharides has exceptionally low activity to sensitize TLR4 due to its unique molecular structure [4]. The maturation of macrophages is also inhibited by vacuolating cytotoxin A (VacA) secreted by *H. pylori*; therefore, the initiation step of adaptive immunity is prevented [4]. Furthermore, the activation of T cells, the main immune effector cells for acquired immunity, are directly affected by *H. pylori* bacterial products, e.g., VacA and arginase, and regulatory T cells (Tregs) are also induced [4, 5].

Tregs, identified from CD4 T cells by the transcriptional factor Foxp3, play an important role in maintaining immune homeostasis [6–9]. Tregs can suppress excessive immune responses using a wide range of molecules, which may contribute to a feasible environment for *H. pylori* infection [4, 6].

Tregs are closely involved in tumor immunity due to their immunosuppressive functions. The frequency of $Foxp3^+$ T cells in peripheral blood mononuclear cells (PBMCs) and tumor tissue-infiltrating lymphocytes (TILs) is strongly associated with tumor malignancy as well as the prognosis of patients in many types of cancer [7]. In in vitro assays with tumor-associated antigenic peptides, the induction of antigen-specific cytotoxic T lymphocytes from the PBMCs was shown to be enhanced by the depletion of Tregs [8]. Among $CD4^+$ Tregs expressing Foxp3, effector Tregs (eTregs) with highly immunosuppressive functions were fractionated in $CD45RA^-$ $Foxp3^{high}$/$CD25^{high}$ by flow cytometry [9]. A minute eTreg analysis of TILs using flow cytometry resolved conflicting findings on the relationship between Tregs and patient prognoses analyzed with immunohistochemistry (IHC) targeting Foxp3 for the identification of Tregs [10–12]. Further analyses by flow cytometry revealed that the profile of antigen expression on Tregs in TILs was both tumor- and intestinal bacteria- specific. In colorectal cancer (CRC), the $CD45RA^-$ $Foxp3^{high}$ fraction was predominantly observed in 50% of patients, and an abundant $CD45RA^-$ $Foxp3^{low}$ fraction in TILs was noted in another 50% of CRC patients from whom Fusobacteria was specifically detected in the intestinal flora [13]. In our previous study on GC, the expression of ICOS was specifically observed in $CD4^+$ Tregs in tumor tissues, patients with abundant $ICOS^+$ $Foxp3^+$ TILs appeared to show advanced GC, short recurrence-free survival (RFS), and positivity for the anti-*H. pylori* antibody (Hp-Ab), and $ICOS^+$ $Foxp3^+$ cells had highly immunosuppressive functions in association with plasmacytoid DC (pDC) expressing TLR9 and ICOS-L [14–17].

H. pylori is a major cause of GC. Cytotoxin-associated gene A is introduced as an oncoprotein that is directly associated with gastric neoplasia [1]; however its relationship with other intestinal tract cancers remains controversial [18]. Furthermore, it currently remains unclear whether *H. pylori* infection plays any role in the prognosis of patients with GC and other intestinal tract cancers [18].

In the present study, we investigated the prognostic relevance of $ICOS^+$ $Foxp3^+$ TILs and *H. pylori* infection in not only GC, but also CRC patients after curative surgical treatments.

Materials and methods

Tissue and blood samples

Fresh tumor tissues and normal mucosa from surgically resected specimens and peripheral blood were obtained prospectively from 81 GC, 50 CRC, 27 esophageal cancer (EC), 31 renal cell carcinoma (RCC), and 34 ovarian cancer (OC) patients between 2016 and 2017. Tissue-infiltrating T cells were purified using the gentle MACS Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany) and a Tumor Dissociation Kit for humans (Miltenyi Biotec), and purified T cells, PBMCs, and sera were frozen and stored in an N_2 bank. In the prognostic analysis, stored TILs obtained from another 44 consecutive patients with GC between 2014 and 2015 were analyzed using a flow cytometer, and stored FFPE specimens obtained from 50 consecutive patients with pStage II/III GC between 2013 and 2014 were retrospectively analyzed by multicolor IHC (Supplementary Table S1). Fifty-six stored FFPE specimens obtained between 2010 and 2012 (Supplementary Table S3) and 128 stored serum samples obtained between 2012 and 2015 from consecutive patients with pStage III CRC (Supplementary Table S4) were also used in the present study.

Antibodies

The following fluorescence-labeled antibodies were purchased for flow cytometry: CD45 (H130), CD3 (UCTH1), CD4 (RPA-T4), CD8 (RPA-T8), CD45RA (cH100), CD25 (cBC96), ICOS (ISA-3), Tim3 (F38-2E2), 4-1BB (4B4-1), CD103 (Ber-ACT8), CD14 (HCD14), CD11c (3.9), ICOS ligand (9F.8A4), IL-17 (BL168) and IFN-γ (4SB3) from BioLegend (San Diego, CA, USA), PD-1 (EH12), OX40

(ACT35), CD19 (HIB1g), CD56 (B15g), HLA-DR (G46-6), IL-10 (JES3-9D7) from BD Biosciences (Franklin Lakes, NJ, USA), Foxp3 (PCH101) from Thermo Fisher Scientific (Waltham, MA, USA), and CD123 (AC145) from Miltenyi Biotec. Zombie NIR (BioLegend) was also used to discriminate live cells. In fluorescent IHC, CD278/ ICOS (SP98; Cell Marque, Roklin, CA, USA), Foxp3 (236A/ E7; Abcam, Cambridge, UK), CD4 (4b12; Thermo Fisher Scientific), and CD303 (124B3.13; Novus Biologicals, Littleton, CO, USA) were used.

Flow cytometry

Cells were stained with fluorophore-conjugated antibodies after an FcR block (Human TruStain FcX Fc Receptor-blocking solution; BioLegend) at 4 °C for 30 min. The BD Pharmingen™ Transcription Factor Buffer Set (BD Biosciences) was used for intracellular staining. Stained cells were analyzed by LSR Fortessa (BD Biosciences), and the frequencies of cell populations were obtained and analyzed with DiVA software (BD Biosciences). To assess positive staining, an isotype control of the primary antibody conjugated with each fluorophore was used.

Intracellular cytokine analysis

T cells purified by FACS Aria II (BD Biosciences) were stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, Saint Louis, MO, USA), 1 μM ionomycin (Sigma-Aldrich), and GolgiStop reagent (BD Biosciences) for 4 h. Harvested cells were washed and stained with antibodies against surface antigens and Zombie NIR (BioLegend) at 4 °C for 20 min. Cells were then washed and permeabilized, and intracellular cytokines were stained [19].

Fluorescent IHC

Fluorescent multi-labeling was performed with FFPE specimens using the Opal 7-Color Fluorescent IHC Kit (Perkin-Elmer, Waltham, USA). Co-localized signals were detected and captured by the Vectra automated quantitative pathology imaging system (Perkin-Elmer). In the quantitative analysis, the number of fluorescent signal-positive cells in a field of 670×500 μm was counted, and the mean cell number in three fields was calculated.

Assessment of *H. pylori* infection

Hp-Ab was detected using an *H. pylori* IgG ELISA Kit (E-plate, Eiken, Japan). In some patients, *H. pylori* infection was confirmed by Giemsa staining [14, 20].

Statistical analysis

The significance of differences in each experimental data set between two groups was assessed using the Student's two-tailed paired *t* test. The Kruskal–Wallis test, Mann–Whitney *U* test, and Chi-squared test were used for univariate analyses. Survival curves were estimated using the Kaplan–Meier method and compared by the log-rank test. All analyses were performed using SPSS for Windows v.10 (SPSS, Chicago, IL). *p* values less than 0.05 were considered to be significant.

Results

GC patients with high % ICOS+ in Foxp3+ CD4+ TILs showed short overall survival

The impact of % $ICOS^+$ in $Foxp3^+$ $CD4^+$ TILs and % $Foxp3^+$ in $CD4^+$ TILs on the prognosis of GC patients was retrospectively analyzed by flow cytometry and multicolor IHC. Forty-four GC patients whose TILs were analyzed using a flow cytometer were divided into two groups based on the median value of % $ICOS^+$ in $Foxp3^+$ $CD4^+$ TILs (10.5–68.6%, median; 36.6%) (Supplementary Table S1). Overall survival (OS) was shorter in patients with high % $ICOS^+$ in $Foxp3^+$ $CD4^+$ than in those with low % $ICOS^+$ ($p = 0.029$, Fig. 1a), while no significant difference was observed when patients were divided based on the median value of % $Foxp3^+$ in $CD4^+$ TILs (1.3–75.2%, median; 21.0%) ($p = 0.72$, Supplementary Figure. S1A). $ICOS^+$ in $Foxp3^+$ $CD4^+$ TILs were analyzed by multicolor IHC using FFPE specimens obtained from 50 GC patients between 2010 and 2012 (Supplementary Figure. S1B). CD4 and ICOS staining was observed on the cell surface, whereas that of Foxp3 was detected in nuclei. The multicolor IHC analysis also showed that OS was shorter in patients with high % $ICOS^+$ in $Foxp3^+$ $CD4^+$ TILs than in those with low % $ICOS^+$ when patients were divided based on the median value of % $ICOS^+$ in $Foxp3^+$ $CD4^+$ TILs (0–98.8%, median; 30.1%) ($p = 0.044$, Fig. 1b, Supplementary Table S1). On the other hand, no significant difference was observed when patients were divided based on the median value of % $Foxp3^+$ in $CD4^+$ TILs (7.6–97.2%, median; 49.9%) ($p = 0.47$, Supplementary Figure. S1C).

GC patients who received pre-operative eradication for *H. pylori* showed low % ICOS+ in CD25+ CD4+ TILs

Tumor tissues, a normal gastric mucosa, and peripheral blood were newly obtained from 81 GC patients, and % $ICOS^+$ in $CD25^+$ $CD4^+$ and Hp-Ab were analyzed prospectively. Between 17.4 and 84.8% $ICOS^+$ in $CD25^+$ $CD4^+$

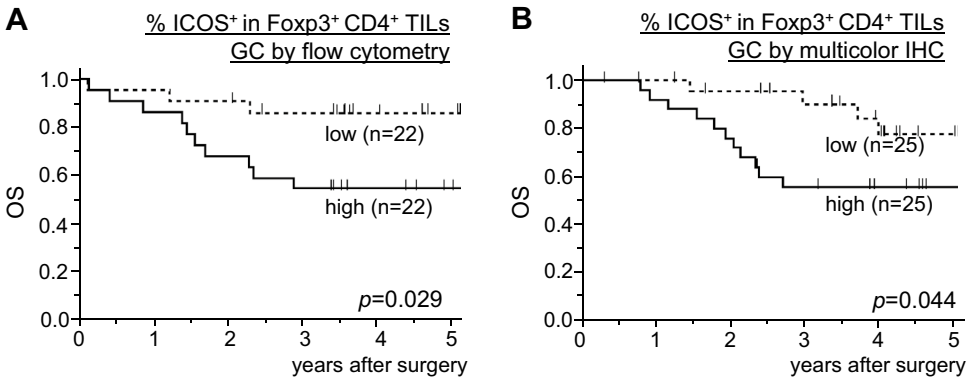

Fig. 1 % ICOS$^+$ in Foxp3$^+$ CD4$^+$ TILs in relation to overall survival in GC patients. Gastric cancer (GC) patients were divided into high (solid line) and low (dotted line) groups based on the median values of % ICOS$^+$ in Foxp3$^+$ CD4$^+$ TILs by flow cytometry (**a**) and multicolor immunohistochemistry (IHC) (Supplementary Table S1) (**b**), and overall survival (OS) curves were compared by the Kaplan–Meier method and log-rank test

TILs were observed in 81 GC tumor tissues and Hp-Ab was detected in 46 GC patients (Supplementary Figure. S1D). Significantly higher % ICOS$^+$ in CD25$^+$ CD4$^+$ TILs was observed in Hp-Ab-positive patients than in Hp-Ab-negative patients (median; 48.1 v.s. 35.5%, $p<0.001$). Hp-Ab-positive patients also had higher % ICOS$^+$ in CD25$^+$ CD4$^+$ in PBMCs and a normal gastric mucosa than Hp-Ab-negative patients (5.6 v.s. 4.0%, $p=0.001$, 28.6 v.s. 13.1%, $p=0.001$, respectively, Supplementary Figure. S1D). Twenty-one out of 35 Hp-Ab-negative patients received *H. pylori* eradication therapy. Eight patients received pre-operative eradication therapy just after their diagnosis of GC (within 1 month before surgery). Hp-Ab-positive patients had not received eradication therapy. % ICOS$^+$ in CD25$^+$ CD4$^+$ TILs in Hp-Ab-negative patients were consistent and lower than those in Hp-Ab-positive patients (Fig. 2a). In PBMCs, % ICOS$^+$ in CD25$^+$ CD4$^+$ of Hp-Ab-negative patients were also lower than those in Hp-Ab-positive patients.

Anti-*H. pylori* Ab and ICOS+ CD25+ CD4+ T cells in CRC

Although % ICOS$^+$ in CD25$^+$ CD4$^+$ TILs in GC was strongly associated with *H. pylori* infection, *H. pylori* colonized the normal gastric mucosa, not GC [21]. Furthermore, % ICOS$^+$ in CD25$^+$ CD4$^+$ in PBMCs was strongly associated with *H. pylori* infection in GC patients. The influence of *H. pylori* infection on ICOS$^+$ CD25$^+$ CD4$^+$ TILs in the cancers of organs other than the stomach was investigated. Tumor tissues, normal tissues, and peripheral blood were obtained from CRC, EC, RCC, and OC patients, and Hp-Ab and % ICOS$^+$ in CD25$^+$ CD4$^+$ TILs were analyzed by ELISA and flow cytometry, respectively (Supplementary Figure. S2A, B). Hp-Ab was detected in 18 out of 50 CRC, 9 out of 27 EC, 8 out of 31 RCC, and 5 out of 34 OC patients. Significantly higher % ICOS$^+$ in CD25$^+$ CD4$^+$ TILs were observed in Hp-Ab-positive CRC patients than -negative patients (40.4 v.s. 31.2%, $p=0.0013$), while no difference was observed in EC, RCC, or OC (EC 70.3 v.s. 67.7%, $p=0.72$; RCC 64.1 v.s. 23.1%, $p=0.09$; OC 48.1 v.s. 38.8%, $p=0.90$, Supplementary Figure. S2B). Among 32 Hp-Ab-negative CRC patients, 5 received *H. pylori* eradication therapy before surgery and their % ICOS$^+$ in CD25$^+$ CD4$^+$ TILs, as well as those of the 27 Hp-Ab-negative CRC patients, were lower than those of 18 Hp-Ab-positive CRC patients (Fig. 2b). % ICOS$^+$ in CD25$^+$ CD4$^+$ in PBMCs from Hp-Ab-positive CRC patients were also higher than those from Hp-Ab-negative patients. On the other hand, no significant differences were observed in the normal colonic mucosa, which is distinguished from GC (Supplementary Figure. S2C). The antigen expression profiles of T cells in the normal colonic mucosa were also consistent between Hp-Ab-positive and -negative CRC patients (Supplementary Table S2).

Immunosuppressive potential of ICOS+ CD25+ CD4+ TILs in CRC

The immunosuppressive functions of ICOS$^+$ CD25$^+$ CD4$^+$ TILs of CRC were analyzed based on the ability for cytokine production. ICOS$^+$ and ICOS$^-$ CD25$^+$ CD4$^+$ TILs were purified from six colorectal tumor tissues, and IL-10-, IFN-γ-, and IL-17-producing cells after a stimulation with PMA/ionomycin were detected individually by flow cytometry. The frequency of IL-10-producing cells was higher in ICOS$^+$ CD25$^+$ CD4$^+$ TILs than in ICOS$^-$ CD25$^+$ CD4$^+$ TILs in all patients examined, while that of IFN-γ and IL-17 was lower in ICOS$^+$ CD25$^+$ CD4$^+$ TILs than in ICOS$^-$ CD25$^+$ CD4$^+$ TILs in all patients (Fig. 3a).

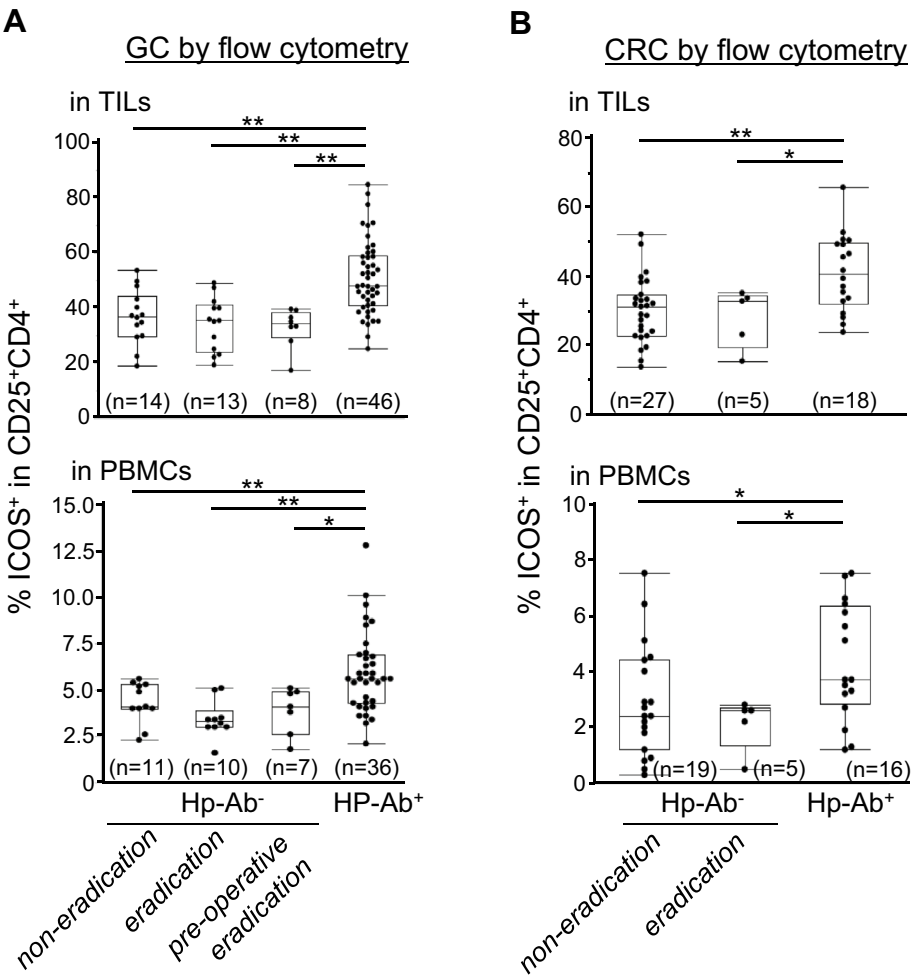

Fig. 2 Relationships between *H. pylori* infection and % ICOS$^+$ in CD25$^+$ CD4$^+$ T cells in patients with GC and CRC. Gastric cancer (GC) patients were divided into four groups; Hp-Ab-negative groups (non-eradication group, eradication group, and pre-operative eradication group) and Hp-Ab-positive group. The pre-operative eradication group received therapy within 1 month before surgery. % ICOS$^+$ in CD25$^+$ CD4$^+$ T cells were analyzed by flow cytometry and plotted according to the *H. pylori* infection status in TILs and PBMCs (**a**). Colorectal cancer (CRC) patients were divided into three groups; Hp-Ab-negative groups (non-eradication, eradication) and Hp-Ab-positive group. % ICOS$^+$ in CD25$^+$ CD4$^+$ T cells were also plotted in TILs and PBMCs. (**b**). $^*p<0.05$, $^{**}p<0.01$

ICOS-L+ in pDCs in CRC

ICOS-L$^+$ in pDCs in colorectal tumor tissue was analyzed by flow cytometry. In our previous study on GC, % ICOS$^+$ Foxp3$^+$ CD4$^+$ TILs were positively associated with % ICOS-L$^+$ in pDCs, % ICOS-L$^+$ in pDCs were higher in tumor tissues from Hp-Ab-positive patients than -negative patients, and ICOS$^+$ Foxp3$^+$ CD4$^+$ T cells were efficiently induced in vitro by the addition of the ICOS-L protein [14]. We then analyzed the influence of *H. pylori* infection on pDCs in CRC tumor tissues. % ICOS-L$^+$ in pDCs in colorectal tumor tissues, which were lower than gastric tumor tissues (19.6 v.s. 55.0%, $p<0.0001$, Supplementary Figure. S3a), were similar between Hp-Ab-positive and -negative CRC patients (20.0 v.s. 19.4%, $p=0.90$, Fig. 3b), and not related to % ICOS$^+$ in CD25$^+$ CD4$^+$ TILs ($r^2=0.024$, $p=0.6$, Fig. 3c). CD303$^+$ pDCs in GC and CRC were analyzed by IHC and their impact on the prognosis of patients was analyzed (Supplementary Figure. S3). 0.7–64 (median; 5.0) and 0.7–158 (median; 10.3) CD303$^+$ cells/field in 50 GC and 56 CRC were observed, respectively, and patients were divided based on the median value of CD303$^+$ cells. A higher CD303$^+$ cell density was associated with a worse prognosis in GC, but not CRC patients ($p=0.022$ and $p=0.94$, respectively, Supplementary Figure. S3B).

Impact of ICOS+ Foxp3+ CD4+ TILs on the prognosis of CRC patients

Multicolor IHC was performed on FFPE specimens from 56 patients with pStage III CRC who underwent curative surgery. 2.9–98.2% (median; 46.8%) ICOS$^+$ in Foxp3$^+$ CD4$^+$ TILs and 3.3–91.7% (median; 44.4%) Foxp3$^+$ in CD4$^+$ TILs were observed. When patients were divided based on the median value, patients with high % ICOS$^+$ in Foxp3$^+$ CD4$^+$ TILs showed shorter RFS ($p=0.059$) and OS ($p=0.035$) than those with low % ICOS$^+$ (Fig. 4a, Supplementary Table S3). When % Foxp3$^+$ in CD4$^+$ TILs were used, no significant differences were observed in RFS or OS between patients with high and low % Foxp3$^+$ in CD4$^+$ TILs (Supplementary Figure. S3C).

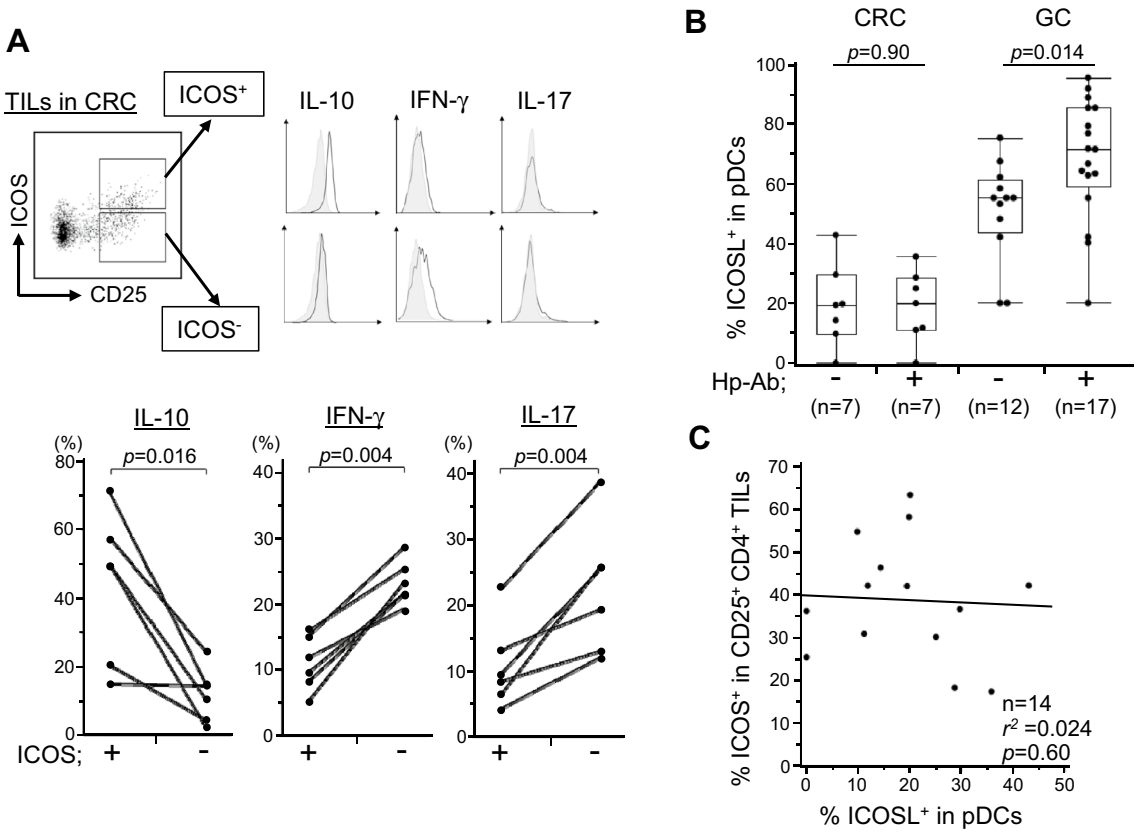

Fig. 3 Immunosuppressive function of ICOS$^+$ CD25$^+$ CD4$^+$ TILs in CRC. The production of IL-10, IFN-γ, and IL-17 in ICOS$^+$ and ICOS$^-$ CD25$^+$ CD4$^+$ TILs from six patients with colorectal cancer (CRC) was detected by intracellular staining and flow cytometry. Representative data from a patient were shown. Gray histograms indicate the isotype control. % IL-10, IFN-γ, and IL-17 in ICOS$^+$ and ICOS$^-$ CD25$^+$ CD4$^+$ TILs from each patient were dotted and connected (**a**). % ICOS-L$^+$ in pDCs were analyzed by flow cytometry and plotted according to Hp-Ap positivity in CRC (Hp-Ab(−); 19.4%, Hp-Ab(+); 20.0%) and gastric cancer (GC) (Hp-Ab(+); 55.0%, Hp-Ab(−); 71.2%) (**b**). % ICOS-L$^+$ in pDCs and % ICOS$^+$ in CD25$^+$ CD4$^+$ TILs from each patient with CRC were plotted, and an approximate straight line was depicted ($n=14$, $p=0.60$, $r^2=0.024$) (**c**). The relationship was analyzed by Pearson's correlation coefficient (r)

Impact of *H. pylori* infection on the prognosis of CRC patients

The influence of *H. pylori* infection on the prognosis of patients with CRC after curative surgical treatment was analyzed. Sera were obtained from 128 patients with pStage III CRC just before surgery, and Hp-Ab was detected by ELISA. Hp-Ab was positive in 65 patients (50.8%) and no correlation was observed between Hp-Ab positivity and clinicopathological factors (Supplementary Table S4). The median follow-up period was 44.3 months. Hp-Ab-positive patients showed shorter RFS than Hp-Ab-negative patients ($p=0.041$), while no significant difference was observed in OS between Hp-Ab-positive and -negative patients ($p=0.77$) (Fig. 4b). The univariate analysis revealed that RFS correlated with Hp-Ab, pT, vascular invasion, and adjuvant chemotherapy (Table 1). The multivariate analysis identified Hp-Ab (hazard ratio (HR) = 2.14, 95% confidence interval (CI) = 1.09–4.39, $p=0.027$), pT (HR = 3.85, 95%CI = 1.36–16.2, $p=0.0085$), vascular invasion (HR = 2.52, 95%CI = 1.24–5.57, $p=0.010$), and adjuvant chemotherapy (HR = 0.28, 95%CI = 0.14–0.56, $p=0.0005$) as independent prognostic factors (Table 1). Additionally, the impact of *H. pylori* infection on the prognosis of GC patients was analyzed. No significant differences were observed in RFS or OS between Hp-Ab-positive and -negative GC patients ($p=0.63$ and 0.63, respectively) (Supplementary Figure. S4).

Discussion

We previously reported that the high frequency of ICOS$^+$ Foxp3$^+$, but not Foxp3$^+$, TILs strongly correlated with short RFS in GC patients, and *H. pylori* infection may be one of the factors inducing ICOS$^+$ Foxp3$^+$ TILs in GC. In the present study, we demonstrated that the frequency of ICOS$^+$ Foxp3$^+$ TILs negatively correlated with OS in GC and CRC

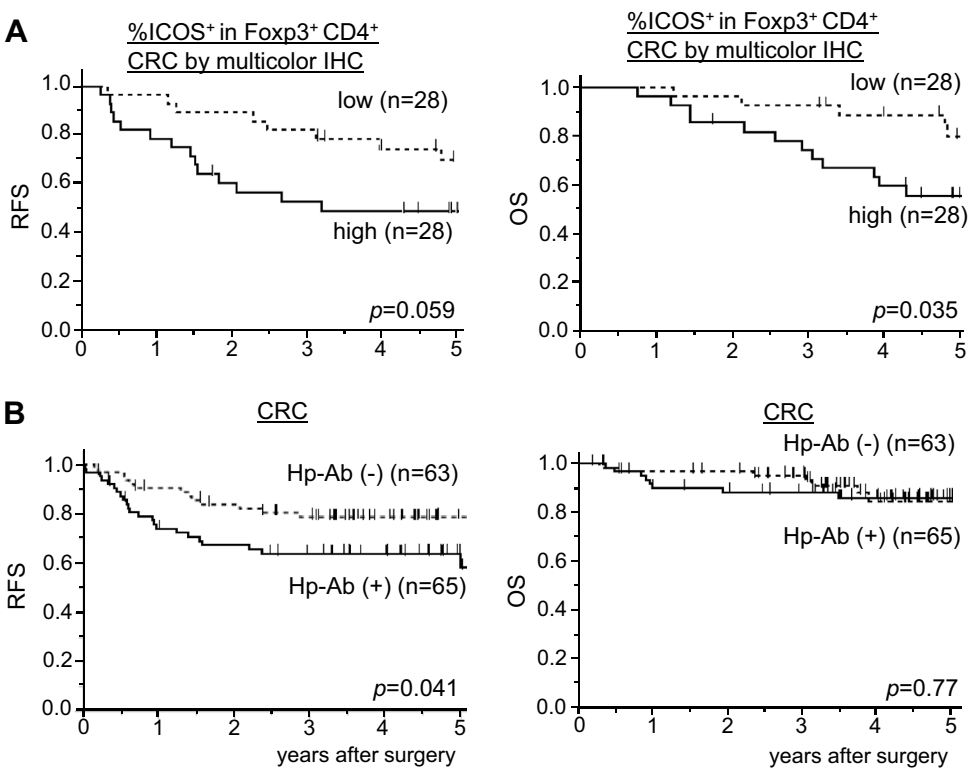

Fig. 4 Relationships between *H. pylori* infection, % ICOS$^+$ in Foxp3$^+$ CD4$^+$ TILs, and the prognosis of CRC patients. Fifty-six patients with pStage III colorectal cancer (CRC) were divided into high (solid line) and low (dotted line) groups based on the median values of % ICOS$^+$ in Foxp3$^+$ CD4$^+$ TILs by multicolor immunohistochemistry (IHC) (Supplementary Table S3) (**a**). A total of 128 patients with pStage III CRC were divided into Hp-Ab-positive (solid line) and -negative (dotted line) groups (**b**) (Supplementary Table S4). Relapse-free survival (RFS) and overall survival (OS) curves were compared by the Kaplan–Meier method and log-rank test

Table 1 Univariate and multivariate analyses between peri-operative variants and recurrence-free survival in 128 CRC patients

	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*p*	HR (95% CI)	*p*
Age, years ≥68 vs < 68	1.65 (0.85–3.26)	0.14		
Sex male vs female	1.54 (0.79–3.07)	0.20		
BMI, kg/m^2 ≥22.3 vs < 22.3	1.62 (0.84–3.25)	0.15		
Alb, g/dL ≥4.0 vs < 4.0	1.25 (0.65–2.47)	0.50		
Hp-Ab status positive vs negative	2.01 (1.03–4.08)	0.040	2.14 (1.09–4.39)	0.027
Site of the primary tumor right vs left colon/rectum	1.13 (0.54–2.25)	0.73		
pT T3-4 vs T1-2	4.32 (1.55–18.0)	0.0031	3.85 (1.36–16.2)	0.0085
Lymphatic invasion yes vs no	0.95 (0.29–5.83)	0.94		
Vascular invasion yes vs no	2.48 (1.23–5.40)	0.010	2.52 (1.24–5.57)	0.010
pN N2-3 vs N1	1.61 (0.77–3.15)	0.20		
Post-operative complications yes vs no	1.46 (0.62–3.07)	0.36		
Adjuvant chemotherapy yes vs no	0.29 (0.15–0.58)	0.0006	0.28 (0.14–0.56)	0.0005

TNM categories were based on the 8th edition of the International Union Against Cancer (UICC) TNM classification

HR hazard ratio, *CI* confidence interval, *BMI* body mass index, *Alb* albumin, *Hp-Ab H. pylori* antibody

patients, possibly due to their highly immunosuppressive function, and eradication therapy reduced ICOS$^+$ Foxp3$^+$ TILs not only in GC, but also CRC patients, suggesting that ICOS$^+$ Foxp3$^+$ TILs are also eTregs in CRC.

Current guidelines recommend the eradication of *H. pylori* after endoscopic resection for early-stage GC to prevent the subsequent development of metachronous GC [20, 22]. However, the present results indicate that pre-operative eradication therapy for not only patients with advanced GC, but also CRC may be beneficial for the prognosis of patients due to reductions in ICOS$^+$ Tregs. It is needless to say that the prevention of subsequent GC will contribute to the better prognosis of patients [23]. Furthermore, the induction of fully functional effector T cells is expected through reductions in immunosuppressive cells while primary tumors with sufficient amounts of tumor antigens exist, indicating the application of eradication therapy prior to surgery and its potential as neo-adjuvant immune therapy [24, 25]. A clinical study with the anti-CCR4 antibody targeting eTregs revealed that cellular and humoral immune responses specific to the cancer/testis (CT) antigens, NY-ESO-1 and XAGE1, were induced and enhanced in advanced cancer patients [26], and, based on these findings, another clinical study that involves the administration of the anti-CCR4 antibody to cancer patients before surgery was planned and is currently in progress (NCT02946671). Additionally, reductions in peripheral eTregs may prevent the development of residual micrometastasis after the primary tumor is surgically removed [24]. Abundant ICOS$^+$ Foxp3$^+$ CD4 T cells were detected not only in gastric tumors, but also in the normal gastric mucosa and PBMCs of patients with Hp-Ab. ICOS$^+$ eTregs induced by *H. pylori* in the stomach may affect the periphery. New clinical research on peripheral ICOS$^+$ Foxp3$^+$ CD4 T cells in Hp-Ab-positive patients without cancer is currently being planned.

Although the increased risk of developing CRC among patients with *H. pylori* infection have been reported [2, 3], the mechanisms by which *H. pylori* infection in the stomach acts as a carcinogen for CRC have not yet been elucidated. Other than virulent molecules secreted upstream of the gastrointestinal tract [27], an altered colorectal microbiome, the excessive production of gastrin, and cyclo-oxygenase-2 due to chronic gastritis, all of which are induced by *H. pylori* colonization, have been reported to be associated with CRC [28–31]. We demonstrated that ICOS$^+$ eTregs strongly correlated with *H. pylori* infection. The present results on eradication therapy appear to support the origin of tumor-infiltrating ICOS$^+$ Tregs in CRC being the stomach, not the colon in relation to *H. pylori* infection. In GC, ICOS$^+$ Tregs have been suggested to be induced by ICOS-L$^+$ pDCs activated by *H. pylori* through TLR9 [14, 15]. In contrast, pDCs in CRC may be irrelevant based on the induction of ICOS$^+$ Tregs. Therefore, colorectal adenocarcinoma may provide a more convenient environment for circulating ICOS$^+$ Tregs to be attracted and reside than other cancers [32]. To clarify this issue, the expression of several chemokines and chemokine receptors in ICOS$^+$ Tregs and tumor tissues were analyzed in many types of cancers; however, a relationship between colorectal tumor tissues and ICOS$^+$ Tregs was not observed (data not shown).

Since CRC patients without *H. pylori* infection or with *H. pylori* eradication showed low ICOS$^+$ Tregs and because CRC patients with low ICOS$^+$ Tregs had long RFS and OS, we expect CRC patients without *H. pylori* infection at surgery to have a better prognosis than those with *H. pylori* infection. Although a longer RFS was noted for Hp-Ab-negative patients than for Hp-Ab-positive patients with CRC, OS was not significantly different between these patients. This may be attributed to *H. pylori* infection not being the only factor affecting the level of ICOS$^+$ Tregs and many types of therapies being available for advanced or relapsed CRC patients [33, 34]. The results of these prognostic analyses need to be compared between Hp-Ab-positive and eradicated cancer patients, but not Hp-Ab-negative because the characteristics of cancer cells induced by *H. pylori* infection differ from "naturally occurring" cancer cells, particularly for GC [35]. However, a sufficient number of GC and CRC patients who were eradicated were not included in the present study. We plan a large clinical study to observe the impact of pre-operative eradication on Hp-Ab-positive GC and CRC patients, which would resolve the inconsistency of patient groups for each analysis in the present study.

In conclusion, ICOS$^+$ Foxp3$^+$ TILs are eTregs that influence the prognosis of GC and CRC patients and are closely associated with *H. pylori* infection. Pre-operative eradication therapy may provide prognostic benefits for GC and CRC patients with *H. pylori* infection in gastric epithelium due to decreases in ICOS$^+$ Tregs.

Acknowledgements We thank all the patients who contributed to this study. We thank Kayoko Maekawa and Junko Yamagishi for their help.

Author contributions Conception of the work: SU, MM, YD, and HW. Data collection and analysis: SU, TM, YK, KY, KG, MH, MH, TM, and ES. Manuscript writing/editing: AK, KI, and HW. Preparation of figures: MY and SU. Critical revision of the manuscript: AM-O, AK, KI, and HW. Final approval: all authors.

Funding This study was supported by Grants-in-Aid for Scientific Research (B) grant no. 19H03729.

Compliance with ethical standard

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval and consent to participate The present study was approved by the Institutional Ethics Committee of Osaka University Hospital (#13266–13, #8226–6) and performed in accordance with the

Declaration of Helsinki. All samples were obtained with the patients' informed consent.

Consent for publication All the patients provided written general consent (#8226–6) to the use of their medical data and publication at the time of their first hospitalization.

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