

# Prognostic value of peripheral and local forkhead box P3<sup>+</sup> regulatory T cells in patients with non-small-cell lung cancer

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Received March 13, 2014; Accepted April 15, 2014

DOI: 10.3892/mco.2014.299

**Abstract.** Several types of immunosuppressive mechanisms in cancer patients have been reported to date. Regulatory T cells (Tregs), which express the master control transcription factor forkhead box P3 (FoxP3), are considered to play a major role in hampering antitumor immune response. However, the clinical significance of Tregs in patients with lung cancer has not been fully elucidated. The aim of this study was to investigate the clinical significance of Tregs in the peripheral blood and in resected cancer tissue specimens. We analyzed peripheral blood mononuclear cells (PBMCs) collected prior to surgery and resected specimens obtained from 67 patients with non-small-cell lung cancer (NSCLC). Peripheral Tregs (pTregs) were detected as CD4<sup>+</sup> and FoxP3<sup>+</sup> cells by flow cytometry. Immunohistochemical staining for CD4, CD8 and FoxP3 expression was also performed quantitatively by analyzing three randomly selected fields from central regions (cCD4, cCD8 and cFoxP3) and interstitial regions of the tumors (iCD4, iCD8 and iFoxP3). The associations between the expression frequencies in selected cells and clinicopathological parameters were statistically analyzed. The frequency of pTregs was found to be significantly higher in patients with pleural invasion ( $P=0.0049$ ), vessel invasion ( $P=0.0009$ ), lymphatic vessel invasion ( $P=0.0053$ ) and recurrent disease ( $P=0.0112$ ). Patients with T1 exhibited a significantly higher frequency of cCD4 ( $P=0.0199$ ) and cCD8 ( $P=0.0058$ ), although cFoxP3 expression was not significant ( $P=0.0935$ ). Patients with low levels of cFoxP3/iFoxP3 exhibited a significantly higher frequency of pTregs ( $P=0.0338$ ) and patients with a high frequency of pTregs exhibited significantly poorer recurrence-free survival ( $P=0.0071$ ). The multivariate analysis identified pTreg frequency as an independent prognostic factor ( $P=0.0458$ ). Although the pathological analysis remains

controversial, the frequency of pTregs in NSCLC patients may be a useful prognostic biomarker.

## Introduction

Lung cancer is the most common type of cancer worldwide, accounting for 13% of all cancers, with an estimated 1.6 million new cases diagnosed annually (1). Despite the recent implementation of multimodality cancer therapy, including surgical techniques and less invasive radiation therapies, such as proton beam therapy and molecular-targeted chemotherapy, the prognosis of lung cancer remains poor and most of these conventional therapies are often harmful. In fact, the 5-year survival rate of lung cancer is only 9-15%, despite these therapies (2). Therefore, to improve lung cancer survival, novel and less toxic therapies are required. Over the last few years, immunotherapy has been applied to lung cancer treatment as an additional modality with fewer adverse events. A previous phase III MAGE-A3 trial used immunotherapy as an adjuvant therapy for patients with non-small-cell lung cancer (NSCLC) following complete pulmonary resection (3); in addition, a phase II L-BLP25 trial investigated a vaccine for patients with advanced disease (4). The clinical effectiveness of those treatment modalities appears to be promising. However, the majority of the results of other immunotherapy trials were not promising (5) and the immunocompromised status of the treated patients was considered to be the major cause of this ineffectiveness.

In a number of previous studies, the patients bearing various types of cancers, particularly advanced cancer, were immunosuppressed (6-8) and the mechanisms underlying this immunosuppression are considered to be one of the major causes of the failure of cancer immunotherapy. The mechanisms involved in immunosuppression, including tumor production of immunosuppressive cytokines [interleukin-10 (IL-10) or transforming growth factor- $\beta$  (TGF- $\beta$ )], were previously investigated (9). Recently, regulatory T cells (Tregs) have attracted the attention of tumor immunology researchers, as they have the ability to suppress or regulate cell-mediated immunity. Tregs express CD4 and CD25 on their surface and also express a master control molecule, the transcription factor forkhead box P3 (FoxP3), in their nucleus (10), which is responsible for their immunosuppressive function. Increased Treg numbers have been reported in various cancer patients

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**Key words:** non-small-cell lung cancer, regulatory T cells, forkhead box P3, immunotherapy, immunosuppression

with poor prognosis. However, to date, the association of clinicopathological characteristics with systemic Tregs in the peripheral blood and local Tregs in the tumors has not been adequately investigated in patients with NSCLC. In the present study, we analyzed Tregs in the peripheral blood and in resected NSCLC cancer tissues to determine the clinical significance of Tregs in NSCLC patients.

## Materials and methods

**Patients and blood samples.** A total of 67 cases of primary NSCLC were investigated in the present study. These samples represented the material obtained from all the patients who had undergone pulmonary resection and regional lymph node dissection at the Department of Thoracic Surgery, Fukushima Medical University Hospital, Fukushima, Japan, between 2007 and 2008. The resected primary tumors were macroscopically and microscopically examined to determine tumor location, size and extent of lymphatic and venous invasion, metastasis to lymph nodes and histological subtype, according to the International Staging System for Lung Tumors, 7th edition (11). Peripheral blood mononuclear cells (PBMCs) were obtained prior to surgery from all the patients participating in this study, as well as from 10 healthy volunteers. The major clinicopathological characteristics of the patients enrolled in this study are summarized in Table I. Lymphatic and venous invasion (ly and v factor, respectively) was determined with Elastica-Masson staining, as well as hematoxylin and eosin staining. The samples were defined as positive or negative for vessel invasion based on the presence or absence, respectively, of cancer cell emboli in the respective vessels after examining all fields of the cancer tissues.

This study was approved by the Ethics Committee of the Fukushima Medical University and informed consent was obtained from all the patients.

**Collection and processing of PBMCs.** Peripheral venous blood (20-30 ml) was drawn and collected into tubes containing EDTA-2Na. The samples were hand-carried to the laboratory and immediately centrifuged on Ficoll-Paque gradients. The PBMCs were then cryopreserved in cell vials dissolved in cell preservation liquid at -80°C. The PBMCs were recovered by flow cytometric analysis, washed in AIM V medium (Invitrogen, Tokyo, Japan), counted in the presence of a trypan blue dye to evaluate viability and used immediately.

**Flow cytometry.** Cell surface and intracellular staining procedures were performed as described previously (12). Briefly, the surfaces of 100  $\mu$ l of cells ( $1 \times 10^6$ ) were stained using 10  $\mu$ l fluorescein isothiocyanate-conjugated anti-CD3, anti-CD8 and anti-CD25, peridinin chlorophyll-conjugated anti-CD4, phycoerythrin-conjugated anti-CD4 and anti-CD8 antibodies, all purchased from BD Pharmingen (San Jose, CA, USA). Isotype control, IgG2a, was included in all the experiments. For intracellular staining, the cells were saponized, washed in cold flow cytometry staining buffer and stained with phycoerythrin-conjugated anti-human FoxP3 and its isotype control Rat IgG2a (eBioscience, San Diego, CA, USA). Flow cytometry was performed using FACSCalibur equipped with CellQuest software (both from BD Biosciences, Franklin

Table I. Patient clinical characteristics.

Characteristics	Patient no. (n=67)	%
Age, years range (mean $\pm$ SD)	45-83 (68.4 $\pm$ 9.0)	
Gender		
Male	44	65.7
Female	23	34.3
Tumor size, cm range (mean $\pm$ SD)	0-14 (3.2 $\pm$ 2.2)	
T factor		
T1	36	53.7
T2	20	29.9
T3	4	6.0
T4	7	10.4
Histology		
Adenocarcinoma	45	67.2
Squamous cell carcinoma	16	23.8
Small-cell carcinoma	2	3.0
Other	4	6.0
N factor		
Negative	17	25.4
Positive	50	74.6
p-Stage		
IA	32	47.8
IB	15	22.4
IIA	4	6.0
IIB	3	4.5
IIIA	6	8.9
IIIB	7	10.4
Ly factor		
Negative	42	62.7
Positive	20	29.9
Unknown	5	7.4
V factor		
Negative	43	64.2
Positive	19	28.4
Unknown	5	7.4
Pl factor		
Negative	50	74.6
Positive	17	25.4
Recurrence		
Yes	15	22.4
No	52	77.6

SD, standard deviation; n, factor, nodal invasion; ly factor, lymphatic invasion; v factor, venous invasion; pl factor, pleural invasion.

Lakes, NJ, USA). The acquisition and analysis gates were restricted to the lymphocyte gate, as determined by their characteristic forward and side scatter properties. Routinely,

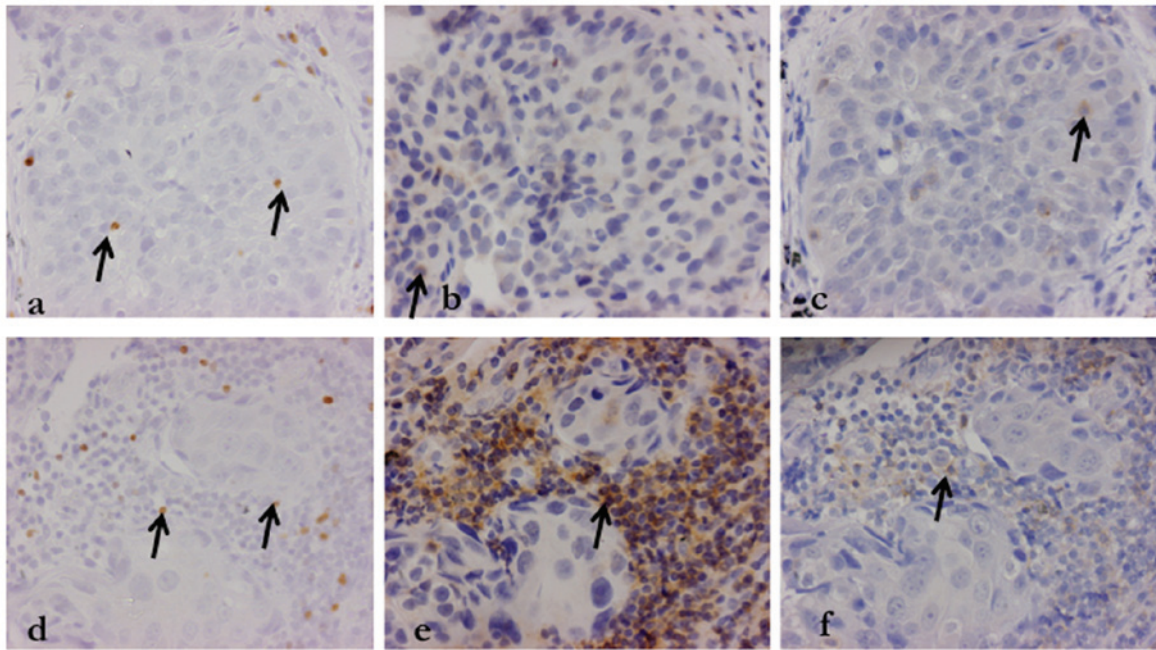


Figure 1. Immunohistochemical analysis of an adenocarcinoma specimen. The arrows indicate positive cells for each stain; (a) cFoxP3, (b) cCD4, (c) cCD8, (d) iFoxP3, (e) iCD4 and (f) iCD8. The same field was examined for each stain for accurate validation. FoxP3, forkhead box P3; c, central; i, interstitial.

the analysis gates were restricted to the CD4<sup>+</sup>CD25<sup>+</sup> T-cell subset. The flow data were analyzed using CellQuest analysis software, version 3.1 (BD Biosciences).

**Immunohistochemistry.** Immunohistochemical analyses were performed according to the methods previously described (13). Briefly, 3- $\mu$ m microtome sections were cut from paraffin-embedded lung cancer tissue specimens. Immunoperoxidase staining by the avidin-biotin-peroxidase complex method was performed. The sections were dewaxed in xylene and dehydrated through graded alcohol solutions. Endogenous peroxidase activity was quenched by 20-min incubation with 0.3% (v/v) solution of hydrogen peroxidase (Wako, Osaka, Japan) in 100% methanol. Following incubation in 5% dried skimmed milk in phosphate-buffered saline (PBS) for 30 min at room temperature, the sections were incubated overnight at 4°C with primary monoclonal antibody to the FoxP3 protein diluted at 1:100 (ab20034; Abcam Inc., Tokyo, Japan), CD4 diluted at 1:50 (NCL-CD4-1F6; Leica Microsystems, Wetzlar, Germany) and CD8 diluted at 1:50 (M7103; Dako, Glostrup, Denmark). The primary antibody was then detected using biotinylated secondary anti-mouse IgG antibody (BA-2000; Vector Laboratories, Burlingame, CA, USA), by the avidin-biotin complex method. The sections were washed several times in PBS after each step and counterstained with Mayer's hematoxylin (Muto Pure Chemicals, Co., Ltd., Tokyo, Japan), dehydrated through graded alcohol solutions and mounted on glass slides.

**Evaluation of immunohistochemical staining.** We selected three random fields from the central and the interstitial regions of the tumor and counted the positively-stained lymphocytes at high-power fields (HPF, 40x objective and 10x eyepiece magnification). We ensured selecting the same

field for each stain (CD4, CD8 and FoxP3) and evaluated the central (cFoxP3, cCD4 and cCD8) and interstitial (iFoxP3, iCD4 and iCD8) field of the tumors. To verify the association between the central and interstitial region of the tumors, the cFoxP3/iFoxP3, cCD4/iCD4 and cCD8/iCD8 ratios were calculated. Similarly, the iFoxP3/iCD4, iFoxP3/iCD8, cFoxP3/cCD4 and cFoxP3/cCD8 ratios were also calculated. The staining of samples from a representative case of adenocarcinoma is shown in Fig. 1.

**Statistical analysis.** Data are expressed as means  $\pm$  standard deviation (SD). The differences between groups were evaluated for statistical significance using the Student's t-test. Survival curves were drawn according to the Kaplan-Meier method. The log-rank test was used to perform univariate survival analysis, whereas multivariate analysis for survival was performed using the Cox's proportional hazard model. All the analyses were performed using SPSS statistics 17.0 (SPSS Inc., Chicago, IL, USA).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Peripheral Tregs are significantly correlated with clinicopathological characteristics on flow cytometric analysis.** We defined peripheral Tregs (pTregs) as CD4<sup>+</sup>FoxP3<sup>+</sup> lymphocytes, since conventional Tregs display CD4 and CD25 on their cell surface. However, a proportion of Tregs are considered to convert from peripheral naïve T cells to Tregs under Treg-proliferative conditions, thus CD4<sup>+</sup>FoxP3<sup>+</sup> Tregs include all Treg populations.

Patients with NSCLC exhibited a significantly higher frequency of peripheral regulatory T cells compared to healthy volunteers (11.2 vs. 5.6%, respectively;  $P = 0.0093$ ). Table II



Table II. Correlation between clinicopathological characteristics and frequency of peripheral regulatory T cells.

Characteristics	CD4 <sup>+</sup> FoxP3 <sup>+</sup> (%)	P-value
Age, years		0.0984
>60	10.4	
≤59	13.8	
Gender		0.5679
Male	11.5	
Female	10.5	
Tumor size, cm		0.0217
≥3.0	13.5	
<3.0	9.6	
T factor		0.0221
T1a-b	9.4	
T2-4	13.3	
Histology		0.0722
Adenocarcinoma	10.1	
Other	13.3	
N factor		0.1735
Negative	10.6	
Positive	13.3	
p-Stage		0.3533
I-II	10.8	
III-IV	13.5	
Ly factor		0.0053
Negative	9.7	
Positive	14.6	
V factor		0.0009
Negative	9.8	
Positive	15.1	
Pl factor		0.0049
Negative	9.9	
Positive	15.0	
Recurrence		0.0112
Yes	14.8	
No	10.2	
Total, range (mean±SD)	0.98-31.9 (11.1±0.8)	

FoxP3, forkhead box P3; n, factor, nodal invasion; ly factor, lymphatic invasion; v factor, venous invasion; pl factor, pleural invasion SD, standard deviation.

shows a significant correlation between peripheral regulatory T cells and tumor size ( $P=0.0217$ ), T factor (T1 vs. T2-4) ( $P=0.0221$ ), lymphatic vessel invasion ( $P=0.0053$ ), venous invasion ( $P=0.0009$ ), pleural invasion ( $P=0.0049$ ) and recurrence ( $P=0.0112$ ). There was no significant association between pathological stage and pTregs, although patients at an advanced pathological stage tended to have a high frequency of pTregs (stage I-II, 10.8% vs. stage III-IV, 13.5%;  $P=0.3533$ ).

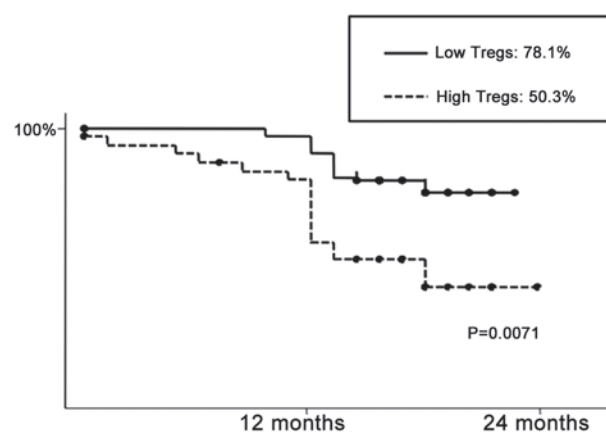


Figure 2. Two-year recurrence-free survival following pulmonary resection. Patients who had a higher frequency of peripheral regulatory T cells (pTregs) exhibited a significantly poorer recurrence-free survival ( $P=0.0071$ ).

*Limited lymphocyte infiltration of larger tumors and restrictive association between number of tumor-infiltrating Tregs and clinicopathological parameters.* The correlation between clinicopathological parameters and tumor lymphocyte infiltration is shown in Table III. The data for each parameter were as follows (presented as range, mean  $\pm$  SD): iFoxP3 (0-48.3,  $5.4\pm1.0$ ), cFoxP3 (0-23.6,  $1.6\pm0.5$ ), iCD4 (3.0-96.0,  $35.4\pm2.9$ ), cCD4 (0-50.4,  $9.3\pm1.4$ ), iCD8 (1.0-66,  $19.0\pm1.8$ ), cCD8 (0-32.6,  $7.0\pm0.98$ ).

The presence of tumor-infiltrating Tregs was not found to be correlated with clinicopathological parameters. However, from the aspect of cFoxP3/iFoxP3 ratio, larger tumors had lower cFoxP3/iFoxP3 ratios ( $\geq 3$  cm, 13.0 vs.  $<3.0$ , 46.2;  $P=0.0301$ ) and patients with pleural invasion also exhibited lower ratios ( $P=0.0156$ ) (Table IIIC).

cCD4 expression was significantly lower in larger tumors ( $P=0.0098$ ), T2-4 tumors ( $P=0.0199$ ), non-adenocarcinoma ( $P=0.0359$ ) and patients with pleural invasion ( $P=0.0244$ ). Similar associations were reported for cCD8: larger tumors, T2-4 tumors and patients with pleural invasion had lower levels of cCD8 lymphocytes ( $P=0.0036$ ,  $P=0.0058$  and  $P=0.0066$ , respectively). The number of tumor-infiltrating lymphocytes was very low, particularly in non-adenocarcinomas. In addition, patients who relapsed had lower cCD4 and cCD8 lymphocytes, although the difference was not statistically significant (Table IIIB).

*pTreg frequency in NSCLC patients correlates with recurrence-free survival.* The median follow-up was 21.3 months and relapse occurred in 15 patients, while 11 patients succumbed to the disease during follow-up. Patients with a high frequency of pTregs exhibited a significantly poorer recurrence-free survival ( $P=0.0071$ ) (Fig. 2). The univariate analysis revealed that tumor size ( $\geq 3$  cm; hazard ratio (HR)=3.773;  $P=0.017$ ), blood vessel invasion (HR=2.589;  $P=0.0205$ ), pleural invasion (HR=3.910;  $P=0.0011$ ) and CD4<sup>+</sup>FoxP3<sup>+</sup> Tregs (HR=2.419;  $P=0.0418$ ) were significant prognostic factors (Table IVA). The multivariate analysis identified CD4<sup>+</sup>FoxP3<sup>+</sup> Tregs ( $P=0.0458$ ), lymphatic vessel invasion ( $P=0.0360$ ), blood vessel invasion ( $P=0.0105$ ) and pleural invasion ( $P=0.0360$ ) as independent prognostic factors (Table IVB).

Table III. Correlation between clinicopathological characteristics and tumor lymphocyte infiltration.

A, Correlation between clinicopathological characteristics and number of interstitially infiltrating lymphocytes in the tumor						
Characteristics	iFoxP3	P-value	iCD4	P-value	iCD8	P-value
Age, years		0.9587		0.2258		0.2316
>70	5.4		31.7		16.6	
≤69	5.3		38.8		21.1	
Gender		0.7112		0.0413		0.3380
Male	5.1		31.1		17.8	
Female	5.9		43.6		21.5	
Tumor size, cm		0.7903		0.7883		0.8905
≥3.0	5.7		34.5		19.4	
<3.0	5.1		36.0		18.8	
T factor		0.6609		0.4704		0.1398
T1a-b	5.7		37.3		21.5	
T2-4	4.8		33.1		16.1	
Histology		0.3714		0.3220		0.5105
Adenocarcinoma	6.0		37.5		19.9	
Other	3.9		31.2		17.3	
N factor		0.7582		0.3828		0.6965
Negative	5.5		34.0		118.7	
Positive	4.7		40.1		20.4	
p-Stage		0.6614		0.9614		0.2397
I-II	5.6		35.5		18.1	
III	4.2		35.1		23.9	
Ly factor		0.2930		0.6320		0.2930
Negative	6.1		34.4		20.3	
Positive	3.6		37.6		16.2	
V factor		0.5860		0.7802		0.8501
Negative	5.7		34.9		19.3	
Positive	4.4		36.7		18.4	
PI factor		0.3873		0.6061		0.1595
Negative	5.9		34.5		20.5	
Positive	3.8		38.0		14.7	
Recurrence		0.9914		0.7614		0.8922
Yes	5.4		37.1		19.5	
No	5.3		34.9		18.9	

B, Correlation between clinicopathological characteristics and number of centrally infiltrating lymphocytes in the tumor

Clinical characteristics	cFoxP3	P-value	cCD4	P-value	cCD8	P-value
Age, years		0.4626		0.7064		0.9857
>70	2.0		8.8		7.0	
≤69	1.2		9.8		7.0	
Gender		0.3777		0.3446		0.4761
Male	1.9		8.4		6.5	
Female	0.9		11.1		8.0	
Tumor size, cm		0.1659		0.0098		0.0036
≥3.0	0.6		5.1		3.7	
<3.0	2.2		12.2		9.3	

Table III. Continued.

B, Correlation between clinicopathological characteristics and number of centrally infiltrating lymphocytes in the tumor						
Characteristics	cFoxP3	P-value	cCD4	P-value	cCD8	P-value
T factor		0.0935		0.0199		0.0058
T1a-b	2.4		12.2		9.5	
T2-4	0.6		5.9		4.2	
Histology		0.6971		0.0359		0.8868
Adenocarcinoma	1.7		11.3		7.1	
Other	1.3		5.2		6.8	
N factor		0.2457		0.2664		0.4223
Negative	1.9		10.1		7.5	
Positive	0.4		6.5		5.5	
p-Stage		0.4334		0.6448		0.7209
I-II	1.8		9.6		7.2	
III	0.6		7.9		6.2	
Ly factor		0.1850		0.6417		0.3064
Negative	2.1		10.9		7.9	
Positive	0.5		5.2		4.7	
V factor		0.1379		0.0607		0.1446
Negative	2.1		10.9		7.9	
Positive	0.2		5.2		4.7	
PI factor		0.1024		0.0244		0.0066
Negative	1.8		11.1		8.6	
Positive	0.1		4.2		2.7	
Recurrence		0.4666		0.3956		0.1743
Yes	0.8		7.0		4.5	
No	1.8		9.9		7.7	

C, Correlation between clinicopathological characteristics and the ratio of centrally/interstitially infiltrating lymphocytes

Characteristics	cFoxP3/iFoxP3, %	P-value	cCD4/iCD4, %	P-value	cCD8/iCD8, %	P-value
Age, years		0.5000		0.9758		0.6371
>70	39.4		37.2		66.4	
≤69	29.2		36.9		55.6	
Gender		0.8283		0.7747		0.9926
Male	35.3		35.9		60.8	
Female	40.0		39.1		60.6	
Tumor size, cm		0.0301		0.0096		0.2192
≥3.0	13.0		21.1		44.0	
<3.0	46.2		48.1		72.4	
T factor		0.1274		0.0292		0.4011
T1a-b	43.5		47.4		69.6	
T2-4	20.2		24.8		50.4	
Histology		0.6591		0.0501		0.4304
Adenocarcinoma	36.0		44.1		67.0	
Other	28.5		22.4		47.9	
N factor		0.3311		0.0604		0.1412
Negative	38.4		42.3		69.9	
Positive	21.8		19.1		30.3	

Table III. Continued.

C, Correlation between clinicopathological characteristics and ratio of centrally/interstitially infiltrating lymphocytes

Characteristics	cFoxP3/iFoxp3, %	P-value	cCD4/iCD4, %	P-value	cCD8/iCD8, %	P-value
p-Stage		0.8674		0.4532		0.3573
I-II	34.6		38.8		65.4	
III	31.3		28.1		37.1	
Ly factor		0.5473		0.8568		0.6802
Negative	37.2		36.3		57.6	
Positive	27.5		38.4		67.8	
V factor		0.0630		0.1287		0.8245
Negative	41.9		50.0		62.3	
Positive	9.7		24.2		57.0	
Pl factor		0.0156		0.0849		0.6691
Negative	43.6		42.3		63.7	
Positive	1.0		22.0		52.6	
Recurrence		0.2634		0.1563		0.1760
Yes	17.4		22.8		31.3	
No	38.3		40.9		68.7	

FoxP3, forkhead box P3; n, factor, nodal invasion; ly factor, lymphatic invasion; v factor, venous invasion; pl factor, pleural invasion; c, central; i, interstitial.

## Discussion

Immunosuppression is commonly associated with patients with advanced-stage cancer. The mechanism underlying this immunosuppressive effect has been gradually elucidated and the presence of Tregs is considered to be a key component in cancer patients. In this study, we focused on the immunocompromised status of patients with lung cancer and observed that increased numbers of pTregs were significantly correlated with worse clinicopathological conditions and disease recurrence. Our data clearly support previous cancer studies, including lung cancer, applying flow cytometric analysis (14-16). Apart from flow cytometric analysis, we used immunohistochemical analysis to demonstrate that patients with a lower cFoxP3/iFoxP3 ratio tend to have larger tumors, pleural invasion and suffer from disease relapse. In addition to FoxP3 staining, we investigated the distribution of cCD4 and cCD8 in the tumors and observed that these effector cells were significantly decreased in larger tumors.

Sakaguchi *et al* (17) previously reported that regulatory T cells, previously referred to as suppressor T cells, express CD4 and IL-2 receptor  $\alpha$ -chain (CD25) on their cell surface and display suppressor functions. Naturally occurring thymus-derived CD4<sup>+</sup>CD25<sup>+</sup> Tregs are a T-cell population with immunosuppressive properties that constitute 5-10% of the total peripheral CD4<sup>+</sup> T cells. The master control gene FoxP3 was later identified and it was revealed that FoxP3 was specific to regulatory T cells and absolutely necessary for their suppressive function (10,18,19). FoxP3 further induces peripheral naive T cells to become regulatory T cells and these induced Tregs also exert a suppressive effect. The precise mechanism of immunosuppression remains unclear.

All Tregs require T cell receptor (TCR) triggering for their suppressive activity. The major pathway of immunosuppression by Tregs may be through direct cell-to-cell suppression of effector T cells, producing soluble factors, such as immunosuppressive IL-10 and TGF- $\beta$  (20). The association between pTregs and various types of cancer has been extensively investigated (14-16,21) and the majority of those studies concluded that the frequency of pTregs in patients with cancer is elevated and is correlated with a poor prognosis, which was consistent with our results. From these collective data, it is most likely that pTregs are involved in cancer progression. However, among those previous studies, data on NSCLC are relatively rare; therefore, our NSCLC data may be valuable.

In contrast to the results referring to pTregs, the immunohistochemical assessment of Tregs in specific tumor locations appears to be controversial. Heimberger *et al* (22) and Grabenbauer *et al* (23) investigated local tumor-infiltrating lymphocytes using tissue microarrays. Their conclusions, however, were conflicting, despite their studies being performed under almost identical conditions and using identical protocols. Hiraoka *et al* (24) investigated patients with pancreatic cancer, premalignant pancreatic lesions, hepatocellular carcinoma, intrahepatic cholangiocarcinoma and metastatic liver tumor at HPF (40x objective and 10x eyepiece magnification) in at least three fields and concluded that an increasing prevalence of Tregs appeared to be an unfavorable prognostic factor (25). Siddiqui *et al* (26) reported that the number of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T cells was not associated with cancer death, whereas CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>-</sup> T cells were significantly associated with outcome in patients with renal cell carcinoma. Badoual *et al* (27) applied immu-

Table IV. Correlation between clinicopathological characteristics, peripheral regulatory T cells and survival by univariate and multivariate analysis.

A, Correlation between clinicopathological characteristics, peripheral regulatory T cells and survival by univariate analysis			
Characteristics	Hazard ratio	95% Confidential interval	P-value
Age, years >70 vs. ≤69	0.677	0.30-1.51	0.3395
Gender Male vs. female	0.910	0.39-2.13	0.8282
Tumor size, cm ≥3.0 vs. <3.0	3.773	1.646-8.647	0.017
T factor T1 vs. T2-4	0.323	0.141-0.740	0.0076
Histology Adenocarcinoma vs. other	0.418	0.186-0.936	0.0340
p-Stage IA vs. other	0.468	0.174-1.261	0.1333
Ly factor Negative vs. positive	1.034	0.442-2.422	0.9382
V factor Negative vs. positive	2.589	1.157-5.791	0.0205
Pl factor Negative vs. positive	3.910	1.726-8.857	0.0011
CD4 <sup>+</sup> /FoxP3 <sup>+</sup> /CD4 <sup>+</sup> High vs. low	2.419	1.033-5.661	0.0418
cFoxP3/iFoxP3 High vs. low	0.744	0.287-1.931	0.5439
iFoxP3 High vs. low	0.908	0.408-2.023	0.8139
iCD4 High vs. low	1.080	0.484-2.413	0.8502
cCD4 High vs. low	0.696	0.310-1.561	0.3792
cCD4/iCD4 High vs. low	0.607	0.265-1.391	0.2380
iCD8 High vs. low	0.646	0.289-1.447	0.2884
cCD8 High vs. low	0.519	0.230-1.169	0.1135
cCD8/cCD8 High vs. low	0.803	0.360-1.794	0.5932
iFoxP3/iCD4 High vs. low	1.067	0.478-2.384	0.8737
iFoxP3/iCD8 High vs. low	1.005	0.994-1.016	0.1887
cFoxP3/cCD4 High vs. low	0.520	0.224-1.205	0.1271
cFoxP3/cCD8 High vs. low	0.535	0.228-1.255	0.1503



Table IV. Continued.

B, Correlation between clinicopathological characteristics, peripheral regulatory T cells and survival by multivariate analysis.

Factor	Coefficient	Standard error	P-value
Age ( $\geq 60$ years)	0.250	0.624	0.6883
Gender	-0.374	0.581	0.5197
T1/T2 or T3/T4	-0.701	0.678	0.3008
N factor	0.445	0.766	0.5610
p-Stage IA or other	-0.079	0.753	0.9165
Ly factor	-2.584	0.805	0.0360
V factor	1.797	0.702	0.0105
Pl factor	1.688	0.805	0.0360
cFoxP3/cCD4 $>2.4\%$	0.802	0.676	0.2360
CD4 <sup>+</sup> FoxP3 <sup>+</sup> Treg	1.087	0.544	0.0458

FoxP3, forkhead box P3; n, factor, nodal invasion; ly factor, lymphatic invasion; v factor, venous invasion; pl factor, pleural invasion; c, central; i, interstitial.

nofluorescence staining to Treg studies and their results indicated that infiltration by regulatory CD4<sup>+</sup>FoxP3<sup>+</sup> T cells was positively associated with better locoregional control in patients with head and neck squamous cell carcinoma. As regards lung cancer, Petersen *et al* (28) reported that the increase in local Tregs correlated with poor prognosis; however, Ishibashi *et al* (29) reported opposite findings; thus, the histological analysis of regulatory T cells in patients with lung cancer yields controversial results. Those previous studies revealed that the clinical significance of local tumor lymphocyte evaluation is contentious. One of the reasons for these variable results of immunohistochemical staining may be the difficulty in evaluation.

Despite these difficulties, our immunohistochemical examination yielded significant findings, one of which was the tendency of non-adenocarcinomas and large-sized tumors to have lower CD4<sup>+</sup> or CD8<sup>+</sup> lymphocyte numbers. Schneider *et al* (30) reported FoxP3<sup>+</sup> Treg accumulation and a decrease in the natural killer cells in the center of adenocarcinomas, whereas squamous cell carcinomas displayed less profound accumulation of Tregs. We demonstrated that the number of cCD4<sup>+</sup> lymphocytes in adenocarcinomas was significantly higher compared to that in other histological types. According to these results, lymphocytes hardly infiltrate the tumor center in larger-sized tumors and squamous cell carcinomas, which usually grow expansively. An additional significant finding was that patients with high numbers of CD4<sup>+</sup> or CD8<sup>+</sup> lymphocytes exhibited relatively better outcomes, although this was not found to be statistically significant. Hiraoka *et al* (31) reported that concurrent infiltration by CD8<sup>+</sup> T cells and CD4<sup>+</sup> T cells is a favorable prognostic factor; Zhang *et al* (32) reported that the number of intratumoral T cells correlates with improved clinical outcome in advanced ovarian carcinoma; our results in this study were similar to other previous studies, as mentioned above (33,34).

Furthermore, we discovered that large tumors may prevent Treg infiltrates entering the tumor site and decreased cFoxP3 expression at the tumor site was significantly correlated with high frequency of pTregs. The mechanism underlying this phenomenon remains unclear, but we hypothesize based on our results that local trapping of Tregs in the tumor caused by unknown factors may affect the peripheral expansion of Tregs and subsequently affect patient outcome. One reason for the regulation of Treg distribution may be local CD4<sup>+</sup> cells.

In this study, we demonstrated the clinical impact of local as well as systemic Tregs. Tregs are clearly correlated with worse clinical conditions; therefore if we manipulate Treg function we may overcome the present shortcomings of clinical immunotherapy. To date, various attempts have been made to reverse this immunosuppressive status using anti-CD25 antibody (35-37), inhibition of the cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) pathway (38), or anti-glucocorticoid-induced tumor necrosis receptor (GITR) family-related protein (39). Yamaguchi *et al* (40) reported that folate receptor 4 expression is also specific to Tregs and may be a candidate for selective Treg elimination. Furthermore, vaccination against FoxP3 was also previously attempted *in vivo* (41); thus, this FoxP3-targeted therapy may be used to treat patients with increased Treg numbers. We may be able to select patients who are suitable for these Treg-targeted therapies using our protocol. Further research and continuous treatment trials may improve lung cancer survival using this immunosuppression-targeted therapy.

#### Acknowledgements

We would like to thank Takuya Tsunoda (Oncotherapy Science, Tokyo, Japan) for his assistance with the manuscript. This study was solely supported by the research expense program of our Institute, with no additional grant support.

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