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Forkhead box P3 regulatory T cells coexisting with cancer associated fibroblasts are correlated with a poor outcome in lung adenocarcinoma

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Recently, an association between tumor infiltrating Forkhead box P3 regulatory T cells (T_{reg}) and an unfavorable prognosis has been clinically shown in some cancers, but the mechanism of T_{reg} induction in the tumor microenvironment remains uncertain. The aims of the present study were to examine the relationship between T_{reg} and patient outcome and to investigate whether T_{reg} induction is influenced by the characteristics of cancer-associated fibroblasts (CAF) in lung adenocarcinoma. The numbers of T_{reg} in both the tumor stroma and the tumor nest were counted in 200 consecutive pathological stage I lung invasive adenocarcinoma specimens. To examine whether the characteristics of CAF influence T_{reg} induction, we selected and cultured CAF from low T_{reg} and high T_{reg} adenocarcinoma. The number of T_{reg} was much higher in the stroma than in the nest ($P < 0.01$). Patients with high T_{reg} had a significantly poorer prognosis than those with low T_{reg} (overall survival: $P = 0.03$; recurrence-free survival: $P = 0.02$; 5-year overall survival: 85.4% *vs* 93.0%). Compared with the CAF from low T_{reg} adenocarcinoma, culture supernatant of the CAF from high T_{reg} adenocarcinoma induced more T_{reg} ($P = 0.01$). Also, CAF from high T_{reg} adenocarcinoma expressed significantly higher mRNA levels of transforming growth factor-β ($P = 0.01$) and vascular endothelial growth factor ($P = 0.01$), both of which are involved in T_{reg} induction. Our studies suggest the possibility that CAF expressing immunoregulatory cytokines may induce T_{reg} in the stroma, creating a tumor-promoting microenvironment in lung adenocarcinoma that leads to a poor outcome. (*Cancer Sci* 2013; 104: 409–415)

Lung cancer is the most common cause of cancer-related death worldwide.[1] Surgery currently plays an important role in the treatment of clinical stage I–IIIA non-small lung cancer (NSCLC). Because of local recurrence and distant metastasis, however, patient outcome remains poor even after complete resection.[2] Effective therapies are needed for individual patients after surgery, and a new prognostic marker for the selection of patients with a high risk of cancer recurrence is required.

Both tumor cell characteristics and patients' immune responses have been shown to affect tumor development and metastasis.[3] Recent studies have shown that the accumulation of immunosuppressive lymphocytes, represented by regulatory T cells (T_{reg}) that suppress autoreactive T cells to maintain immunological self-tolerance and inhibit autoimmunity, is associated with advanced tumor growth and a poor outcome in several types of malignant tumors, including lung cancer.[4–8] Forkhead box P3 (Foxp3) is a member of the forkhead/winged-helix family of transcriptional factors that is critically involved in the development and function of T_{reg}.[9,10]

Cancer cells coexist with several stromal cell types that together create a cancer microenvironment. The main constituents of the stromal cell types are inflammatory cells, including lymphocytes and fibroblasts. Several recent reports have provided compelling experimental evidence indicating that the progression of tumors toward a malignant phenotype does not depend exclusively on the cell-autonomous properties of the cancer cells themselves; it is also deeply influenced by cancer associated fibroblasts (CAF).[11–13] Activated CAF contribute not only to inducing tumor progression, but also to creating the tumor microenvironment and inducing endothelial cells and other stromal cells via extracellular matrix proteins, proteases, cytokines and growth factors such as transforming growth factor (TGF)-β, human growth factor, vascular endothelial growth factor (VEGF), and fibroblast growth factor.[14] However, the correlation between tumor-infiltrating T_{reg} cells and CAF that express immunoregulatory cytokines has not been thoroughly investigated.

The aims of this study were to investigate the relationship between the T_{reg} number and the outcome of patients with p-stage I lung adenocarcinomas and to examine the possible correlation between T_{reg} induction in the tumor microenvironment and the characteristics of CAF.

Materials and Methods

Patients. The present study group comprises 200 consecutive patients with adenocarcinoma of the lung who underwent a complete resection at the National Cancer Center Hospital East, Kashiwa, Japan. All patients were diagnosed as having pathological stage I disease between January 2004 and December 2005, and all had a solitary lesion. Patients who had received preoperative chemotherapy or preoperative thoracic radiotherapy and whose tumor was diagnosed as a pure bronchioloalveolar carcinoma were excluded. All patients underwent a lobectomy or pneumonectomy for the resection of the primary lesion. We surveyed the patients at 3-month intervals for the first 2 years and at 6-month intervals thereafter.

The present research was approved by the Internal Review Board of the National Cancer Center Hospital East. The research consisted of a retrospective chart review in March 2012. No personally identifiable information was included.

Histopathological analysis and evaluation of clinicopathological factors. The available pathology slides from all 200 surgical specimens were coded, masked for identity, and then reviewed by two pathologists (T.K. and G.I.). The cases were reviewed according to the current (third) edition of the World Health

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Organization's histological classification and were staged according to the TNM classification of the seventh edition of the Union for International Cancer Control. Furthermore, per the WHO classification, we organized all the cases into the four subtypes focused on their histological predominance: bronchioloalveolar carcinoma, acinar, papillary, or solid adenocarcinoma with mucin production.

Antibodies and immunohistochemistry. After the pathologic assessment of the H&E-stained slides of the surgical specimens, 4-μm thick sections were made from formalin-fixed, paraffin-embedded specimens. Each slide was then incubated overnight at 4°C with mouse antihuman Foxp3 antibodies (ab20034 clone 236A/E7 diluted at 1:200; Abcam Inc, Cambridge, MA, USA). The slides were incubated with EnVision™ (Dako, Glostrup, Denmark) for 30 min at room temperature for visualization of bound primary antibody. They were visualized in 2% 3,3′-diaminobenzidine in 50-mM Tris buffer (pH 7.6) containing 0.3% hydrogen peroxidase.

Immunohistochemical scoring. All the stained tissue sections were semiquantitatively scored and evaluated independently under a light microscope by two pathologists (T.K. and G.I.) who did not know any clinicopathological information regarding the cases. Among tumor-infiltrating lymphocytes, T_{reg} were detected based on the presence of positive nuclear staining for Foxp3. The absolute numbers of T_{reg} in the stroma and in the nest were counted in five different high-power fields (HPF, ×400 magnification). We counted T_{reg} in the clear hot spot, and average cell count was defined as the T_{reg} number.

Statistical analysis. Overall survival (OS) was measured from the date of surgery until the date of death from any cause or the date on which the patient was last known to be alive. The recurrence-free survival (RFS) time was measured as the interval between the date of surgery and the date of recurrence, the date of death from any cause, or the most recent date on which the patient was last known to be disease-free. Survival curves were plotted according to the Kaplan–Meier method and were compared using the log-rank test. Two-category comparisons were performed using the Pearson chi-squared test or the Mann–Whitney *U*-test for quantitative data. All the tests were two sided, and *P*-values <0.05 were considered statistically significant. The statistical analysis was performed using StatView version 5.0 for Windows (SAS Institute Inc., Cary, NC, USA).

Fibroblast culture. Both CAF and non-cancer-associated fibroblasts were prepared from human lung cancer tissue and non-cancerous lung tissue obtained from the same specimen as previously reported.[15,16] Briefly, an approximately 5-mm^3 sample of carcinoma from each tissue specimen was cut into about 15 subdivisions and placed in an minimum essential medium alpha modification (α-MEM; Sigma, St. Louis, MO, USA) culture containing 10% heat-inactivated FBS and antibiotics (penicillin and streptomycin). The medium was changed every other day until the tissue was surrounded by adherent fibroblasts. After 10–20 days of growth, the fibroblasts were separated from the epithelial and endothelial cells using differential trypsinization. When the cells reached 80% confluence, they were harvested with 0.25% trypsin and 1-mmol/L ethylene-diamine-tetra-acetic acid and replated at a density of 1×10^4 cells/cm^2.

CD4+CD25− cell purification and induction of Foxp3. For induction of CD4+Foxp3+ Treg cells, CD4+CD25−CD45RA+ naive conventional T cells were purified from the peripheral blood cells obtained from two healthy volunteers with a naive CD4+ T cell isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany). Then, 5×10^5 naive T cells were cultured in the culture supernatant from CAF with 4×10^7 beads/mL CD3/CD28 beads-T cell expander (Invitrogen, Carlsbad, CA, USA) and 10-U/μL recombinant human interleukin (hIL)-2 (Roche, Penzberg, Germany) for 144 h at 37°C in a humidified atmosphere of 5% CO_2. For the control experiments, naive CD4+ T cells were cultured in RPMI1640 containing 10% FBS and 1% penicillin/streptomycin/glutamine with 50-ng/μL recombinant human transforming growth factor-β1 (Peprotech, Rocky Hill, NJ, USA), CD3/CD28 beads-T cell expander, and recombinant hIL-2. The fresh medium was replaced every other day. After 6 days of culture, living T cells were collected from them.

Real-time RT-PCR. The cultured T cells, CAF, and non-cancer-associated fibroblasts were washed with PBS, suspended in 1 mL TRIzol (Invitrogen), and then stored at −80°C. Total RNA was purified from the thawed samples with standard techniques, and cDNA was synthesized with the PrimeScript RT Reagent Kit (TaKaRa, Shiga, Japan), according to the manufacturer's instructions. Real-time RT-PCR was performed using a Smart Cycler System (TaKaRa) and SYBR Premix Ex Taq (TaKaRa), according to the manufacturer's instructions. Next, Foxp3 mRNA expression in the incubated T cells was analyzed with RT-PCR. We compared each Foxp3 expression level in proportion to the total cell number. To normalize the mRNA expression of cytokines such as TGF-β, VEGF, interleukin-10, and COX-2, we calculated the expression ratio (CAF/non-cancer-associated fibroblasts) and defined this ratio relative to the cytokine expression in CAF.

Results

Immunohistochemical staining. We detected T_{reg} among the tumor-infiltrating lymphocytes in the tumor stroma and the tumor nest based on the presence of positive nuclear staining for Foxp3. The average number of T_{reg} ranged from 0.0 to 23.0 (mean: 6.0, median: 5.5) per HPF in the stroma. The typical staining results for T_{reg} in the stroma are shown in Figure 1(a,b). In contrast, the average number of T_{reg} in the nest ranged from 0.0 to 6.4 (mean: 0.4, median: 0) per HPF. The typical staining results for T_{reg} in the nest are shown in Figure 1(c,d). Clearly, the absolute number of T_{reg} was much higher in the stroma than in the nest ($P < 0.01$) (Fig. 1e).

Correlations between number of T_{reg} and clinicopathological features. The study cohort included 88 men and 112 women, with a median of age of 65 years (range: 20–84 years, SD: 9.5 years). Five (2.5%) underwent a pneumonectomy and 195 (97.5%) underwent a lobectomy. The follow-up periods ranged from 3 to 87 months (median follow-up for surviving patients: 73 months).

We examined the clinicopathological characteristics of the cases according to the T_{reg} number in the tumor stroma and tumor nest. To assess the correlation between the clinicopathological characteristics and the T_{reg} number, we divided the patients into two groups according to their mean T_{reg} count (6/HPF). The group in which the T_{reg} count in the stroma was lower than 6/HPF ($n = 107$) was the low T_{reg} group, and the group with a T_{reg} count of 6/HPF or higher ($n = 93$) was the high T_{reg} group. The mean number of T_{reg} in the nest was 0.4/HPF, which was much lower than that in the stroma. The group in which the T_{reg} count in the nest was lower than 0.4/HPF ($n = 124$) was the low T_{reg} group, and the group with a T_{reg} count of 0.4/HPF or higher ($n = 76$) was the high T_{reg} group. For the stroma data, a large tumor diameter ($P = 0.04$), a high serum carcinoembryonic antigen level ($P = 0.03$), the presence of vascular invasion ($P < 0.01$), and the presence of pleural invasion ($P < 0.01$) were significantly more common among the high T_{reg} group than among the low T_{reg} group. For the nest data, a significant difference in vascular invasion ($P = 0.03$) was noted between the two groups, but no apparent differences in the tumor diameter, lymphatic permeation, or pleural invasion were seen (Table 1).

Comparison of T_{reg} number in the stroma according to predominant histological subtypes. To examine the difference in the T_{reg} number according to the predominant histologic subtype,

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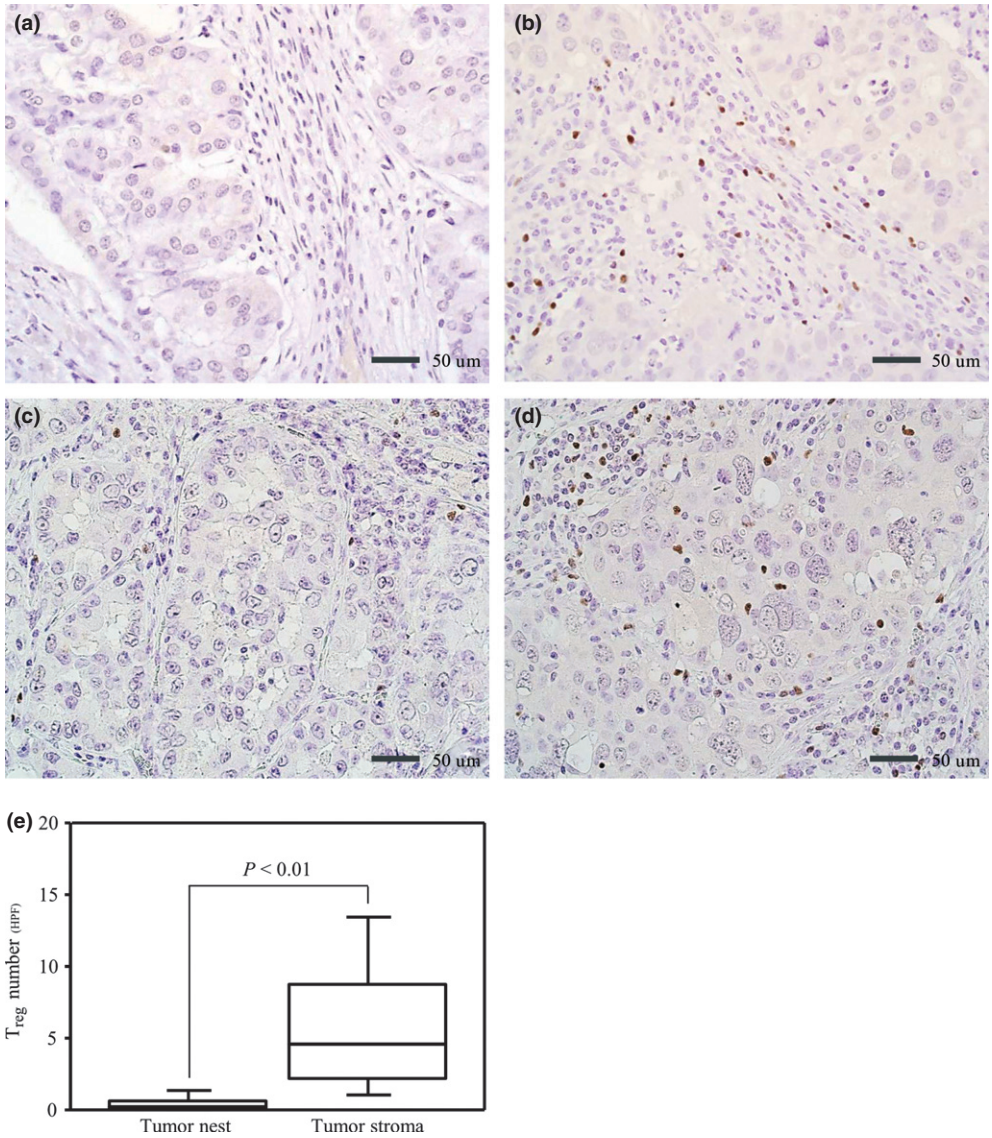

Fig. 1. Representative immunohistochemical findings for Forkhead box P3 (Foxp3+) expression in lymphocytes: (a) low T_{reg} in the stroma, (b) high T_{reg} in the stroma, (c) low T_{reg} in the nest, and (d) high T_{reg} in the nest. (e) Comparison of T_{reg} counts in the nest and the stroma. All analyses were performed using the Mann–Whitney *U*-test. T_{reg}, regulatory T cells.

we divided the cases into four groups: bronchioloalveolar carcinoma, papillary, acinar, and solid (Fig. 2). A difference in the T_{reg} number between the predominantly papillary tumors (n = 83, range: 0.2–27.4, mean: 6.7, median: 5.4) and the predominantly acinar tumors (n = 14, range: 1.0–14.4, mean: 6.9, median: 6.4) was not apparent (P = 0.79). However, the T_{reg} number in the predominantly bronchioloalveolar carcinoma tumors (n = 79, range: 0.2–22.9, mean: 4.0, median: 2.6) was much lower than that in the other histological types ($P < 0.01$), and that in the predominantly solid tumors (n = 24, range: 3.6–22.4, mean: 11.8, median: 11.6) was significantly higher than those in the other groups (P = 0.01). In the tumor nest, these differences could not be observed (data not shown).

Survival analysis according to number of T_{reg} in the tumor stroma. Figure 3(a) shows the OS curves according to the results of the T_{reg} count (high T_{reg} *vs* low T_{reg}) in the tumor stroma. The 5-year OS rates of the high T_{reg} and low T_{reg} groups were 85.4% and 93.0%, respectively. The OS of the high T_{reg} group was significantly shorter than that of the low T_{reg} group (P = 0.03). Also, RFS curves were plotted according to the results of the T_{reg} count (Fig. 3b). The 5-year RFS rates of the high T_{reg} and low T_{reg} groups were 76.5% and 87.0%, respectively. The RFS of the high T_{reg} group was significantly shorter than that of the low T_{reg} group (P = 0.02).

Induction of T_{reg} from naive CD4+ T cells by CAF. Because we found that the tumor stroma, which is mainly composed of CAF, was the main location of the T_{reg}, we investigated whether soluble factors secreted by CAF influenced the induction of T_{reg}. We selected CAF from 12 cases: six from low T_{reg} adenocarcinomas and six from high T_{reg} adenocarcinomas (Fig. 4a). Next, we cultured the naive T cells according to the above-described method using supernatant samples from these CAF. The results are shown in Figure 4(b). Compared with the low T_{reg} CAF, a significantly higher number of Foxp3+ T cells were induced by the supernatant from the high T_{reg} CAF (P = 0.01).

Correlations between number of T_{reg} and mRNA levels of immunoregulatory cytokines in CAF. We examined whether a correlation was present between the T_{reg} number in the tumor

Table 1. Correlation between T_{reg} number and clinicopathological factors

	Stroma			Nest		
	Low T_{reg} n = 107 (%)	High T_{reg} n = 93 (%)	P-value	Low T_{reg} n = 124 (%)	High T_{reg} n = 76 (%)	P-value
Sex						
Men	48 (44.9)	40 (43.0)	0.89	54 (43.5)	34 (44.7)	0.88
Women	59 (55.1)	53 (57.0)		70 (56.5)	42 (55.3)	
Age (year)						
<65	51 (47.7)	55 (59.1)	0.12	67 (54.0)	39 (51.3)	0.77
≥65	56 (52.3)	38 (40.9)		57 (46.0)	37 (48.7)	
Smoking status						
Never	50 (46.7)	47 (50.5)	0.67	60 (48.4)	36 (47.4)	>0.99
Ever	57 (53.3)	46 (49.5)		64 (51.6)	40 (52.6)	
Tumor diameter (cm)						
≤3	84 (78.5)	60 (64.5)	0.04	90 (72.6)	54 (71.1)	0.87
>3	23 (21.5)	33 (35.5)		34 (27.4)	22 (28.9)	
Serum CEA (ng/mL)						
<5	83 (77.6)	59 (63.4)	0.03	95 (76.6)	47 (61.8)	0.07
≥5	24 (22.4)	34 (36.6)		29 (23.4)	29 (38.2)	
Lymphatic permeation						
Negative	101 (94.4)	80 (86.0)	0.05	114 (91.9)	67 (88.2)	0.45
Positive	6 (5.6)	13 (14.0)		10 (8.1)	9 (11.8)	
Vascular invasion						
Negative	93 (86.9)	59 (63.4)	<0.01	101 (81.5)	51 (67.1)	0.03
Positive	14 (13.1)	34 (36.6)		23 (18.5)	25 (32.9)	
Pleural invasion						
Negative	99 (92.5)	69 (74.2)	<0.01	109 (87.9)	59 (77.6)	0.07
Positive	8 (7.5)	24 (25.8)		15 (12.1)	17 (22.4)	

Two-category comparison was performed using the Pearson's χ^2. CEA; carcinoembryonic antigen; T_{reg}, regulatory T cells.

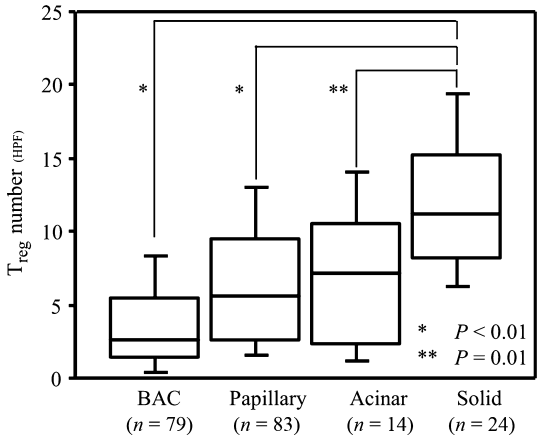

Fig. 2. Comparison of T_{reg} number in the stroma between predominant histological subtypes. All analyses were performed using the Mann–Whitney U-test. BAC, bronchioloalveolar carcinoma; T_{reg}, regulatory T cells.

stroma and the CAF-induced expression levels of immunoregulatory cytokines. RNA samples from cultured fibroblasts isolated from lung cancer tissue (CAF) in 12 adenocarcinoma patients were analyzed by RT-PCR. The results are shown in Figure 5. The relative TGF-β expression levels (median ± SD) induced by the CAF were 1.8 ± 0.4 in the high T_{reg} group and 0.9 ± 0.5 in the low T_{reg} group. In the same way, the relative VEGF expression levels induced by the CAF were 2.6 ± 1.1 in the high T_{reg} group and 1.0 ± 0.4 in the low T_{reg} group. The relative interleukin-10 expression levels induced by the CAF were 1.9 ± 3.2 in the high T_{reg} group and 1.0 ± 1.2 in the low T_{reg} group. The relative COX-2 expression levels induced by the CAF were 1.9 ± 3.2 in the high T_{reg} group and 1.0 ± 1.3 in the low T_{reg} group. No apparent difference in the expression levels of interleukin-10 (P = 0.52) or COX-2 (P = 0.78) were seen, but significant differences in the TGF-β (P = 0.01) and VEGF (P = 0.01) levels were detected between the two groups.

Discussion

The accumulation of tumor-infiltrating T_{reg} has been reported to be an unfavorable prognostic marker in several types of carcinomas.[4–8] Petersen *et al.* reported that infiltrating T_{reg} were associated with the recurrence of pathological stage I NSCLC, but they did not mention the influence of T_{reg} on overall survival.[7] We first found that the T_{reg} number in the tumor stroma was a significant indicator of a poor outcome with regard to overall, recurrence-free and disease-specific survival in p-stage I lung adenocarcinoma. Although the T_{reg} number was not an independent prognostic factor in multivariate analysis (data not shown), the presence of T_{reg} in the tumor stroma may encourage an unfavorable prognosis in patients with lung adenocarcinoma.

Growing evidence suggests that T_{reg} play an important role in suppressing T cell-mediated immunity in patients with cancer.[17,18] The number of T_{reg} in the tumor stroma was much larger than in the tumor nest. Commonly, CAF are located in the stroma and are distinctively detected in invasive carcinomas, including NSCLC. Therefore, we focused on the relationship between T_{reg} and the characteristics of CAF. We first hypothesized that cytokines secreted by CAF may have an important role in T_{reg} induction in the stroma, and we examined whether T_{reg} could be induced by soluble factors secreted by CAF derived from high T_{reg} and low T_{reg} adenocarcinomas. Compared with the low T_{reg} CAF, the high T_{reg} CAF induced a

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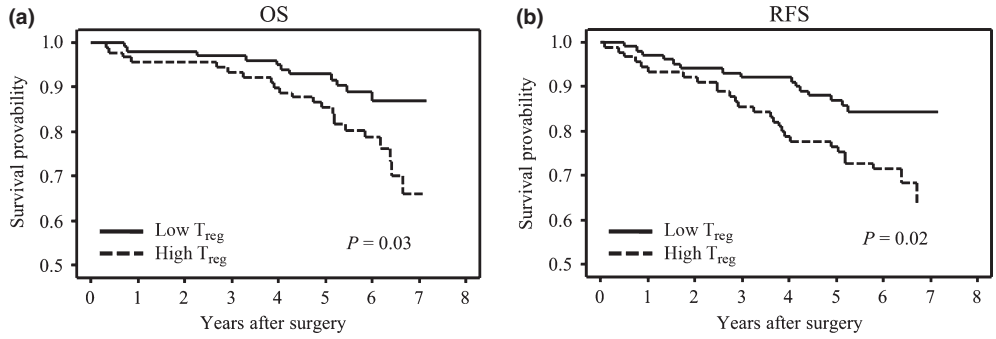

Fig. 3. Kaplan–Meier (a) overall survival curve and (b) recurrence-free survival curve for patients with p-stage I invasive lung adenocarcinoma according to the T_{reg} number in the stroma. The log-rank test was used for all the survival analyses. OS, overall survival; RFS, recurrence free survival; T_{reg}, regulatory T cells.

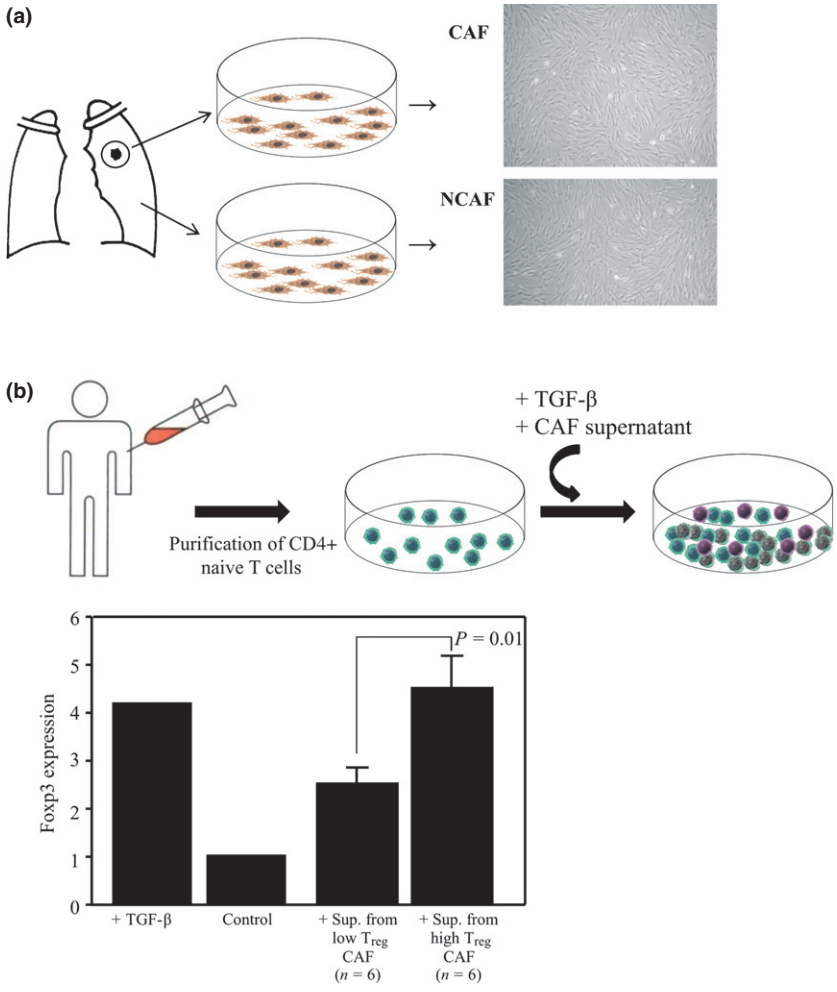

Fig. 4. (a) CAF and NCAF were prepared from human lung cancer tissue and non-cancerous lung tissue obtained from the same samples. After 10 to 20 days of growth, the fibroblasts were cultured as previously reported. (b) Induction of T_{reg} from naive CD4+ T cells by CAF. We selected CAF and cultured the naive T cells according to the above-described method using supernatant samples from 12 cases: six from low T_{reg} adenocarcinomas and six from high T_{reg} adenocarcinomas. CAF, cancer-associated fibroblasts; NCAF, non-cancer-associated fibroblasts; Sup., supernatant; TGF-β, transforming growth factor-β; T_{reg}, regulatory T cells.

significantly larger number of T_{reg} from CD4+ naive T cells. Furthermore, we examined the expression of four kinds of immunoregulatory cytokines from 12 adenocarcinoma cases. The expressions of TGF-β and VEGF, which reportedly induce T_{reg} from naive T cells in the periphery,[19,20] were significantly higher in the CAF from high T_{reg} adenocarcinomas than in the CAF from low T_{reg} adenocarcinomas. CAF are known to produce mainly TGF-β and VEGF, compared with cancer cells.[21,22] We suggest that the CAF in high T_{reg} adenocarcinomas may have a higher immunoregulatory cytokine-secreting capacity, leading to T_{reg} induction. Although we did not evaluate the influence of cytokine expression from tumor cells and tumor-associated macrophages in the present study. Saji *et al.* confirmed that tumor-infiltrating stromal cells were major sources of TGF-β based on the results of an immunohistochemical analysis in NSCLC.[23] Thus, the characteristics of CAF may have a great influence on tumor progression via the recruitment of other types of tumor-promoting stromal cells. There have been no reports demonstrating the correlation between the T_{reg} induction/recruitment and characteristics of CAF. It is

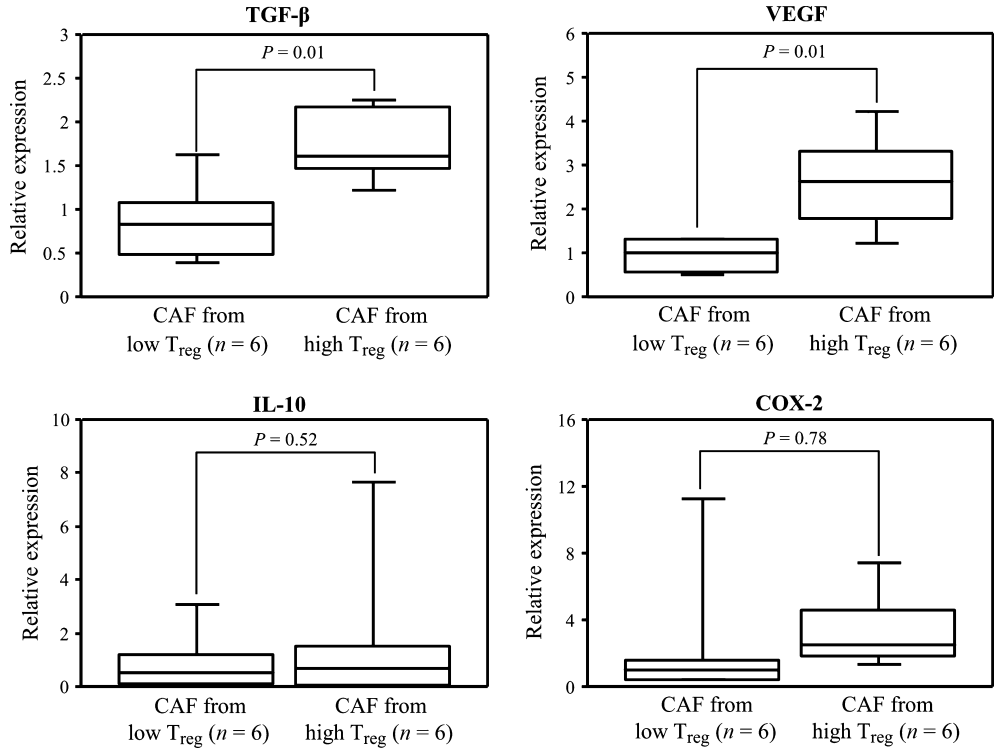

Fig. 5. Relative expression levels of immunoregulatory cytokines expressed by CAF (CAF/NCAF). Total RNA was purified from thawed samples, and cDNA was synthesized. All the analyses were performed using the Student's *t*-test. CAF, cancer-associated fibroblasts; IL-10, interleukin-10; NCAF, non-cancer-associated fibroblasts; TGF-β, transforming growth factor-β; T_{reg}, regulatory T cells; VEGF, vascular endothelial growth factor.

well-known that cytokines, other than TGF-β and VEGF, are also concerned with the T_{reg} induction. Examining the gene expression profile through microchip analysis will be helpful for elucidating which cytokines are upregulated in high T_{reg} CAF.

Recently, Miyao *et al.* reported that there was a minor population of non-regulatory Foxp3+ T cells exhibiting promiscuous and transient Foxp3 expression that were induced from the naive T cells in the peripheral lymph nodes.[24] In order to demonstrate whether these induced Foxp3+ T cells have immunoregulatory function, we would need to divide induced T_{reg} into groups according to CD25 expression levels and compare the immunosuppressive ability thereafter. In the current study, we did not examine the influence of chemokines. In the tumor microenvironment, there are many kinds of stromal cells such as macrophages and monocytes. It is possible these stromal cells educate CAF, which could create some chemokines and the T_{reg}-abundant microenvironment.

In lung cancer, Tao *et al.* reported the influence of T_{reg} in tumor stroma on OS and RFS, but did not mention the correlation between the T_{reg} count and tumor malignant parameters.[6] In the current study, we found that the high T_{reg} group, more frequently than the low T_{reg} group, had predictors of a poor outcome such as a large tumor diameter, vessel invasion, and pleural invasion. These differing results may have occurred because we examined T_{reg} in all stage of NSCLC, including squamous cell carcinomas and large cell carcinomas.

Additionally, the impact of the histological features of lung adenocarcinomas on T_{reg} accumulation has not been previously reported. In this study, the T_{reg} count was highest among patients with a predominantly solid adenocarcinoma subtype. Lung adenocarcinoma patients with a solid adenocarcinoma component are known to have a poorer prognosis than patients without this component.[25] The tumor microenvironment of a solid component probably recruits and induces more T_{reg} than other histological subtypes, enabling both the tumor cells to evade the immune system and the tumor to progress easily. Thus, we suggest that tumor cells acquire the ability to survive and metastasize as a result of a tolerance in antitumor immunity induced by T_{reg} in the tumor stroma as the tumor progresses.

In conclusion, we showed that lung adenocarcinoma with a large number of T_{reg} in the tumor stroma was associated with a poor outcome among patients with p-stage I lung adenocarcinoma after complete resection. Additionally, CAF overexpressing immunoregulatory cytokines, such as TGF-β and VEGF, may play an important role in T_{reg} induction. Recently, numerous reports have described the treatment of patients with colorectal cancer, breast cancer, or lung cancer using humanized monoclonal anti-VEGF antibody therapy (bevacizumab). To confirm the effectiveness of anti-VEGF antibody as an adjuvant therapy in patients with abundant T_{reg} in the tumor stroma, further studies are needed to elucidate the relationship between T_{reg} and CAF. Understanding this relationship will enable the creation of efficacious follow-up plans and improved therapeutic options for patients.

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Disclosure Statement

The authors have no conflict of interest.

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