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The Prevalence of FOXP3$^+$ Regulatory T-Cells in Peripheral Blood of Patients with NSCLC

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Abstract

We have studied CD4$^+$CD25^{high}FOXP3$^+$ regulatory T-cells (T_{regs}) from 51 patients with non–small-cell lung cancer (NSCLC) and 33 healthy donors. Regulatory T-cells were identified by fluorescence-activated cell sorting by using a panel of antibodies and by reverse transcriptase polymerase chain reaction analysis for FOXP3 expression. Functional studies were done to analyze their inhibitory role. Finally, regulatory T-cells were analyzed in malignant pleura effusion (PE) from patients with NSCLC. Patients with NSCLC have increased numbers of CD4$^+$CD25^{high} FOXP3$^+$ T_{regs} in their peripheral blood and pleura effusion (PE), which express high levels of CTLA-4, GITR. These cells were anergic toward T-cell receptor stimulation and, when cocultured with activated CD4$^+$CD25$^-$ cells, potently suppressed their proliferation and cytokine secretion. Our data suggest that in NSCLC patients, there is an increase of CD4$^+$CD25^{high}FOXP3$^+$ regulatory T-cells in the peripheral blood and tumor microenvironment. These T-cells might prevent effective antitumor immune responses, and the increase in frequency of CD4$^+$CD25^{high}FOXP3$^+$ Tregs might play a role in the modulation of the immune response against NSCLC and could be important in the design of immunotherapeutic approaches.

Key words: non-small-cell lung cancer (NSCLC), CD4$^+$ CD25^{high} FOXP3$^+$ T-cells, regulatory T cells (T_{regs})

Introduction

Lung cancer has become the number-one killer among cancers worldwide. Although lung cancer remains the leading cause of cancer-related mortality in the United States, its incidence is increasing; in 2008, 215,020 new cases are expected, and 161,840 persons are projected to die from the disease in the United States.[1] Non-small-cell lung cancer (NSCLC) accounts for 85% of all lung cancer cases in the United States. After the initial diagnosis, accurate staging of NSCLC is crucial for determining appropriate therapy. When feasible, surgical resection remains the single most consistent, successful option for cure. However, close to 70% of patients with lung cancer present with locally advanced or metastatic disease at the time of diagnosis. Chemotherapy is beneficial for patients with metastatic disease, and the administration of concurrent chemotherapy and radiation is indicated for stage III lung cancer,[2] but the overall 5-year survival rate is a dismal 15%.[1] Therefore, identifying and establishing alternative approaches for the treatment of NSCLC is quite a challenge and is of high interest.

The clinical behavior of any given tumor is unpredictable, but it most likely depends on a complex relation between the tumor genotype and the host response to disease. Previous studies have investigated the changes in morphology, genetics, and molecular biology of epithelial cells during tumorigenesis. Recently, many studies have suggested that the tumor microenvironment also plays an important role in the establishment and progression of tumors. Lymphocytes contribute to the tumor microenvironment through

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immunity and inflammation. $CD8^+$ CTLs can directly kill target cells by releasing granules, including membrane-lytic materials, such as perforin and granzymes in acquired immune responses, thereby playing a central role in antitumor immunity. Indeed, a high frequency of $CD8^+$ T-cells infiltrating cancer tissue can be a favorable prognostic indicator in ovarian cancer[3] and colorectal cancer.[4]

In contrast to $CD8^+$ CTL, which generally exerts a suppressive influence on tumor growth, (T_{regs}) are thought to have a positive effect on tumor growth through the suppression of antitumor immune cells. $CD4^+CD25^+$ T_{regs} are a minor, but functionally unique, population of T-cells, which maintain immune homeostasis in immune tolerance and the control of autoimmunity. T_{regs} can inhibit immune responses mediated by $CD4^+CD25^-$ and $CD8^+$ T cells *in vitro* by a contact-dependent and cytokine-independent mechanism,[5] although more recent reports suggest that the immune suppression mechanisms of T_{regs} *in vivo* are more complex. The forkhead or winged helix family of transcription factor P3 (FOXP3) is critical for the development and function of T_{regs} in mice and humans,[6] and is still the only marker for evaluating real T_{regs} that have a suppressive function. In murine models, it has been described that T_{regs} inhibit the antitumor immune response.[7] Recently, Baecher-Allan et al.[8] reported the identification of a $CD4^+$ population of regulatory T-cells in the circulation of healthy humans expressing high levels of CD25 ($CD4^+CD25^{high}$) that exhibit *in vitro* characteristics identical with those of the $CD4^+CD25^+$ regulatory cells isolated in mice. This $CD4^+CD25^{high}$ T-cell subset in humans comprises 1%–2% of circulating $CD4^+$ T-cells, unlike that in rodents, where 6%–10% of $CD4^+$ T-cells demonstrate regulatory function. Whereas the entire population of $CD4^+$ $CD25^+$ T-cells expressing both low and high CD25 levels exhibit regulatory function in the mouse, only the $CD4^+$ $CD25^{high}$ population exhibits a similarly strong regulatory function in humans.

Involvement of $CD4^+CD25^+$ T_{regs} in human cancer has been observed in peripheral blood and tumor tissues from patients with several types of cancer.[9–12] Early studies detected T_{regs} not as $FOXP3^+$ T cells, but as $CD4^+CD25^+$ T-cells, although recent studies have revealed that $CD4^+CD25^+$ T-cells consist of T_{regs} and activated effector T-cells, the latter being increased in inflammatory lesions.[13]

In the present study, we investigated the clinical values of $CD4^+CD25^{high}$ $FOXP3^+$ T_{regs} in both the peripheral blood and PE of NSCLC.

Materials and Methods

Patients and samples

The study protocol was approved by the Ethics Committee of Union Hospital affiliated to Tongji Medical School of Hua-Zhong University Of science and Technology (Wuhan, China) for human studies, and informed consent was obtained from all subjects.

Blood samples were collected from a total 51 NSCLC patients, who were males ($n = 34$) and females ($n = 17$) with a mean age of 54.25 ± 13.17 years (range, 25–89) from February 2007 to November 2008, seen at the Cancer Center of our hospital. Tumors were classified with a tumor-node metastasis (TNM) classification, according to the World Health Organization (WHO) classification.[14] NSCLC was diagnosed by pathology or cytology, including 15 cases of squamacarcinoma, 29 of adenocarcinoma, 4 of squama-adenocarcinoma, 1 of large-cell carcinoma, and 2 of nondifferentiation. Of these, 6 were stage I–II, 20 were stage III, and 25 stage IV.

Table 1. Characteristics of NSCLC Patients and Healthy Donors

	NSCLC	Healthy donors
Age (y)	54.25 ± 13.17	51.80 ± 13.60
Range	25–89	24–71
Gender		
Male	34	18
Female	17	15
Total	51	33
Clinical stage		
Stage I-II	6	
Stage III	20	
Stage IV	25	
Pathology		
Squamacarcinoma	15	
Adenocarcinoma	29	
Squama-adenocarcinoma	4	
Large-cell	1	
Nondifferentiated	2	

NSCLC, non–small cell lung cancer.

Blood samples were also taken from 33 healthy volunteers, 15 of whom were males and 15 females with a mean age of 51.8 ± 13.6 (range, 24–71). Patient characteristics and disease classification are shown in Table 1.

In addition, 18 PE samples were collected from stage IV NSCLC patients, who were males ($n = 10$) and females ($n = 8$) with a mean age of 54.50 ± 12.90 years (range, 26–89). In total, 15 nonmalignant PE samples were collected as a control from the Department of Respirtory Disease in our hospital, who were males ($n = 9$) and females ($n = 6$) with a mean age of 56.8 ± 17.8 years (range, 20–78).

At the time of sample collection, none of the patients had received any anticancer treatment, corticosteroids, or other nonsteroidal anti-inflammatory drugs (NSAIDs).

Cell isolation and sorting

Peripheral blood mononuclear cells (PBMCs) were isolated from freshly obtained blood by Ficoll density gradient (Biochrom, Berlin, Germany), as described before.[8] For the isolation of $CD4^+CD25^+$ and $CD4^+CD25^-$ T-cells, PBMCs were further separated by using the regulatory T-cell isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany) and MiniMACS separation unit (Miltenyi Biotech), according to the manufacturer's instruction. For sorting of the cells, $CD4^+$ T-cells were purified from freshly isolated PBMCs using the $CD4^+$ T-cell isolation kit and sorted into $CD4^+$ $CD25^+$ and $CD4^+CD25^-$ cells, using CD25 microbeads and PE-conjugated antibody 25 (Miltenyi Biotec). Eventually, $CD4^+$ $CD25^+$ T-cells were obtained with a purity ranging from 85% to 90%. PE was collected at the time of paracentesis. The fluid was centrifuged at 1200 rpm for 10 minutes, and the cell pellet was separated on a Ficoll density gradient, as described previously.[15] Enriched cells were >90% pure, as determined by flow cytometry. The lymphocyte layer was collected,

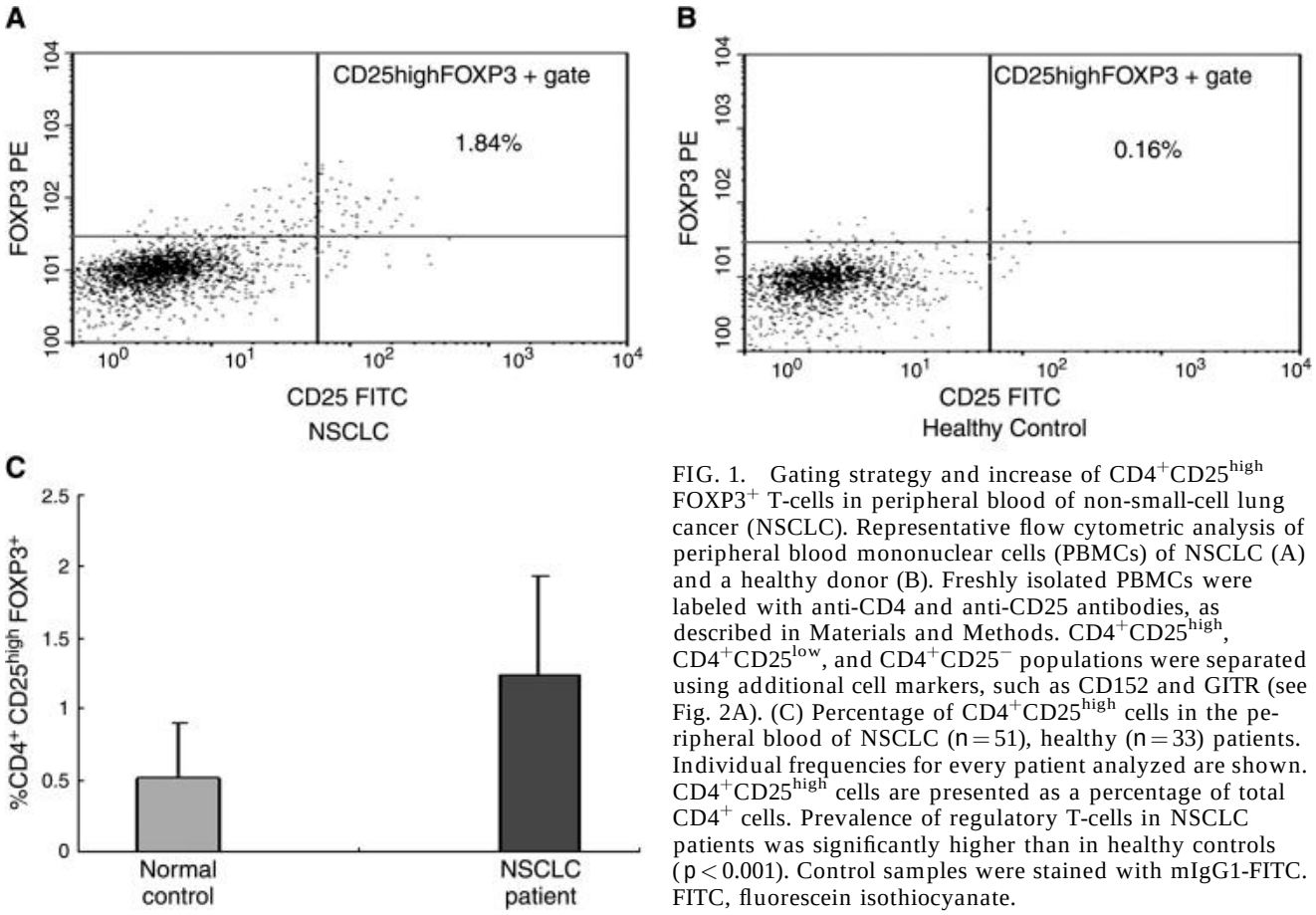

FIG. 1. Gating strategy and increase of $CD4^+CD25^{high}$ $FOXP3^+$ T-cells in peripheral blood of non-small-cell lung cancer (NSCLC). Representative flow cytometric analysis of peripheral blood mononuclear cells (PBMCs) of NSCLC (A) and a healthy donor (B). Freshly isolated PBMCs were labeled with anti-CD4 and anti-CD25 antibodies, as described in Materials and Methods. $CD4^+CD25^{high}$, $CD4^+CD25^{low}$, and $CD4^+CD25^-$ populations were separated using additional cell markers, such as CD152 and GITR (see Fig. 2A). (C) Percentage of $CD4^+CD25^{high}$ cells in the peripheral blood of NSCLC ($n = 51$), healthy ($n = 33$) patients. Individual frequencies for every patient analyzed are shown. $CD4^+CD25^{high}$ cells are presented as a percentage of total $CD4^+$ cells. Prevalence of regulatory T-cells in NSCLC patients was significantly higher than in healthy controls ($p < 0.001$). Control samples were stained with mIgG1-FITC. FITC, fluorescein isothiocyanate.

washed, and further stained for regulatory T-cells, as described above.

Flow cytometry analysis

To determine the frequency and phenotype of $FOXP3^+$ regulatory T-cells in PBMCs and pleura effusion mononuclear cells (PEMCs), multicolor-fluorescence-activated cell-sorting analysis was done, using the following antibodies: anti-CD4-Percp (BD Biosciences, San Jose, CA), anti-CD25-FITC (BD Biosciences), PE-conjugated FOXP3 (BD Biosciences), anti-GITR-APC (eBiosciences, CA), and anti-CD152 (CALT4)-PE (eBiosciences, San Diego, CA). Before use, all mAbs were titrated using normal resting or activated PBMCs and PEMCs to establish optimal staining dilutions. Flow cytometry was done by using a FACScan flow cytometry (BD Biosciences) equipped with software. The acquition and analysis gates were restricted to the lymphocyte gate, as determined by characteristic forward- and side-scatter properties of lymphocytes (Fig. 1a.); forward and side scatters were set in a linear scale. For analysis, 1×10^5 lymphocytes were acquired. The analysis gates were restricted to the $CD3^+CD4^+$, $CD4^+CD25^+$ and $CD4^+CD25^{high}$ T-cell subsets (e.g., Fig. 1). Isotype-matched antibodies were used with all the samples as controls. Cells expressing T_{reg} markers were acquired and analyzed.

To determine the frequency of $CD4^+CD25^{high}$ T-cells and the "T_{reg}" surface marker profile, PBMCs and PEMCs (at least $0.2\times10^6\times$cell/tube, respectively) were stained with monoclonal antibodies (mAbs) in the above-described panel for 15 minutes at 48C.

Intracellular staining for FOXP3, CD152 (cytotoxic lymphocyte-associated antigen-4; CTLA-4), surface cellular staining for glucocorticoid-induced tumor necrosis factor (TNF): receptor family–related receptor (GITR). Briefly, samples were first incubated with mAbs against surface markers CD4, CD3, and CD25, fixed with 4% (v/v) formaldehyde in phosphate buffered saline (PBS) for 20 minutes at room temperature, stained with anti-CTLA-4-PE, anti-FOXP3-FTIC, for 30 minutes at room temperature, then resuspended in flurescence-activated cell-sorting flow solution, and immediately analyzed by flow cytometry. Appropriate isotope controls were included for each sample. Appropriate isotope antibody controls were used for each sample. Cells were washed and examined by four-color flow cytometry.

Analysis of FOXP3mRNA expression by RT-PCR

$CD4^+CD25^-$ and $CD4^+CD25^+$ cells were sorted, as described above. Total RNA was extracted from cells isolated from magnetic beads with Trizol reagent (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized with a cDNA synthesis kit (Toyobo, Japan). Reverse transcription polymerase chain reaction (RT-PCR) was performed with lower than 1 mg of total RNA for cDNA synthesis. The following primers were used in PCR reactions. FOXP3: 5′-primer

GAAACAGCACATTCCCAGAGTTC:3′-primer CACC ACC CTG T TGCTGAT (expected size, 100 bp). GAPDH: 5′-primer TATGA TGAC ATC AA GAAGGTGG; 3′-primer CACC ACCC TGTTGC TGAT (expected size, 213 bp). PCR reactions were done at 37 cycles in a 25-mL volume, and PCR products were analyzed in an agarose gel stained with ethidium bromide. Results are shown as relative FOXP3 mRNA levels.

Western blot analysis

$CD4^+$ T-cells were purified from freshly isolated PBMC of NSCLC patients and healthy controls. Nuclear extracts were prepared, as described previously[16] and run on a 10% sodium dodecyl sulfate (SDS) gel. FOXP3 protein was detected by using a polyclonal anti-FOXP3 antibody (Abcam, Cambridge, United Kingdom).

Proliferation and immunosuppression assays

T_{regs} obtained from PBMCs were tested for regulatory function by coculture analysis at the suppressor ($CD4^+CD25^+$ regulatory T-cells) to responder ($CD4^+CD25^-$ effector T-cells) ratios of 1:1, 1:2, and 1:4 with 5 mmol/L of carboxyfluorescein diacetate, succinimidyl ester (CFSE; Molecular Probes, Carlsbad, CA)-labeled autologous $CD4^+CD25^-$ T-responder cells per well. Soluble purified antihuman CD3 (BioLegend) (10 ug/mL) and soluble purified antihuman CD28mAb (5 ug/mL) (BioLegend) were used for stimulation for 6 days.

CFSE labeling of responder T-cells was done, as described previously.[15] Briefly, $CD4^+CD25^-$T-cells (5×10^4/well) separated by single-cell sorting were stained with 5 mmol/L carboxyfluorescein diacetate succinimidyl ester (CFSE) for 10 minutes at room temperature. The CFSE label was quenched by the addition of an equal volume of prewarmed PBS (100%, filtered; Wuhan Sanli, China). Cells were centrifuged for 5 minutes at 400 g. Discarded supernatant and vortex pellets were added to obtain a single-cell suspension. Cells were washed by suspending them in l mL of 2% FBS/PBS and were washed 3 times. Cells were set in standard 96-well plates for proliferation assays.

Cells were cultured in complete RPMI-1640 media (Hyclone, Logan, UT), supplemented with 10% FCS, 100 U/mL of penicillin G sodium, and 100 u/mL of streptomycin. After 6 days of incubation at 378C/5% CO_2, they were analyzed by flow cytometry.

All CFSE data were analyzed through ModFit (ModFit LT2.0) software provided by the Verity Software House. The percentages of suppression were calculated based on the proliferation index of responder cells alone, compared with the proliferation index of cultures containing responder plus T_{reg}. The program determines the percentage of proliferating cells within each peak, and the sum of all peaks in the control culture (no T_{reg}) is taken as 100% of proliferation and 0% of suppression.

ELISA

Cell culture supernatants obtained from the T-cell proliferation and immunosuppression assays were analyzed for interleukin (IL)-2, transforming growth factor-beta (TGF-b) and interferon-gamma (IFN-g) levels by an enzyme-linked immunosorbent essay (ELISA) kit (Jingmei, China) according to the manufacturer's instructions.

Confocal microscopy

Mononuclear cells from PB or PE of NSCLC patients were incubated with affinity-purified monoclonal mouse antihuman-CD25 (1:200 dilution; eBioscience) and polyclonal rabbit antihuman-FOXP3 (1:300 dilution; eBioscience), followed by rhodamine-goat antimouse and Fluorescein isothiocyanate (FITC)-mouse antirabbit (1:100 dilution; Santa Cruz), cells were tested by confocal microscopy (Olympus).

Statistical analysis

Date were analyzed by using the SPSS 11.5 for windows software (SPSS Inc., Chicago, IL). Values are presented as mean value ± standard deviation. The statistical significance of different study groups was assessed by using the Student's two-tailed t-test. p-values <0.05 were considered significant.

Results

NSCLC patients have increased numbers of $CD4^+CD25^{high}$ $FOXP3^+$ T-Cells in their peripheral blood

We analyzed the peripheral blood of 51 patients with NSCLC, 33 healthy donors for the prevalence of $CD4^+$ $CD25^{high}$ $FOXP3^+$ T-cells. The population of $CD4^+CD25^+$ T-cells as a percentage of total $CD4^+$ T-cells was identified by flow cytometry after cell-surface labeling for the expression of CD4 and CD25 molecules. We analyzed the $CD4^+$ cells with the highest level of CD25 expression ($CD4^+CD25^{high}$), which protrudes as a tail from the major population of $CD4^+$ $CD25^{low}$ cells (Fig. 1). Intracellular staining for FOXP3, CD152 (CTLA-4), and cellular surface staining for GITR were used to distinguish $CD25^-$, $CD25^{low}$, and $CD25^{high}$ T-cells.

The $CD4^+CD25^{high}$ $FOXP3^+$ population represents 0.06%–1.38% of the $CD4^+$ T-cell population in healthy dornors; however, $CD4^+CD25^{high}$ $FOXP3^+$ T-cells compromise 0.13%–6.33% of $CD4^+$ T-cells in NSCLC group. Representative dotplots of NSCLC patients and healthy donors are shown (Fig. 1). Individual frequencies of $CD4^+CD25^{high}$ $FOXP3^+$ T-cells as well as the cumulative data for all the patients and healthy donors analyzed are represented as scatter plots (Fig. 1). As shown, the frequency of $CD4^+CD25^{high}$ $FOXP3^+$ T-cells in NSCLC patients was significantly higher in NSCLC patients (1.24% ± 0.69%) than in healthy donors (0.52% ± 0.38%).

Phenotypic analysis of $CD4^+CD25^{high}$ $FOXP3^+$ T-Cells

The $CD4^+CD25^{high}$ T-cells were analyzed for expression of cell markers. Freshly isolated PBMCs from all patient populations were labeled with CD4, CD25, and a series of cell markers to further characterize these cells. Representative histograms of the surface marker expression analysis of GITR and intracellular marker CD152 (CTLA-4)] on $CD4^+CD25^{high}$, $CD4^+CD25^{low}$, and $CD4^+CD25^-$ cells are shown in Figure 2. GITR, a member of the TNF-receptor superfamily, which is a surface marker predominantly expressed on $CD4^+CD25^{high}$ regulatory T-cells,[17] was present up to 8.41% in $CD4^+$ $CD25^{high}$ cells. $CD4^+CD25^{high}$ cells are also distinguishable from $CD4^+CD25^-$ cells by their elevated expression of CTLA-4 (CD152; 24.56%) (Fig. 2), which has been reported previ-

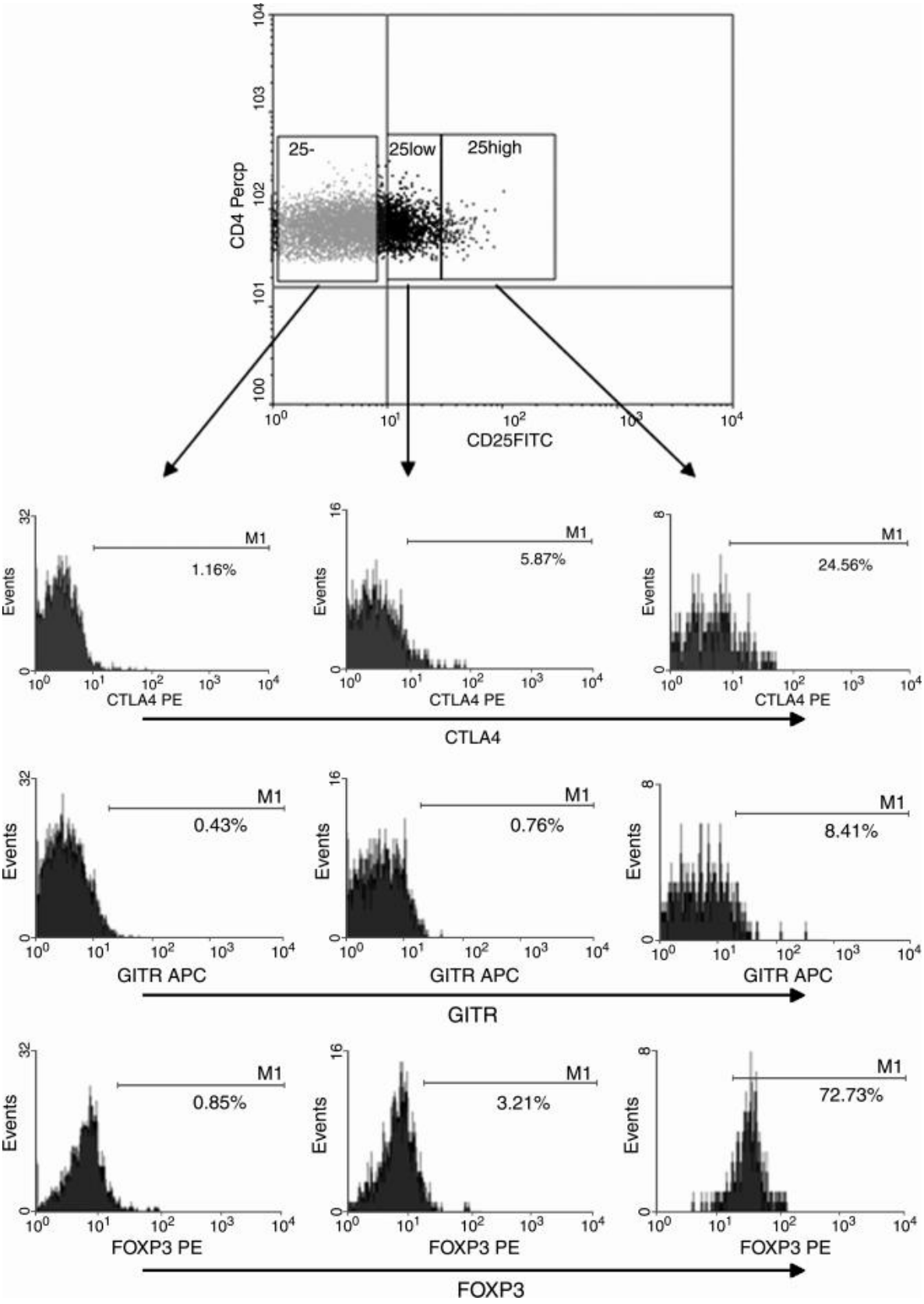

FIG. 2. Phenotypic analysis of regulatory T-cells and FOXP3 expression. Freshly isolated peripheral blood mononuclear cells from non-small-cell lung cancer patients or healthy donors were labeled with anti-CD4, anti-CD25, anti-FOXP3, anti-GITR, and anti-CTLA4. Control samples were stained with mIgG1-FITC, mIgG2a-FITC, or IgG2b-FITC. $CD4^{+}CD25^{high}$, and $CD4^{+}CD25^{-}$ (see Fig. 1A). Cell populations were analyzed separately. FITC, fluorescein isothiocyanate.

ously for human circulating regulatory T-cells.[18] Intracellular staining FOXP3 was positive in the $CD4^+CD25^{high}$ T_{regs}. Cell-surface expression of regulatory T-cells for healthy donors was similar to NSCLC patients (data not shown). Thus, the $CD4^+CD25^{high}$ T-cells in NSCLC patients and healthy donors were phenotypically similar to regulatory T-cells described previously.

Overall, our data show that a high frequency of circulating $CD4^+CD25^{high}$ cells from NSCLC patients express GITR, CTLA-4, and FOXP3. Therefore, the $CD4^+CD25^{high}$ cells detected in peripheral blood of NSCLC patients are, indeed, T_{regs}.

Expression of FOXP3 in $CD4^+CD25^+$ cells

FOXP3 has been described recently as an important transcription factor and the most specific molecular marker for regulatory T-cells known thus far.[19] We analyzed the expression of FOXP3 mRNA in $CD4^+CD25^-$ and $CD4^+CD25^+$ cells from NSCLC patients and healthy donors by using RT-PCR. As shown in Figure 3A, sorted $CD4^+CD25^+$ cells from both NSCLC and healthy donors expressed FOXP3 mRNA, whereas $CD4^+CD25^-$ cells expressed no, or very little, FOXP3 mRNA. Additionally, Western blot analysis of $CD4^+$ cells from NSCLC patients and healthy donors also confirmed the expression of FOXP3 in nuclear extracts (Fig. 3B). Overall, our data show that a high frequency of circulating $CD4^+CD25^+$ cells from NSCLC patients express FOXP3 mRNA. These characteristics are highly indicative of regulatory $CD4^+CD25^+$ T-cells already described in both healthy donors and cancer patients.[10–12]

Suppressor activity of $CD4^+CD25^+$ T_{reg} cells isolated from PBMC of NSCLC

To evaluate the suppressor activity of $CD4^+CD25^+$ T_{reg} cells in PBMC, single-cell sorting was used for their isolation, followed by cocultures with CFSE-labeled autologous $CD4^+CD25^-$ T-cells as responders. The cocultures were set up at the suppressor to responder ratios of 1:1, 1:2, and 1:4, using responder proliferating in response to anti-CD3 and anti-CD28 antibodies. To analyze the proliferation of responder cells, gating was set to include $CD4^+CFSE^+$ T-cells. A strong suppression of proliferation ($p < 0.01$) was observed (Fig. 4).

Cytokine profile of $CD4^+CD25^+$ T-cells

The cytokine-expression profile of $CD4^+CD25^+$ T-cells from NSCLC patients and healthy donors were analyzed and compared with each other. The culture supernatants were then assayed for IFN-g, TGF-b, and IL-2 production by ELISA. $CD4^+CD25^-$ T-cells from NSCLC patients ($n = 4$) predominantly secrete IFN-g and IL-2 with little secretion of TGF-b, whereas $CD4^+CD25^+$ T-cells mainly produce TGF-b (which is a cytokine known to inhibit T-cell proliferation) and very low levels of IFN-g and IL-2. The secretion of IFN-g and IL-2 of $CD4^+CD25^-$ T-cells was also suppressed (Table 2 and Fig. 5).

Prevalence of $CD4^+CD25^{high}$ $FOXP3^+$ T-Cells in PE

We determined the prevalence of $CD4^+CD25^{high}$ $FOXP3^+$ regulatory T-cells also in 18 PE of NSCLC patients and compared them with $CD4^+CD25^{high}$ $FOXP3^+$ regulatory T-cells in 15 nonmalignant PE. We observed a small fraction of $CD4^+$ $CD25^{high}$ $FOXP3^+$ T-cells in nonmalignant PE from individuals with infection (0.24%–0.96%; mean, $0.44 \pm 0.22\%$; $n = 15$). $CD4^+CD25^{high}$ $FOXP3^+$ regulatory T-cells were more abundant in PE with advanced NSCLC (0.30%–6.13%; mean $2.85\% \pm 1.49\%$; $n = 18$, $p < 0.01$) (Fig. 6A), supporting the idea that $CD4^+CD25^+$ T-cells accumulate in a tumor-specific manner in malignant PE. Meanwhile, the $CD4^+CD25^{high}$ $FOXP3^+$ regulatory T-cells in PE of 8 NSCLC patients were

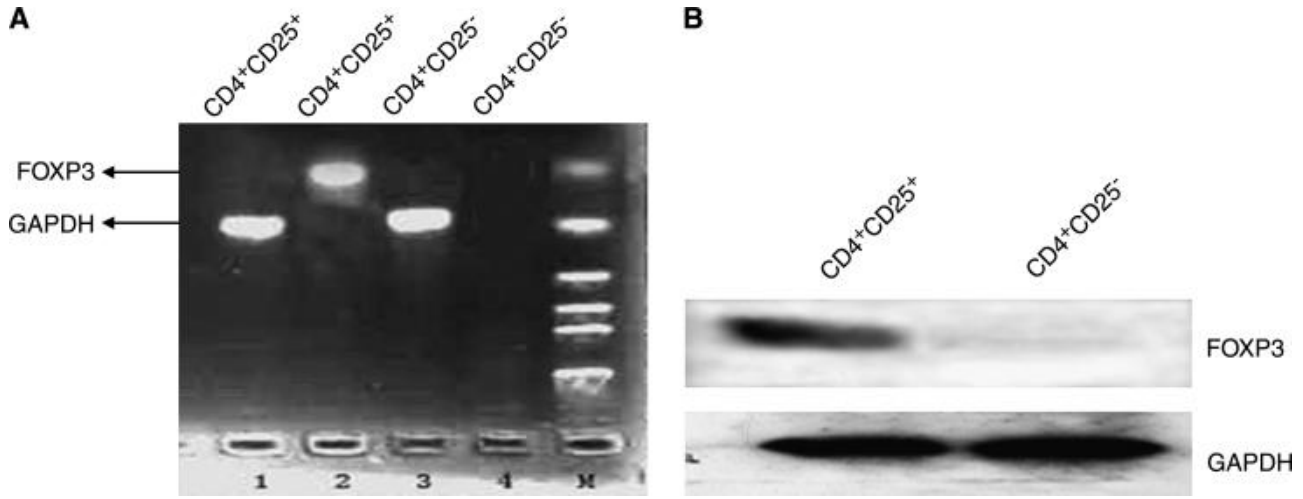

FIG. 3. (A) The expression of FOXP3 in regulatory T-cells as determined in B, cDNA obtained from sorted and or magnetically purified populations of $CD4^+CD25^-$ and $CD4^+CD25^+$ cells were subjected to reverse transcriptase polymerase chain reaction analysis by using primers specific for FOXP3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (M: marker). One (1) GAPDH in $CD4^+CD25^+$ T_{reg} cells; 2. FOXP3 in $CD4^+CD25^+$ T_{reg} cells; 3 GAPDH in $CD4^+CD25^-$ T cells; 4 FOXP3 not expressed in $CD4^+CD25^-$ T-cells. (B) Nuclear extracts from $CD4^+$ T-cells isolated from peripheral blood of hepatocellular carcinoma patients and healthy donors were prepared. Expression of FOXP3 was assessed in the nuclear extracts by Western blotting.

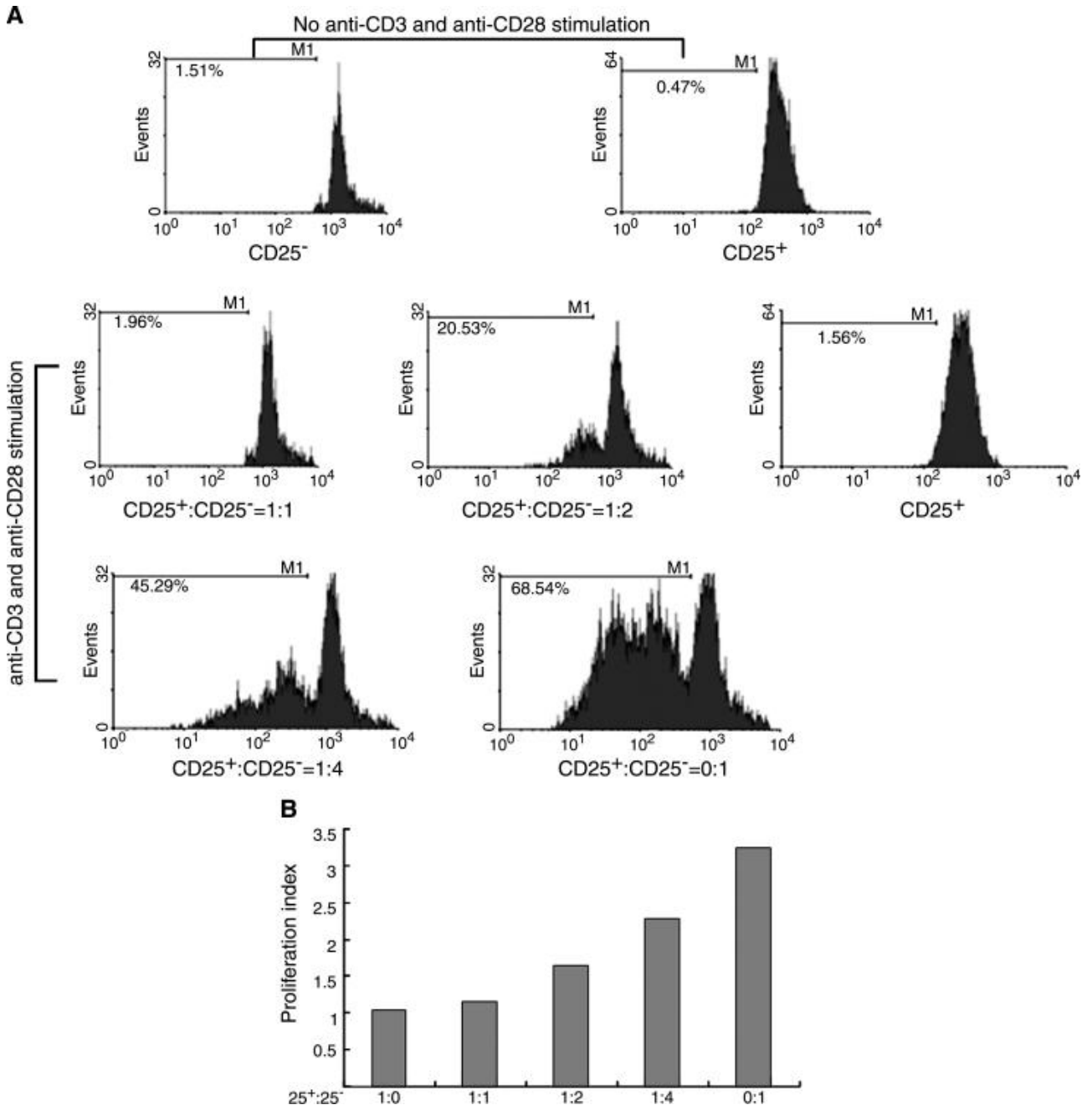

FIG. 4. $CD4^+CD25^+$ T-cells from peripheral blood were suppressive in vitro. For sorting of the cells, $CD4^+$ T-cells were purified from freshly isolated peripheral blood mononuclear cells by using the $CD4^+$ T-cell isolation kit and sorted into $CD4^+CD25^+$ and $CD4^+CD25^-$ cells, using CD25 microbeads and pleura-effusion-conjugated antibody 25 (Miltenyi Biotec, Bergisch Gladbach, Germany) and isolated over magnetic columns, as described in Materials and Methods. Freshly isolated $CD4^+CD25^-$ (50,000 cells/well) effector T-cells or $CD4^+CD25^+$ regulatory T-cells (T_{regs}) were labeled with 5 mmol/L of carboxyfluorescein diacetate succinimidyl ester (CFSE) at ratios of 1:1, 1:2, and 1:4 and stimulated with anti-CD3 and anti-CD28. Cell culture conditions are indicated. After 6 days of incubation at 378C/5% CO_2, they were analyzed by flow cytometry. All CFSE data were analyzed throught ModFit software provided by the Verity Software House. The percentages of suppression were calculated based on the proliferation index of responder cells alone, compared with the proliferation index of cultures containing responder plus T_{reg}. The program determines the percentage of proliferating cells within each peak, and the sum of all peaks in the control culture (no T_{reg}), is taken as 100% of proliferation and 0% of suppression. Representative histograms (A) and the corresponding proliferation index (B) of these cells of a patient are shown.

further compared with $CD4^+CD25^{high}$ $FOXP3^+$ regulatory T-cells in their peripheral blood, $CD4^+CD25^{high}$ $FOXP3^+$ regulatory T-cells were more abundant in PE with advanced NSCLC than in their blood [mean: $2.86\% \pm 1.1\%$(1.02% * 5.55%) and $0.95\% \pm 0.48\%$ (0.53% * 1.59%) respectively; $n = 8$; $p < 0.05$] (Fig. 6B). These data showed that $CD4^+$ $CD25^{high}$ $FOXP3^+$ regulatory T-cell frequencies were increased not only in peripheral blood, but also in PE,

Table 2. Cytokine profiles

	$CD4^+CD25^-$ T-cells	$CD4^+CD25^+$ T-cells	Mixture of $CD4^+CD25^-$ with $CD4^+CD25^+$ T-cells at the ratio of 1/1
IL-2 (pg/mL)	909.2 ± 272.4	27.7 ± 22.9	34.4 ± 30.28
IFN-g(pg/mL)	1158.3 ± 380	20.0 ± 23.3	37.4 ± 41.3
TGF-b (pg/mL)	230.7 ± 27.2	1444.7 ± 468.8	1750.0 ± 438.7

IL, interleukin, IFN, interferon, TGF, tumor growth factor.

suggesting that these T-cells might suppress immune activation at the tumor site.

$CD4^+CD25^{high}$ $FOXP3^+$ T-cells from PE were also analyzed for the expression of cell-surface and intracellular markers, such as CALT-4 and GITR, and a similar pattern, as seen in the peripheral blood of these patients, was found (Fig. 6C). We identified a substantial accumulation of $CD4^+CD25^+$ $FOXP3^+$ T-cells by multicolor confocal microscopic analysis in malignant PE (Fig. 6D).

Discussion

Recent evidence has demonstrated that the pathologic interactions between cancer cells and host immune cells in the tumor microenvironment create an immunosuppressive network that promotes tumor growth, protects the tumor from immune attack, and attenuates immunotherapeutic efficacy. Poor tumor-associated antigen (TAA)-specific immunity is not simply due to a passive process, whereby adaptive immunity is shielded from detecting TAAs, but there is also an active process of "tolerization" taking place in the tumor microenvironment. Tumor tolerization is the result of imbalances in the tumor microenvironment, including alterations in antigen-presenting cell subsets, costimulatory and coinhibitory molecule alterations, and altered ratios of effector T-cells and T_{regs}. More and more evidence showed that regulatory-T-cell-mediated immunosuppression is one of the crucial tumor immune-evasion mechanisms and the main obstacle of successful tumor immunotherapy.[20,21]

Lung cancer is the first most common cancer worldwide with a poor prognosis. Even when diagnosed at an early stage, patients relapse at a rate as high as 50% after surgical resection.[22] Petersen et al.[23] examined the relation between T_{reg} cells and total tumor-infiltrating T-cell lymphocytes (TILs) to determine whether they correlated with recurrence. They found that patients with stage I NSCLC who have a higher proportion of tumor T_{reg} cells relative to TIL had a significantly higher risk of recurrence. So, a better understanding of the role of regulatory T-cells in NSCLC is important for the design of future immunotherapy-based clinical protocols.

Because only the $CD4^+CD25^{high}$ population exhibits a similarly strong regulatory function in humans,[8] this study presents evidence for an increase in frequency of $CD4^+$ $CD25^{high}$ T_{reg} cells in the peripheral blood of NSCLC patients. We used several cell-surface and intracellular markers

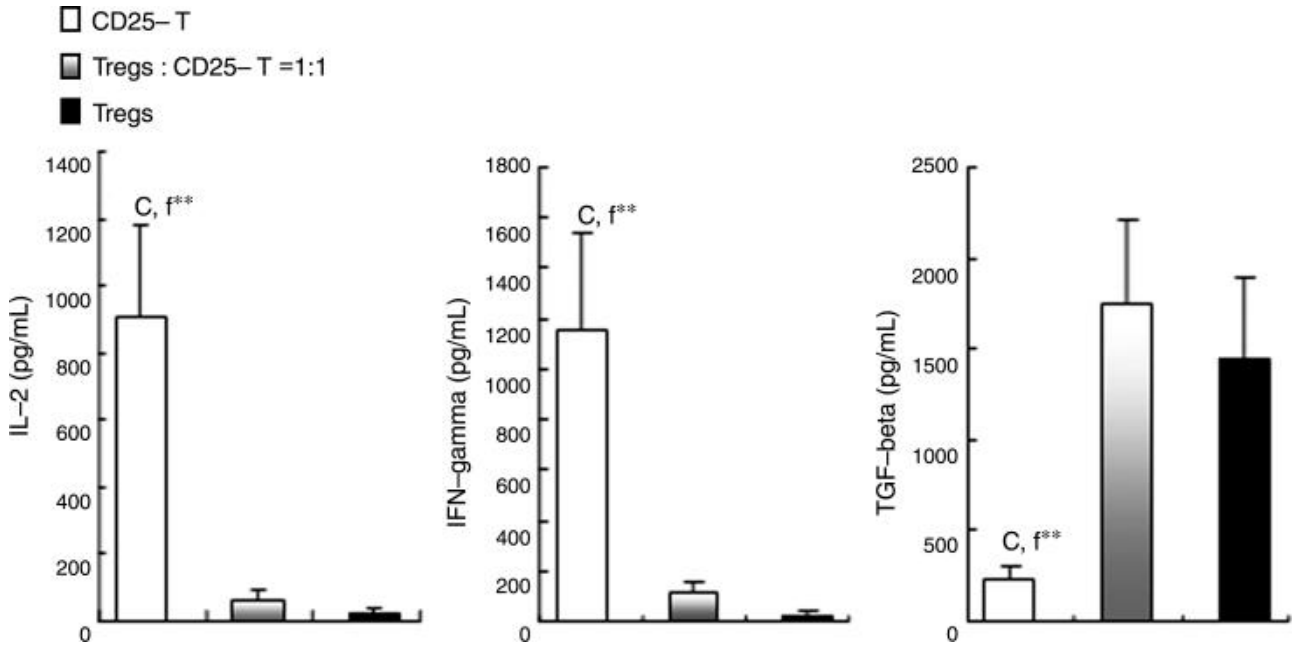

FIG. 5. T_{regs} suppress the production of interleukin-2 and interferon-gamma by $CD4^+CD25^-$ T-cells, but increase transforming growth factor beta production. Isolated $CD4^+CD25^-$ T-cells, regulatory T-cells (T_{regs}), and the mixing of these two types of cells at the ratio of 1;1 were stimulated, respectively, for 6 day, as indicated. The supernatants were analyzed by enzyme-linked immunosorbent assay ($n = 4$), $^{**}p < 0.01$, compared to the T_{regs} group and compared to the mixture group.

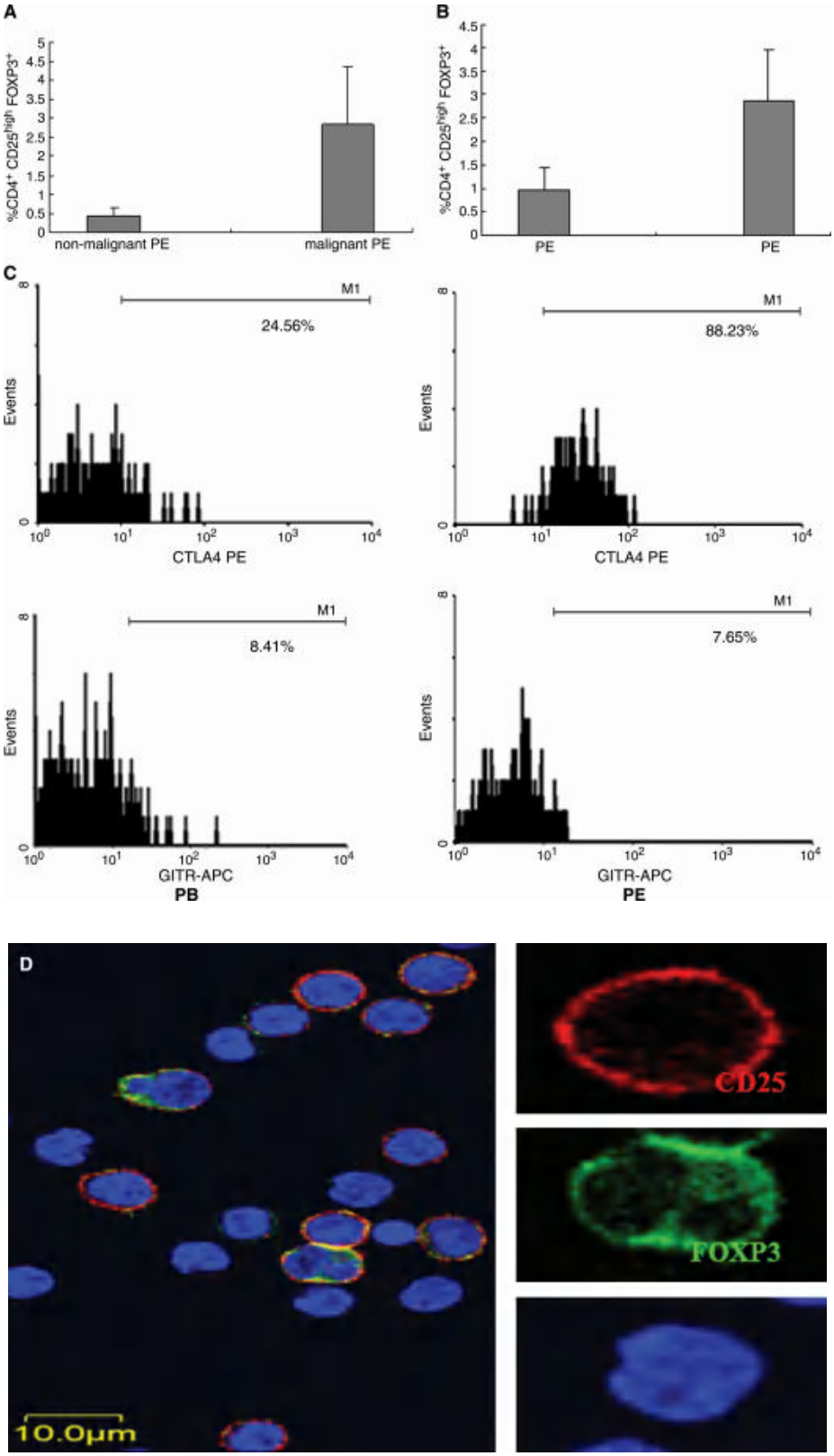

FIG. 6. Analysis of $CD4^{+}CD25^{high}$ $FOXP3^{+}$ T-cells in pleural effusion (PE). (A) PE from advanced non–small-cell lung cancer (NSCLC) patients ($n = 8$) were tested in parallel to peripheral blood for the presence of $CD4^{+}CD25^{high}$ $FOXP3^{+}$ T_{reg} cells. (B) PE from advanced non–small-cell lung cancer (NSCLC) patients ($n = 18$) were compared to nonmalignant PE ($n = 15$). (C) T_{regs} from peripheral blood (PE) showed similar expression patterns for CTLA-4 and GITR expression. (D) a substantial accumulation of $CD4^{+}CD25^{+}FOXP3$ T-cells in PE were identified by multicolor confocal microscopic analysis shown here in black & white.

to distinguish $CD4^+CD25^{high}$ T_{reg} cells from activated cells. Although CD25 is the typical cell-surface marker used to identify T_{reg} cells, its specificity is not limited to regulatory T-cells, but also to other types of activated T-cells. Therefore, we decided to include other, more specific markers, such as GITR and CD152(CTLA-4), and FOXP3, to identify T_{reg} cells by flow cytometry.[8,17] This allowed for the distinguishing of T-cells with regulatory properties from other activated $CD4^+$ T-cells in the peripheral blood from NSCLC patients and controls. The stringent conditions used in our study led to sorting out a smaller population of $CD4^+CD25^{high}$ $FOXP3^+$ T-cells, which represent 0.06%–1.38% of $CD4^+$ T-cells in healthy people. In addition, we have analyzed FOXP3 expression in $CD4^+CD25^{high}$ cells from NSCLC patients and healthy donors by RT-PCR, because it has been shown recently that $CD4^+CD25^{high}$ T_{regs} specifically express this transcription factor,[23] and it is currently considered to be the most accurate marker to identify T_{regs}. We also found FOXP3 to be expressed almost exclusively by the $CD4^+$ $CD25^{high}$ T-cell population. The cell-surface and intracellular-marker analysis, combined with FOXP3 expression, led us to believe that the population detected in the peripheral blood of NSCLC patients is, indeed, $CD4^+CD25^{high}$ T_{regs}.

In *ex vivo* assays, we also studied the function of $FOXP3^+$ T_{regs} in NSCLC patients by analyzing their proliferative and suppressive abilities as well as cytokine profile. $FOXP3^+$ T_{reg} cells from NSCLC patients had a potent suppressive activity, as they inhibited the proliferative response of $CD4^+CD25^-$ T-cells significantly upon T-cell-receptor stimulation. In addition, these cells secreted inhibitory cytokines, such as TGF-b. Several groups have found the suppressive effect of regulatory T-cells to be cell-contact dependent and not mediated by cytokines. However, other studies have shown that TGF-b are responsible for the suppressive effect of regulatory T-cells.[24] This study presents that immune tolerization is predominant in the immune system in patients with advanced NSCLC. It is time to consider combinatorial tumor therapies, including those that subvert the immune-tolerating conditions within the tumor.

To analyze the role of the regulatory T-cells in the tumor environment, we have also analyzed $CD4^+$ $CD25^{high}$ $FOXP3^+$ T-cell population isolated from PE. The $CD4^+$ $CD25^{high}$ T-cells in malignant PE from NSCLC were CTLA-4 positive and GITR positive and also expressed FOXP3. Our data clearly showed that $CD4^+$ $CD25^{high}$ $FOXP3^+$ regulatory T-cell frequencies were increased not only in peripheral blood, but also in tumor microcircumstance, suggesting that these T-cells might suppress immune activation at the tumor site.

Several experimental models have shown that the elimination of $FOXP3^+$ T-cells can lead to effective antitumor immune responses. In mice, treatment with anti-CD25 mAb led to the regression of leukemia and fibrosarcoma.[25] In another study, the depletion of regulatory T-cells resulted in a slower growth of B16 melanoma.[26]

It remains to be shown as to how the increase in $CD4^+$ $CD25^{high}$ $FOXP3^+$ regulatory T-cells in humans contributes to immune tolerance or inhibition of effective antitumor immune responses in NSCLC. It is possible that the presence of $CD4^+$ $CD25^{high}$ $FOXP3^+$ regulatory T-cells at the tumor site promote tumor growth. On the other hand, the secretion of inhibitory factors by tumors might cause an expansion of regulatory T-cells.

Conclusions

In conclusion, our data suggest that in NSCLC patients, there is an increase of $CD4^+CD25^{high}$ $FOXP3^+$ regulatory T-cells in peripheral blood and PE. These T-cells might prevent effective antitumor immune responses, and in designing immunotherapy protocols for NSCLC, $CD4^+CD25^{high}$ $FOXP3^+$ regulatory T-cells are one more obstacle to overcome. These findings will prove to be important for the design of immunotherapeutic approaches to NSCLC.

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Disclosure Statement

No competing financial interests exist.

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