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ORIGINAL ARTICLE

Non-small-cell lung cancer-induced immunosuppression by increased human regulatory T cells via *Foxp3* promoter demethylation

Xing Ke[1,2] · Shuping Zhang[1,2] · Jian Xu[1,2] · Genyan Liu[1,2] · Lixia Zhang[1,2] · Erfu Xie[1,2] · Li Gao[1,2] · Daqian Li[1,2] · Ruihong Sun[1,2] · Fang Wang[1,2] · Shiyang Pan[1,2]

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Abstract Patients with non-small-cell lung cancer (NSCLC) have immune defects that are poorly understood. Forkhead box protein P3 (Foxp3) is crucial for immunosuppression by $CD4^+$ regulatory T cells (Tregs). It is not well known how NSCLC induces Foxp3 expression and causes immunosuppression in tumor-bearing patients. Our study found a higher percentage of $CD4^+$ Tregs in the peripheral blood of NSCLC compared with healthy donors. NSCLC patients showed demethylation of eight CpG sites within the *Foxp3* promoter with methylation ratios negatively correlated with $CD4^+CD25^+Foxp3^+$ T levels. Foxp3 expression in $CD4^+$ Tregs was directly regulated by *Foxp3* promoter demethylation and was involved in immunosuppression by NSCLC. To verify the effect of tumor cells on the phenotype and function of $CD4^+$ Tregs, we established a coculture system using NSCLC cell line and healthy $CD4^+$ T cells and showed that SPC-A1 induced IL-10 and TGF-β1 secretion by affecting the function of $CD4^+$ Tregs. The activity of DNA methyltransferases from $CD4^+$ T was decreased during this process. Furthermore, eight CpG sites within the *Foxp3* promoter also appeared to have undergone demethylation. Foxp3 is highly expressed in $CD4^+$ T cells, and this may be caused by gene promoter demethylation. These induced Tregs are highly immunosuppressive and dramatically inhibit the proliferative activity of naïve $CD4^+$ T cells. Our study provides one possible mechanism describing *Foxp3* promoter demethylation changes by which NSCLC down-regulates immune responses and contributes to tumor progression. Foxp3 represents an important target for NSCLC antitumor immunotherapy.

Keywords Non-small-cell lung cancer · Regulatory T cells · Forkhead box protein P3 · DNA demethylation · Immunosuppression

Abbreviations

AP-1	Activator protein-1
DNMTs	DNA methyltransferases
ELISA	Enzyme-linked immunosorbent assay
Foxp3	Forkhead box protein P3
IL	Interleukin
NF-AT	Nuclear factor of activated T cells
NSCLC	Non-small-cell lung cancer
PBMCs	Peripheral blood mononuclear cells
SAM	*S*-adenosylmethionine
SCC	Squamous cell carcinoma
TGF	Transforming growth factor
Tregs	Regulatory T cells
TSDR	Treg-specific demethylated region
TSS	Transcriptional start site

Xing Ke and Shuping Zhang have contributed equally to this work.

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✉ Fang Wang
wangfang@njmu.edu.cn

✉ Shiyang Pan
sypan@njmu.edu.cn

[1] Department of Laboratory Medicine, The First Affiliated Hospital of Nanjing Medical University, No. 300 of Guangzhou Road, Nanjing 210029, China

[2] National Key Clinical Department of Laboratory Medicine, No. 300 of Guangzhou Road, 210029 Nanjing, China

Introduction

Lung cancer is the leading cause of cancer death and affects public health in China and throughout the world [1, 2]. Furthermore, the morbidity and mortality of lung cancer are rising every year. More than 80 % of all lung cancer cases are diagnosed as non-small-cell lung cancer (NSCLC). Local and systemic immune defects in patients with NSCLC often occur and are associated with poor outcomes in patients [3, 4]; thus, it is critical to understand the contribution of genetic and environmental interactions in the development of NSCLC.

T-cell-related immune responses protect the host from tumorigenesis and tumor progression and have become the core of tumor immunotherapy [5]. Regulatory T cells (Tregs) generate down-regulating signals to inhibit immune response and play an important role in the formation and maintenance of immune tolerance. CD25 is the interleukin (IL)-2 receptor α-chain and a phenotypic marker of $CD4^+$ Tregs [6]. $CD4^+CD25^+$ Tregs have been shown to infiltrate tumor tissues and hinder immune response against tumor cells [7]. The transcription factor forkhead box protein P3 (Foxp3) is an important intracellular marker of $CD4^+CD25^+$ Tregs [9, 10]. *Foxp3* gene is located on the X chromosome at Xp11.23 and is essential for differentiation processes of Tregs [8]. Furthermore, it is reported that naïve murine $CD4^+CD25^+$ T cells can convert to the phenotype of Tregs after *Foxp3* gene transfer [11]. CD127, α-chain of the IL-7 receptor, is another important maker of Tregs and plays a significant role in the maintenance of immune self-tolerance [12]. Tregs accumulate in tumor microenvironment, effectively suppress immune responses and generally contribute to the poor outcomes seen in NSCLC patients [13, 14]. Previous studies have critically discussed the roles of a complex network of cytokine interactions in the development and progression of primary malignant tumors [15, 16]. Among the cytokines secreted by Tregs, the immune-modulating cytokine transforming growth factor-β1 (TGF-β1) or IL-10 regulate immune functions in the host [17] by down-regulating the immune reactivity of effector T cells [18]. Immunosuppression induced by Tregs has become one of the important mechanisms of tumor immune escape and the main difficulty of successful tumor immunotherapy [19]. In the tumor immunotherapy field, it is necessary to identify specific and functionally relevant surface molecules associated with Tregs to facilitate the development of effective therapeutic strategies against tumor-promoting Tregs.

Epigenetic modification, such as DNA methylation, often occurs in CpG islands of many gene promoters and is involved in numerous pathological processes in tumors [20–22]. DNA methyltransferases (DNMTs) catalyze the methylation process by using *S*-adenosylmethionine (SAM) to add a methyl molecule to cytosine [23]. DNMT1 is involved in maintaining methylation patterns and prefers hemi-methylated substrates [24, 25]. DNMT3a and DNMT3b function as de novo methyltransferases, as they mainly methylate completely unmethylated DNA [26, 27]. When promoter regions contain 5-methylcytosine in their CpG islands, transcription factors are unable to bind DNA, preventing gene transcription and leading to gene silencing [28, 29]. Nevertheless, DNA demethylation is very important in regulating Foxp3 expression. Studies have shown a Treg-specific demethylated region (TSDR) in the *Foxp3* promoter is closely related with Foxp3 expression. Furthermore, cytosine demethylation in the *Foxp3* promoter within CpG islands would likely result in the binding of transcription factors and lead to enhanced expression of Foxp3 [30, 31].

In the present study, we identified an important subpopulation, $CD4^+CD25^+Foxp3^+$ Tregs in the peripheral blood of NSCLC patients and analyzed the demethylation status of the *Foxp3* promoter in $CD4^+$ T cells. The correlation between *Foxp3* promoter demethylation and gene expression was further established. We also investigated the effect of NSCLC tumor cell line on the phenotype and immunosuppressive function of $CD4^+$ Tregs. The link between *Foxp3* gene promoter demethylation and Foxp3 expression was further confirmed in the coculture environment using NSCLC tumor cell line.

Materials and Methods

Patients and samples

The use of blood from patients and healthy donors and the clinical data was approved by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University (Nanjing, China). Twenty-eight cases with newly diagnosed NSCLC (14 males and 14 females, mean age of 63.3 ± 7.7 years) who received no prior treatment, 9 new cases with benign lung tumor (5 males and 4 females, mean age of 51 ± 10.4 years) with no other known medical conditions and 26 age-matched healthy donors (14 males and 12 females, mean age of 52.8 ± 9.3 years) with no family history of autoimmune diseases or tumors were enrolled in this study from the First Affiliated Hospital of Nanjing Medical University (Nanjing, China).

Of the 28 NSCLC samples, 20 samples were lung adenocarcinoma and 8 samples were lung squamous carcinoma. The 9 benign lung tumor samples comprised 3 lung hematomas, 3 benign nodules, 2 adenomatous hyperplasias and 1 lung spindle cell tumor.

Blood sample collection and CD4$^+$ T cells isolation

Peripheral blood (10 ml) was collected in EDTA-K_2 anticoagulant tubes from lung cancer patients, benign lung tumor patients and healthy donors. Serum was stored at −70 °C. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll–Hypaque density gradient centrifugation (GE Health Care Life Sciences, Piscataway, NJ, USA). Subsequently, CD4$^+$ T cells were separated using Dynabeads Untouched Human CD4 T Cells Kit and the manufacturer's instructions (Dynal, Oslo, Norway) [3]. PE-conjugated anti-CD4 and PC5-conjugated anti-CD3 monoclonal antibodies (Beckman, Marseille, France) were used to determine the purity of isolated CD4$^+$ T cells by flow cytometry.

Flow cytometry analysis

Foxp3 was labeled using Human Foxp3 Buffer Set (BD Biosciences, New York, NY, USA) according to the manufacturer's instructions. Cells (10^5) were labeled with fluorochrome-conjugated monoclonal antibodies for Tregs (CD4$^+$CD25$^+$Foxp3$^+$ T and CD4$^+$CD127$^-$ T). The antibodies were FITC-conjugated anti-CD4, APC-conjugated anti-CD25, PE-conjugated anti-Foxp3 and relevant isotype control, PE-conjugated anti-CD4 and FITC-conjugated anti-CD127 and relevant isotype control (all obtained from eBioscience, San Diego, CA, USA). Samples were detected on a Beckman-Coulter Gallios flow cytometer (Beckman-Coulter, Brea, CA, USA) and analyzed using Kaluza 1.3 software (Beckman-Coulter).

Enzyme-linked immunosorbent assay (ELISA)

After 5 days of coculture, CD4$^+$ T cells were collected and cultured in 96-well plates (5×10^4 cells per well). About 1 mg/ml of anti-CD3 (eBioscience, San Diego, CA, USA) and anti-CD28 (eBioscience, San Diego, CA, USA) was added for 12 or 24 h. After stimulation, the levels of IL-10 and TGF-β1 in the culture supernatants were tested using an ELISA Kit (eBioscience, San Diego, CA, USA) and instructions recommended by the manufacturer.

DNA isolation and methylation analysis

Bisulfite sequencing was used to conduct methylation analysis. Genomic DNA from CD4$^+$ T cells was extracted and modified according to the instructions of QIAamp Mini Kit (Qiagen, Hilden, Germany) and CpGenome DNA modification Kit (Millipore Corporation, Billerica, MA, USA). *Foxp3* promoter was amplified in ABI 2720 Thermal Cycler (Applied Biosystems/Life Technologies, Grand Island, NY, USA) by using Takara Ex Taq Hot Start (Takara, Shigaken, Japan) at the concentration recommended. The primers used to amplify the *Foxp3* promoter were 5′-TGGTGAAGTGGATTGATAGAAAAGG-3′ and 5′-TATAAAAACCCCCCCCCACC-3′. Subsequently, the DNA was treated with TA cloning using DNA A-Tailing (Takara) and sequenced on an ABI 3730 (Applied Biosystems/Life Technologies, Grand Island, NY, USA) in GeneScript Corporation (a Sino-America joint venture, Nanjing, China). Fifteen clones were detected and sequenced for each sample. Sequences were analyzed using the bioinformatics software Chromas version 1.45 (Technelysium, South Brisbane, Australia) and QUantification tool for Methylation Analysis (QUMA; http://quma.cdb.riken.jp/) online analysis system.

Cell lines and culture conditions

The human NSCLC cell lines SPC-A1 and A549, and the human fetal lung fibroblast cell line HFL-1 were purchased from the Chinese Academy of Sciences in Shanghai. They were cultured at 37 °C, with 5 % CO_2 in RPMI 1640 media (Gibco, Gaithersburg, MD, USA) containing 10 % fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), penicillin G (50 U/ml) and streptomycin (50 mg/ml).

Coculture of NSCLC cell line and CD4$^+$ T cells

Transwell coculture experiments were carried out in 24-well plates with a 0.4-μm pore inner well (Greiner, Frickenhausen, Germany) to physically separate CD4$^+$ T cells and tumor cells. Tumor cells were prior added in the outer wells of the plates at 2×10^5 cells per well. Isolated human CD4$^+$ T cells were seeded into the inner wells at 6×10^5 cells per well. After 5 days of incubation, CD4$^+$ T cells were collected for further analysis.

Real-time PCR for mRNA expression

Total RNA was prepared and subjected to first-strand cDNA synthesis using RNeasy Micro Kit (Qiagen, Hilden, German) and PrimeScript RT Master Mix (Takara). Gene expression levels were measured by an ABI 7500 Real-time PCR system (Applied Biosystems/Life Technologies, Grand Island, NY, USA) using SYBR Premix DimerEraser (Takara). The sequences of the primers were Foxp3: 5′-CAGCACATTCCCAGAGTTCCTC-3′ and 5′-GCGTGTGAACCAGTGGTAGATC-3′; DNMT1: 5′-GATGTGGCGTCTGTGAGGT-3′ and 5′-CCTTGCAGGCTTTACATTTCC-3′; DNMT3a: 5′-CTCCTGTGGGAGCCTCAATGTTACC-3′ and 5′-CAGTTCTTGCAGTTTTGGCACATTCC-3′; IL-10: 5′-GCTGGAGGACTTTAAGGGTTACCT-3′ and 5′-CTTGATGTCTGGGTCTTGGTTCT-3′; TGF-β1: 5′-CAGCAACAATTCCTGGCGATAC-3′ and 5′-TCAACCACTGCCGCACAACT-3′; β-actin: 5′-GAGCTACGAGCTGCCTGACG-3′ and 5′-GTAGTTTCG

TGGATGCCACAG-3′. Cycle threshold (CT) values were estimated by normalizing these values of detected samples against CT values of β-action and converted to be relative expression levels to show the fold change.

Western blot analysis

Cytoplasmic and nuclear protein was extracted from cells using NE-PER nuclear and cytoplasmic extraction reagents (Thermo scientific, Waltham, MA, USA). Total protein was separated by SDS-PAGE and transferred onto PVDF membranes (Bio-Rad, Hercules, CA, USA). Appropriate primary antibody (rabbit anti-human Foxp3, 1:1000 dilution, Cell signaling technology, Danvers, MA, USA; mouse anti-human GAPDH, 1:1000 dilution, ZSGB, Beijing, China) and horseradish peroxidase (HRP)-conjugated secondary antibody (ZSGB) were used to detect specific protein. Protein expression was determined using the enhanced chemiluminescence reagents (Millipore, Billerica, MA, USA) on X-ray film.

Proliferation assay of naïve $CD4^+$ T cells

Naïve $CD4^+$ T cells were isolated from healthy donors by using Dynabeads Untouched Human CD4 T Cells Kit (Dynal, Oslo, Norway) and Dynabeads FlowComp Human CD45RA Kit (Dynal). Different numbers of $CD4^+$ T cells collected from the transwell system were cocultured with naïve $CD4^+$ T cells (1×10^5) and irradiated autologous PBMCs (5×10^4) and stimulated with 500 ng/ml anti-CD3 antibody (eBioscience, San Diego, CA, USA) for 56 h. The proliferative activity of naïve $CD4^+$ T cells was detected by measuring [^{3}H]-thymidine [32].

Nuclear DNMTs activity assay

Nuclear protein from $CD4^+$ cells was extracted using NE-PER nuclear and cytoplasmic extraction reagents (Thermo scientific, Waltham, MA, USA) after 5 days of incubation. Nuclear protein (20 μg) was used in the total DNMTs activity assay, which was performed using the total DNMTs activity detection Kit (Genmed Scientifics, USA) according to the manufacturer's instructions.

Statistics

SPSS 16.0 software (IBM, Chicago, IL, USA) was used to analyze all data. Student's two-tailed *t* test was performed for statistical analysis of group differences. Results were shown as mean ± SD of at least three independent experiments. Pearson correlation analysis was performed to test the correlation. Statistical significance was set as $p < 0.05$.

Results

Elevated level of Tregs in patients with lung cancer

The level of Tregs in peripheral blood of patients was analyzed by flow cytometry. We found that the percentage of $CD4^+CD25^+$ T cells, $CD4^+CD25^+Foxp3^+$ T cells and $CD4^+CD127^-$ T cells was significantly higher in peripheral blood samples of patients with lung cancer than those with benign tumor or healthy control (Fig. 1a). The proportion of total $CD4^+CD25^+$ T cells in patients with lung cancer ($n = 28$) was increased compared with benign tumor ($n = 9$) or healthy control ($n = 26$) [(19.79 ± 7.10 %) vs. (6.58 ± 4.67 %) (mean ± SD), $p < 0.05$ and 19.79 ± 7.10 vs. 7.90 ± 5.51 %, $p < 0.05$, respectively] (Fig. 1b). A statistically significant increase in $CD4^+CD25^+Foxp3^+$ T cells in lung cancer was also found in comparison with benign tumor or healthy control (6.04 ± 5.12 vs. 1.7 ± 1.21 %, $p < 0.05$ and 6.04 ± 5.12 vs. 1.35 ± 0.90 %, $p < 0.05$, respectively) (Fig. 1b). Additionally, the percentage of $CD4^+CD127^-$ T cells in patients with lung cancer was higher than in patients with benign tumor or healthy control (1.82 ± 1.19 vs. 1.12 ± 0.59 %, $p < 0.05$ and 1.82 ± 1.19 vs. 1.18 ± 0.77 %, $p < 0.05$, respectively) (Fig. 1b). There was no obvious difference in the percentage of $CD4^+CD25^+$ T cells, $CD4^+CD25^+Foxp3^+$ T cells and $CD4^+CD127^-$ T cells between benign tumor and healthy control groups.

High levels of IL-10 and TGF-β1 in patients with lung cancer

ELISA was used to detect cytokines levels in the serum of tumor-bearing patients (Fig. 2). The IL-10 and TGF-β1 levels in lung cancer patients ($n = 21$) were significantly higher than in healthy control ($n = 23$) and benign tumor ($n = 8$) (26.3 ± 10.32 vs. 14.04 ± 2.83 pg/ml, $p < 0.05$ and 26.3 ± 10.32 vs. 17.42 ± 9.57 pg/ml, $p < 0.05$ for IL-10; 88.98 ± 49.82 vs. 55.81 ± 22.26 pg/ml, $p < 0.05$ and 88.98 ± 49.82 vs. 61.26 ± 22.23 pg/ml, $p < 0.05$ for TGF-β1).

Correlation between demethylation status of *Foxp3* promoter and gene expression in patients with lung cancer

The *Foxp3* gene has an associated CpG island that incorporates the promoter and transcriptional start site (TSS) important for *Foxp3* gene expression. This CpG island contains eight CpG sites (−138, −126, −113, −77, −65, −58, −43 and −15), TATA box and other transcription-enhancing factors, such as nuclear factor of activated T cells (NF-AT) and activator protein-1 (AP-1) regions (Fig. 3a). $CD4^+$ T cells

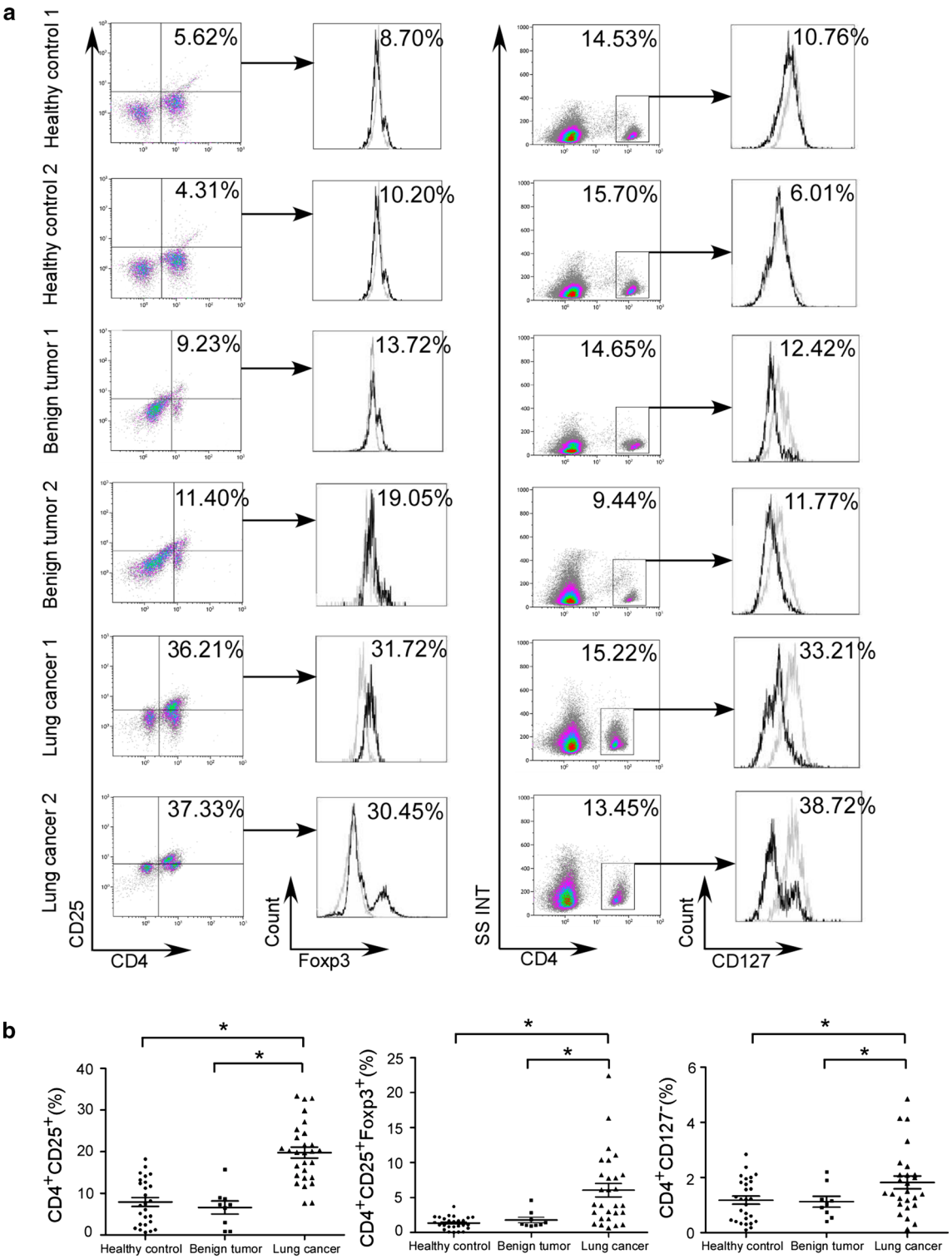


Fig. 1 High percentage of Tregs in patients with lung cancer. **a** Flow charts of Foxp3$^+$ T cells from the CD4$^+$CD25$^+$ T cells gate and CD127$^-$ T cells from CD4$^+$ T cells gate in lung cancer, benign tumor and healthy control group. The *horizontal axis* and *vertical axis* represent the corresponding fluorescence degree of phenotype expressions. *Gray* and *black histograms* represent flow cytometry results of isotype control and clinical samples, respectively. **b** Percentage of CD4$^+$CD25$^+$ T cells, CD4$^+$CD25$^+$Foxp3$^+$ T cells and CD4$^+$CD127$^-$ T cells in lung cancer (n = 28), benign lung tumor (n = 9) and healthy control (n = 26) by flow cytometry. Percentage of CD4$^+$CD25$^+$Foxp3$^+$ T cells is obtained by multiplying percentage of CD4$^+$CD25$^+$ T cells by Foxp3$^+$ T cells. Percentage of CD4$^+$CD127$^-$ T cells is obtained by multiplying percentage of CD4$^+$ T cells by CD127$^-$ T cells. *$p < 0.05$ compared with benign lung tumor or healthy control group. *SS* side scatter

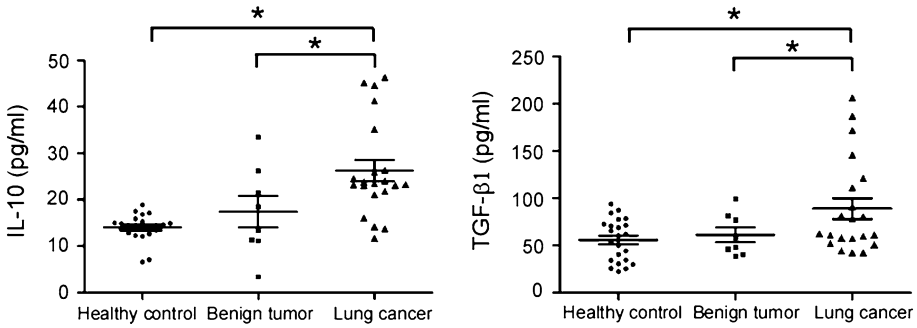


Fig. 2 High levels of IL-10 and TGF-β1 in patients with lung cancer. Levels of IL-10 and TGF-β1 in serums of lung cancer ($n = 23$), benign lung tumor ($n = 8$) and healthy control ($n = 21$) by ELISA. $^{*}p < 0.05$ compared with benign lung tumor or healthy control group

DNA was isolated from PBMCs from 17 cases of healthy control, 8 cases of benign tumor and 17 cases of lung cancer. These samples underwent bisulfite-sequencing PCR. *Foxp3* promoter of $CD4^+$ T cells from lung cancer patients overall demonstrated a low methylation ratio at the CpG sites investigated compared with benign tumor or healthy control samples (Fig. 3b).

The methylation ratio of total CpG sites in lung cancer was significantly lower than benign tumor and healthy control samples (50.22 ± 14.43 vs. 67.10 ± 8.29 %, $p < 0.05$ and 50.22 ± 14.43 vs. 70.64 ± 8.71 %, $p < 0.05$, respectively) (Fig. 3c). *Foxp3* promoter in $CD4^+$ T cells from lung cancer displayed a low methylation ratio and differed significantly from healthy controls at CpG positions −138, −126, −113, −77, −65, −58 and −43 (Fig. 3c). The results also showed that all the methylation sites in lung cancer, except sites −77 and −15, decreased relative to benign tumors. Pearson correlation analysis showed that the percentage of $CD4^+CD25^+Foxp3^+$ T cells negatively correlated with the total methylation ratios ($r = -0.721$, $p < 0.05$). $CD4^+CD25^+Foxp3^+$ T cells also negatively correlated with methylation ratios at CpG positions −126, −113, −77 and −43 ($r = -0.724$, $p < 0.05$; $r = -0.692$, $p < 0.05$; $r = -0.623$, $p < 0.05$; $r = -0.755$, $p < 0.05$, respectively) (Fig. 3d).

Enhanced Foxp3 expression in $CD4^+$ T cells from the coculture system

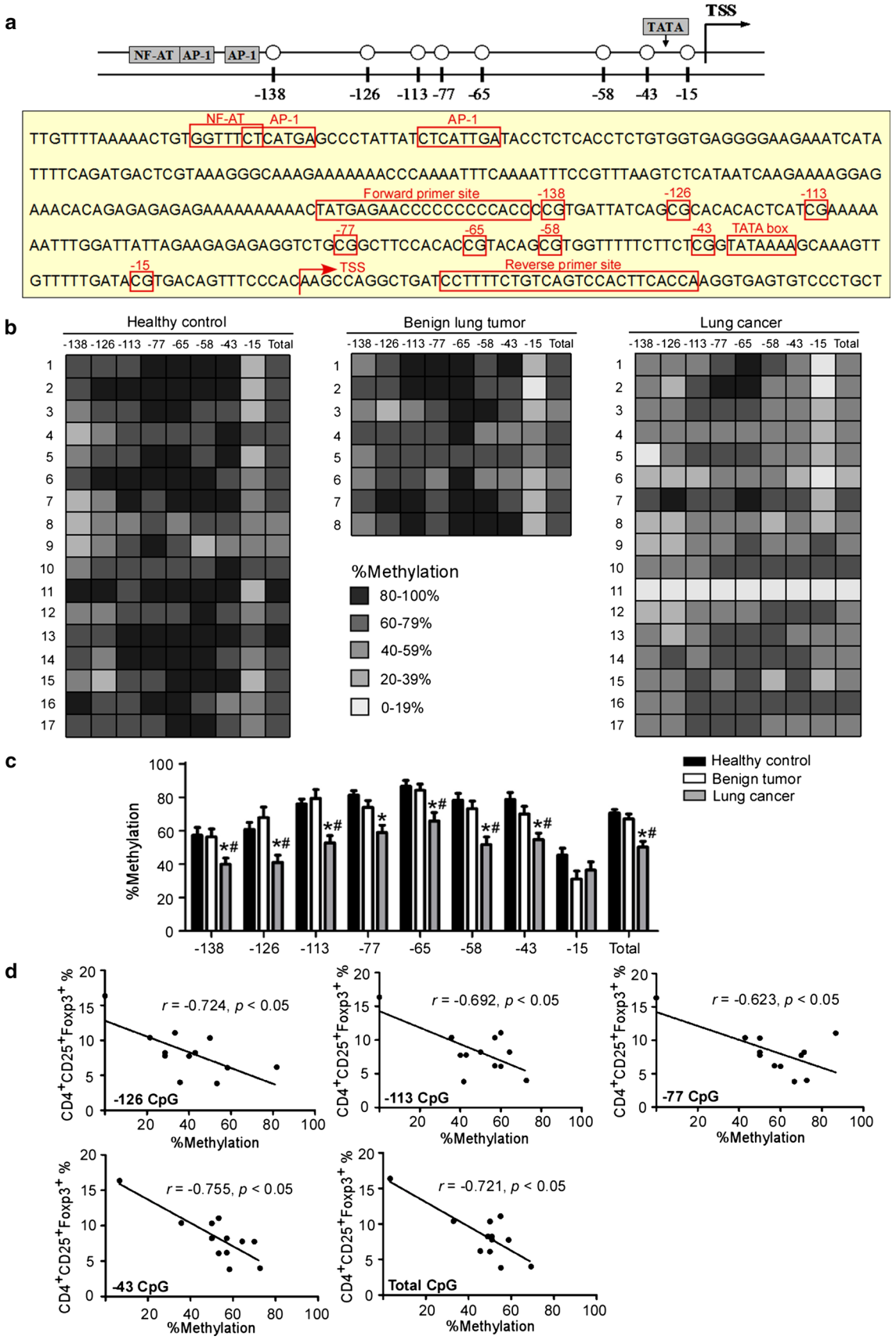
A transwell coculture system was used to investigate the effect of NSCLC cell line on Foxp3 level in $CD4^+$ T cells (Fig. 4a). As shown in Fig. 4b, we observed significantly increased levels of Foxp3 mRNA in $CD4^+$ T cells after being cocultured with SPC-A1 for 5 days when compared with HFL-1 coculture (1.89-fold, $p < 0.05$) or $CD4^+$ T cells cultured alone (2.11-fold, $p < 0.05$). Foxp3 protein levels in cytoplasmic and nuclear fractions were increased in $CD4^+$ T cells after being cocultured with SPC-A1 (Fig. 4c). Gray-level analysis also indicated Foxp3 protein was increased in SPC-A1 coculture compared to HFL-1 coculture group

Fig. 3 Demethylation status of the *Foxp3* promoter in patients with lung cancer. **a** Schematic representation of the conserved region upstream of the transcription start site (TSS) indicating the location of promoter elements and CpG sites. The CpG island contains eight CpG sites, TATA box, transcriptional start site and other transcription-enhancing factors such as NF-AT and AP-1 region. The *arrow* shows the position of TSS. **b** Gene map of the methylation status of the *Foxp3* promoter, which contains eight CpG sites in $CD4^+$ T cells from lung cancer ($n = 17$), benign lung tumor ($n = 8$) and healthy control ($n = 17$). The methylation degree at each CpG site is depicted by the strength of shading. **c** Statistical analysis of the methylation ratio at each CpG site among lung cancer ($n = 17$), benign lung tumor ($n = 8$) and healthy control ($n = 17$) groups. $^{*}p < 0.05$ compared with the healthy control group. $^{\#}p < 0.05$ compared with the benign lung tumor group. **d** Pearson correlation analysis between the percentage of $CD4^+CD25^+Foxp3^+$ T cells and methylation ratios. Levels of $CD4^+CD25^+Foxp3^+$ T cells in patients with NSCLC were negatively correlated with the methylation ratios of the *Foxp3* promoter (CpG site −126, −113, −77, −43 and total methylation ratios)

(2.27-fold, $p < 0.05$ and 1.67-fold, $p < 0.05$) or $CD4^+$ T cells cultured alone (2.5-fold, $p < 0.05$ and 1.5-fold, $p < 0.05$).

To identify the amount of $CD4^+$ T cells with the Tregs' phenotype, we evaluated the levels of $CD4^+CD25^+Foxp3^+$ T cells and $CD4^+CD127^-$ T cells in the coculture system by flow cytometry. The percentage of $CD4^+CD25^+$ T cells was increased after being cocultured with SPC-A1 relative to HFL-1 coculture or $CD4^+$ T cells cultured alone (14.20 ± 2.42 vs. 7.12 ± 1.66 and 14.20 ± 2.42 vs. 4.98 ± 3.20 %, respectively) (Fig. 4d). The numbers of $CD4^+CD25^+Foxp3^+$ T cells were also increased after being cocultured with SPC-A1 relative to HFL-1 coculture or $CD4^+$ T cells cultured alone (3.72 ± 1.55 vs. 0.78 ± 1.02 %, $p < 0.05$ and 3.72 ± 1.55 vs. 0.32 ± 0.95 %, $p < 0.05$, respectively). Coculture with SPC-A1 increased the prevalence of $CD4^+CD127^-$ T cells more than coculture with HFL-1 or $CD4^+$ T cells cultured alone (17.50 ± 3.56 vs. 7.15 ± 2.05 %, $p < 0.05$ and 17.50 ± 3.56 vs. 5.13 ± 1.87 %, $p < 0.05$, respectively).

Furthermore, we also observed Foxp3 mRNA in $CD4^+$ T cells was significantly increased in A549 coculture group relative to $CD4^+$ T cells cultured alone


a
TSS
TATA
NF-AT
AP-1
AP-1
-138
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Forward primer site
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-126
-113
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-43
TATA box
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Reverse primer site
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-126 CpG
r = -0.692, p < 0.05
-113 CpG
r = -0.623, p < 0.05
-77 CpG
r = -0.755, p < 0.05
-43 CpG
r = -0.721, p < 0.05
Total CpG

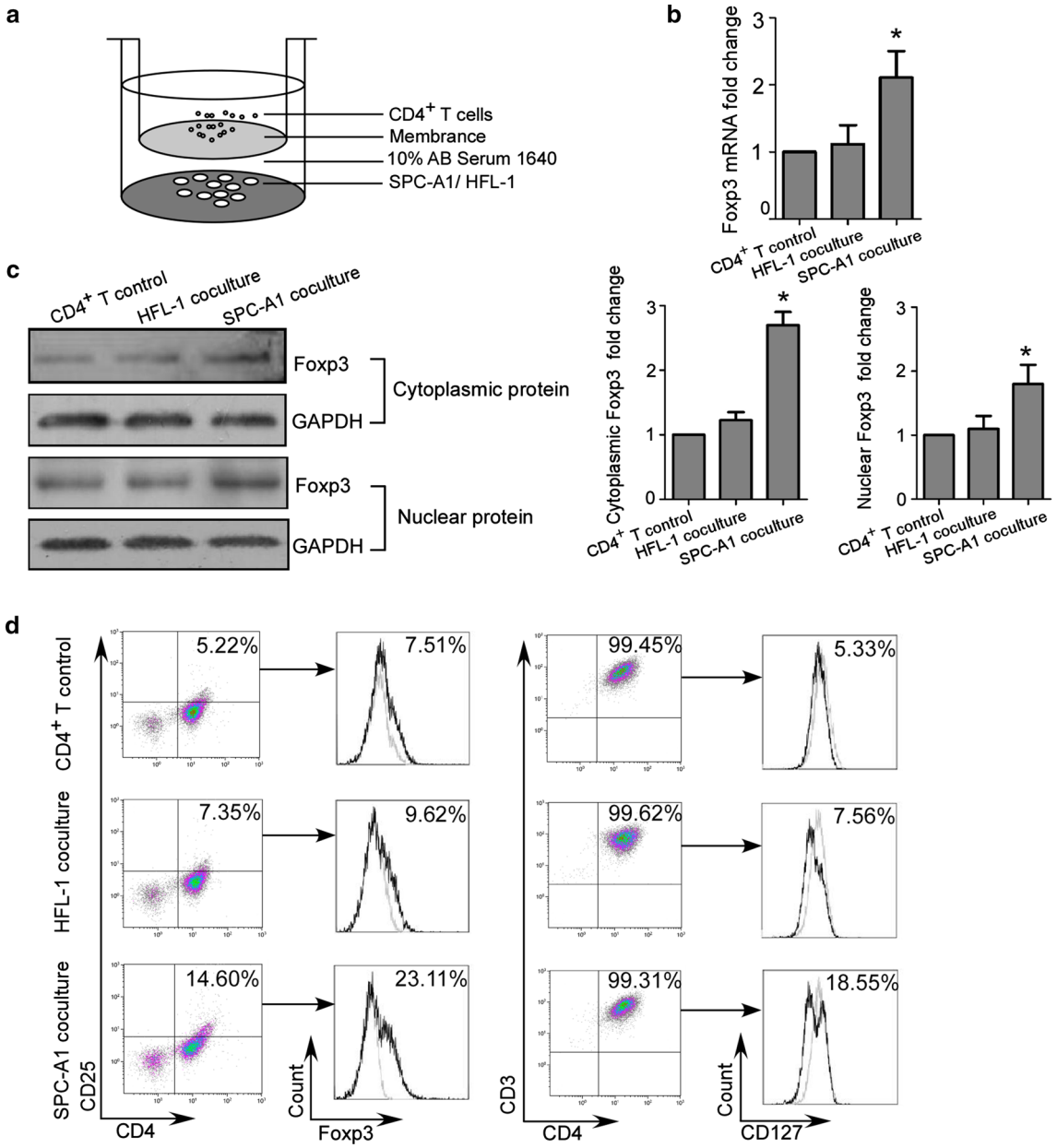

Fig. 4 Enhanced Foxp3 expression in $CD4^+$ T cell from the coculture system. **a** $CD4^+$ T cells were cocultured with SPC-A1 or HFL-1 cells. $CD4^+$ T cells (6×10^5 cells/well) and SPC-A1 or HFL-1 cells (2×10^5 cells/well) were grown separated by a 0.4-μm pore insert in 24-well culture plates. SPC-A1 or HFL-1 cells were grown in the outer wells. $CD4^+$ T cells were grown in suspension in the inner wells. **b** Expression of Foxp3 mRNA in $CD4^+$ T cells from SPC-A1 coculture, HFL-1 coculture and $CD4^+$ T cells cultured alone. $^*p < 0.05$ compared with HFL-1 coculture and $CD4^+$ T cells cultured alone. **c** Cytoplasm and nuclear protein expression and gray-level analysis of Foxp3 in $CD4^+$ T cells from SPC-A1 coculture, HFL-1 coculture and $CD4^+$ T cells cultured alone. $^*p < 0.05$ compared with HFL-1 coculture and $CD4^+$ T cells cultured alone group. **d** Flow charts of $Foxp3^+$ T cells from $CD4^+CD25^+$ T cells gate and $CD127^-$ T cells from $CD3^+CD4^+$ T cells gate in SPC-A1 coculture, HFL-1 coculture and $CD4^+$ T cells cultured alone. The *horizontal axis* and *vertical axis* represent the corresponding fluorescence degree of phenotype expressions. *Gray* and *black histograms* represent flow cytometry results of isotype control and clinical samples

(2.81-fold, $p < 0.05$) (Suppl. Fig. 1a). The percentages of $CD4^+CD25^+$ T cells, $CD4^+CD25^+Foxp3^+$ T cells and $CD4^+CD127^-$ T cells were increased after being cocultured with A549 cells compared with $CD4^+$ T cells cultured alone (Suppl. Fig. 1b).

Immunosuppressive function of $CD4^+$ T cells from the coculture system

To study the expression of immunosuppressive cytokines in the coculture system, we analyzed IL-10 and TGF-β1

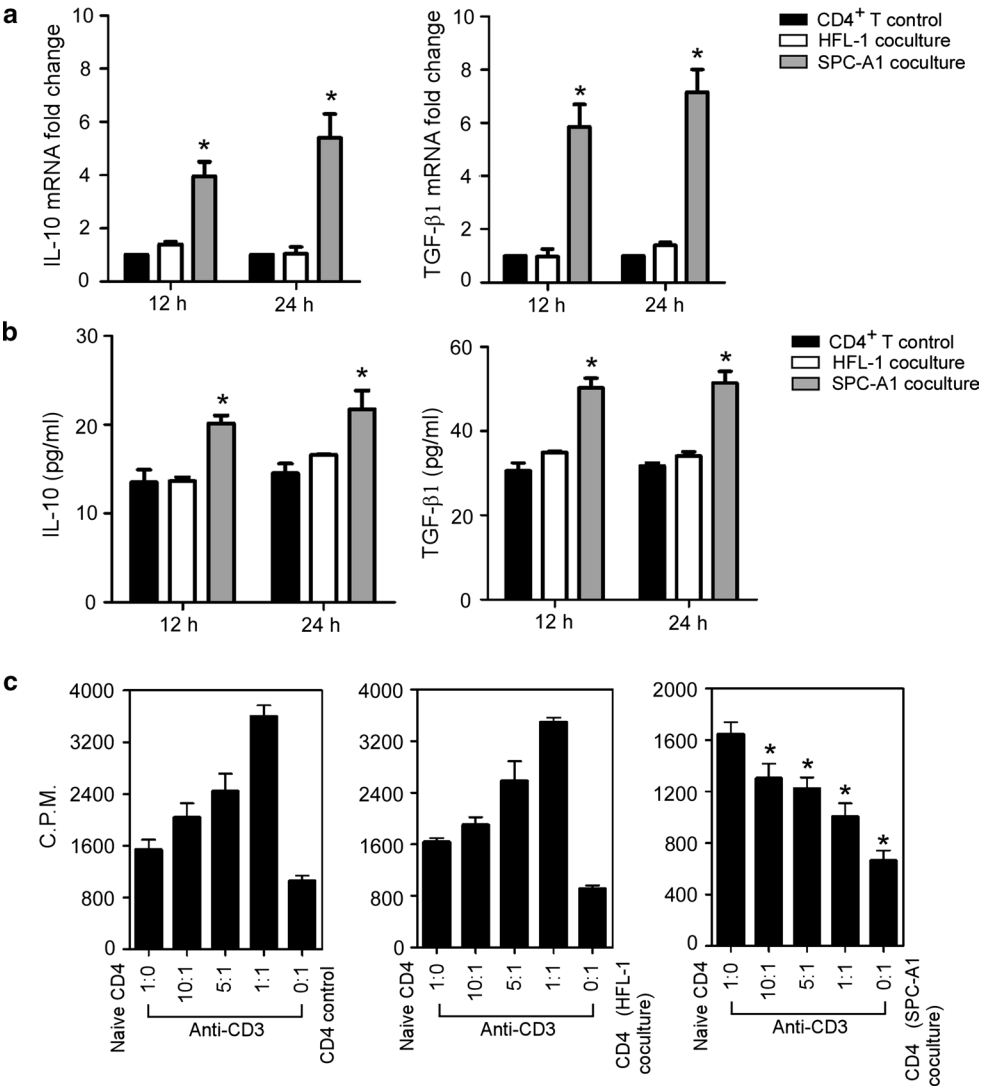

Fig. 5 Immunosuppressive function of $CD4^+$ T cells from the coculture system. **a** mRNA expression of IL-10 and TGF-β1 in $CD4^+$ T cells isolated from the coculture system after co-stimulation with anti-CD3 and anti-CD28 for 12 or 24 h. *$p < 0.05$ compared with HFL-1 coculture and $CD4^+$ T cells cultured alone. **b** Supernatant levels of IL-10 and TGF-β1 in $CD4^+$ T cells isolated from the coculture system after co-stimulation with anti-CD3 and anti-CD28 for 12 or 24 h. *$p < 0.05$ compared with HFL-1 coculture and $CD4^+$ T cells cultured alone. **c** Suppressive effect of $CD4^+$ Tregs on naïve $CD4^+$ T cells. Naïve $CD4^+$ T cells were cultured at a ratio of 10:1 with either naive $CD4^+$ T cells or SPC-A1 $CD4^+$ T cells in the presence of anti-CD3 antibody. SPC-A1 $CD4^+$ T cells possess significant suppressive activity for naïve $CD4^+$ T cells. *$p < 0.05$ compared to the 1:0 group

mRNA and supernatant protein after anti-CD3 and anti-CD28 stimulation for 12 and 24 h. Interestingly, $CD4^+$ T cells showed higher IL-10 and TGF-β1 responses after being cocultured with SPC-A1. The results demonstrated that mRNA levels of IL-10 and TGF-β1 in SPC-A1-cocultured $CD4^+$ T cells were significantly increased compared with HFL-1-cocultured $CD4^+$ T cells (4.68-fold, $p < 0.05$ and 2.82-fold, $p < 0.05$ for 12 h; 5.11-fold, $p < 0.05$ and 5.14-fold, $p < 0.05$ for 24 h) or $CD4^+$ T cells cultured alone (5.85-fold, $p < 0.05$ and 3.95-fold, $p < 0.05$ for 12 h; 7.15-fold, $p < 0.05$ and 5.40-fold, $p < 0.05$ for 24 h) (Fig. 5a). Additionally, IL-10 and TGF-β1 protein found in supernatants was also significantly enhanced in SPC-A1-cocultured $CD4^+$ T cells compared with HFL-1-cocultured $CD4^+$ T cells ($p < 0.05$ for 12 and 24 h) and $CD4^+$ T cells cultured alone ($p < 0.05$ for 12 and 24 h) (Fig. 5b).

We next determined whether SPC-A1-cocultured $CD4^+$ T cells had suppressive effects on naïve $CD4^+$ T cells. Figure 5c showed the proliferative activity of naïve $CD4^+$ T cells cultured at different ratios with naïve $CD4^+$ T cells or SPC-A1-cocultured $CD4^+$ T cells. 10:1 represented that the ratio of naïve $CD4^+$ T cells and cocultured $CD4^+$ T cells. We found that SPC-A1-cocultured $CD4^+$ T cells showed low proliferative activity in response to anti-CD3 antibody stimulation. When cocultured with purified naïve $CD4^+$ T cells, these cells dramatically inhibited the proliferative activity of naïve $CD4^+$ T cells following the addition of anti-CD3 antibody, in a dose-dependent manner. In contrast, neither HFL-1-cocultured $CD4^+$ T cells or $CD4^+$ T cells cultured alone showed any suppressive effects on naive $CD4^+$ T cells, with both groups enhancing the proliferative activity of naïve $CD4^+$ T cells.

Demethylation status of *Foxp3* promoter in $CD4^+$ T cells from the coculture system

To evaluate the underlying mechanism of methylation regulation of *Foxp3* gene expression, we detected the

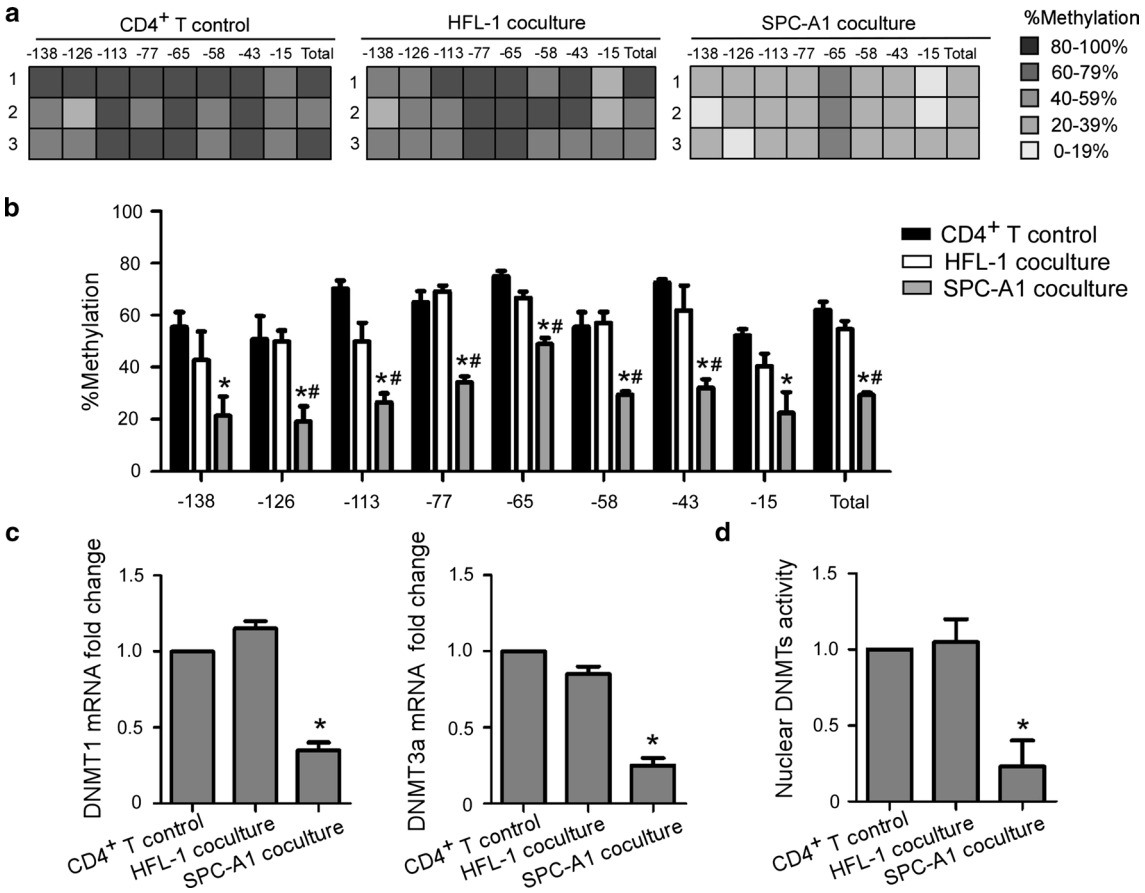

Fig. 6 Demethylation status of the *Foxp3* promoter in CD4$^+$ T cells from the coculture system. **a** A gene map of methylation status of the *Foxp3* promoter, which contains eight CpG sites in CD4$^+$ T cells from SPC-A1 coculture, HFL-1 coculture and CD4$^+$ T cells cultured alone. The methylation degree at each CpG site is depicted by the strength of shading. **b** Statistical analysis of the methylation ratio of each CpG site in SPC-A1 coculture, HFL-1 coculture and CD4$^+$ T cells cultured alone. *$p < 0.05$ compared with CD4$^+$ T cells cultured alone group. $^{\#}p < 0.05$ compared with HFL-1 coculture group. **c** mRNA expression of DNMT1 and DNMT3a in SPC-A1 coculture, HFL-1 coculture and CD4$^+$ T cells cultured alone. *$p < 0.05$ compared with HFL-1 coculture or CD4$^+$ T cells cultured alone. **d** Nuclear DNMTs activity levels of CD4$^+$ T cells from SPC-A1 coculture, HFL-1 coculture and CD4$^+$ T cells cultured alone. *$p < 0.05$ compared with HFL-1 coculture or CD4$^+$ T cells cultured alone

methylation status of the *Foxp3* promoter in CD4$^+$ T cells after coculture with tumor cells. We found that the *Foxp3* promoter displayed demethylation changes after coculture with SPC-A1 compared with HFL-1-cocultured CD4$^+$ T cells or CD4$^+$ T cells cultured alone in the repeat tests of three times (Fig. 6a). The percentage of total CpG sites in SPC-A1-cocultured CD4$^+$ T cells was significantly lower than HFL-1-cocultured CD4$^+$ T cells (29.23 ± 2.06 vs. 54.7 ± 5.22 %, $p < 0.05$) or CD4$^+$ T cells cultured alone (29.23 ± 2.06 vs. 62.13 ± 5.32 %, $p < 0.05$) (Fig. 6b). Additionally, Fig. 6b showed that all the CpG sites displayed obvious differences between SPC-A1 coculture group and CD4$^+$ T control group. In SPC-A1 coculture group, the methylation ratio of the *Foxp3* promoter showed a remarkable decrease in CpG positions −126, −113, −77, −65, −58 and −43 relative to HFL-1 coculture group (Fig. 6b). Furthermore, all the CpG sites of the *Foxp3* promoter displayed demethylation changes in A549 coculture group relative to CD4$^+$ T cells cultured alone (Suppl. Fig. 2a, b).

Additionally, we detected that mRNA expression of DNMT1 and DNMT3a was decreased in CD4$^+$ T cells after being cocultured with SPC-A1 relative to HFL-1 cocultured CD4$^+$ T cells (0.30-fold, $p < 0.05$ and 0.29-fold, $p < 0.05$, respectively) or CD4$^+$ T cells cultured alone (0.35-fold, $p < 0.05$ and 0.25-fold, $p < 0.05$, respectively) (Fig. 6c). Nuclear DNMTs activity in CD4$^+$ T cells was also decreased after being cocultured with SPC-A1 relative to HFL-1 coculture (0.22-fold, $p < 0.05$) or CD4$^+$ T cells cultured alone (0.23-fold, $p < 0.05$) (Fig. 6d).

Discussion

$CD4^+CD25^+$ T cells play a vital role in tumor immunity and peripheral tolerance and have become a significant research focus [33, 34]. Tumor-induced Tregs maintain immune self-tolerance in tumor patients and enable tumor cells to develop special mechanisms to escape immune surveillance and anti-tumor immune response [35–37].

It was previously thought that the inhibitory state in lung cancer patients was partially due to the increase in Tregs observed in our study. Ju et al. [38] identified the percentage of $CD13^+CD4^+CD25^{hi}$ Tregs was significantly increased and was related to pathological stages in NSCLC. Research by Schneider et al. [39] showed a high density of $Foxp3^+$ Tregs was found in the tumor center of adenocarcinomas. We hypothesized that the effect of the increased $CD4^+CD25^+Foxp3^+$ Tregs and $CD4^+CD127^-$ Tregs in our experiments were important for NSCLC tumor cells escaping the immune system. Our study also demonstrated that the levels of IL-10 and TGF-β1 were significantly higher in serums of NSCLC patients. Yang et al. also demonstrated a high level of IL-10 and TGF-β1 in malignant pleural effusion, which predicted a poor prognosis in patients with lung cancer [40]. Indirect suppression via the secretion of immunosuppressive mediators, such as IL-10 and TGF-β1, plays a particularly significant role for Tregs-mediated immunosuppression in the tumor microenvironment [41].

Considering the importance of Foxp3 in the immunosuppressive microenvironment mainly established by $CD4^+CD25^+$ T cells [42], the factors that control and regulate *Foxp3* gene expression needed to be determined. Epigenetic mechanisms have some relationships with the regulation of various genes in the process of cell differentiation and development [43, 44]; thus, it was hypothesized that the methylation status of the *Foxp3* promoter may lead to the differences in Foxp3 expression level. In this study, the methylation status of *Foxp3* gene promoter region (198 bp) of $CD4^+$ T cells in human peripheral blood was determined. The methylation ratio of the *Foxp3* promoter in $CD4^+$ T cells from lung cancer displayed was low and significantly differed from healthy control and benign tumor samples. Interestingly, levels of $CD4^+CD25^+Foxp3^+$ T cells negatively correlated with the methylation ratios. Our data suggested that the enhanced expression of Foxp3 in $CD4^+$ T cells from NSCLC patients maybe a direct consequence of the demethylation at the *Foxp3* promoter region.

To elucidate the effects of tumor cells on *Foxp3* promoter demethyation, we cocultured $CD4^+$ T cells and NSCLC cell line. It was found that the expression of Foxp3 in $CD4^+$ T cells was increased in NSCLC cell line coculture group. Additionally, the levels of $CD4^+CD25^+$ T cells, $CD4^+CD25^+Foxp3^+$ T cells and $CD4^+CD127^-$ T cells were remarkably increased in the coculture system with the NSCLC cell line. However, HFL-1 coculture or $CD4^+$ T culture alone did not display these changes. These results suggest that the phenotype of T cells can be influenced and changed by NSCLC tumor cells. Additionally, indirect inhibition by Tregs through the secretion of anti-inflammatory cytokines, such as IL-10 or TGF-β1, would be in effect. Moreover, NSCLC cell line coculture induced T cells, which could significantly suppress the proliferative activity of naïve $CD4^+$ T cells in a dose-dependent manner. It was possible that the presence of Tregs may improve the survival of NSCLC tumor cells.

The demethylation status of *Foxp3* promoter was further analyzed to understand the regulation mechanism of *Foxp3* gene expression. Demethylation of *Foxp3* promoter appeared to be influenced by SPC-A1 cells. Nuclear DNMTs activity of $CD4^+$ T cells was decreased after being cocultured with NSCLC cell line. It is possible that DNMT1 and DNMT3a participate in *Foxp3* promoter demethylation process, as they can catalyze DNA methylation [45]. Elevated expression of Foxp3 in $CD4^+$ T cells is thought to be regulated by demethylation status changes in the CpG island of the *Foxp3* promoter. This provided a strict mechanism for the regulation of Tregs' phenotypic marker expression. The tumor-related $Foxp3^+$ Tregs, in combination with cytokine secretion, showed immunosuppressive potential and suggested a vital role in the formation and development of an immunosuppressive tumor microenvironment.

In this study, we demonstrated that NSCLC tumor cells constitutively influence function of $CD4^+$ T cells from healthy donors. By down-regulating the activation of DNMTs, NSCLC tumor cells demethylated the *Foxp3* gene promoter and enhanced the transcription and expression of *Foxp3* gene. These $Foxp3^+$ T cells were likely responsible for the secretion of immunosuppressive cytokines, IL-10 and TGF-β1, to increase immunosuppression in tumor-bearing patients. Tregs showed a crucial role in establishing and maintaining the immunosuppressive microenvironment of NSCLC. NSCLC tumor cells have the potential to generate different regulation mechanisms to evade anti-tumor immunity and defeat conventional tumor immunotherapy. Therefore, specific activity inhibition of Tregs may become an effective therapeutic focus to develop successful strategies for NSCLC immunotherapy.

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Compliance with ethical standards

Conflict of interest The authors declared no financial or commercial conflict of interest.

References

1. He YQ, Bo Q, Yong W, Qiu ZX, Li YL, Li WM (2013) Foxp3 genetic variants and risk of non-small cell lung cancer in the Chinese Han population. Gene 531:422–425
2. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM (2010) Estimates of worldwide burden of cancer in 2008: gLOBOCAN 2008. Int J Cancer 127:2893–2917
3. Wang F, Xu J, Zhu Q, Qin X, Cao Y, Lou J, Xu Y, Ke X, Li Q, Xie E, Zhang L, Sun R, Chen L, Fang B, Pan S (2013) Downregulation of IFNG in $CD4^+$ T cells in lung cancer through hypermethylation: a possible mechanism of tumor-induced immunosuppression. PLoS ONE 8:e79064
4. Forde PM, Reiss KA, Zeidan AM, Brahmer JR (2013) What lies within: novel strategies in immunotherapy for non-small cell lung cancer. Oncologist 18:1203–1213
5. Mockler MB, Conroy ML, Lysaght J (2014) Targeting T cell immunometabolism for Cancer Immunotherapy; understanding the impact of the tumor microenvironment. Front Oncol 4:107
6. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M (1995) Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chain (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol 155:1151–1164
7. Nishikawa H, Sakaguchi S (2010) Regulatory T cells in tumor immunity. Int J Cancer 127:759–767
8. Wang L, Liu R, Li W, Chen C, Katoh H, Chen GY, McNally B, Lin L, Zhou P, Zuo T, Cooney KA, Liu Y, Zheng P (2009) Somatic single hits inactivate the X-linked tumor suppressor FOXP3 in the prostate. Cancer Cell 16:336–346
9. Braga WM, da Silva BR, de Carvalho AC, Maekawa YH, Bortoluzzo AB, Rizzatti EG, Atanackovic D, Colleoni GW (2014) FOXP3 and CTLA4 overexpression in multiple myeloma bone marrow as a sign of accumulation of CD4(+) T regulatory cells. Cancer Immunol Immunother 63:1189–1197
10. da Silva Martins M, Piccirillo CA (2012) Functional stability of $Foxp3^+$ regulatory T cells. Trends Mol Med 18:454–462
11. Yagi H, Nomura T, Nakamura K, Yamazaki S, Kitawaki T, Hori S, Maeda M, Onodera M, Uchiyama T, Fujii S, Sakaguchi S (2004) Crucial role of FOXP3 in the development and function of human $CD25^+CD4^+$ regulatory T cells. Int Immunol 16:1643
12. Hall BM, Verma ND, Tran GT, Hodgkinson SJ (2011) Distinct regulatory $CD4^+$T cell subsets; differences between naïve and antigen specific T regulatory cells. Curr Opin Immunol 23:641–647
13. Beyer M, Schultze JL (2006) Regulatory T cells in cancer. Blood 108:804
14. Landskron J, Helland Ø, Torgersen KM, Aandahl EM, Gjertsen BT, Bjørge L, Taskén K (2015) Activated regulatory and memory T-cells accumulate in malignant ascites from ovarian carcinoma patients. Cancer Immunol Immunother 64:337–347
15. Khaghanzadeh N, Samiei A, Ramezani M, Mojtahedi Z, Hosseinzadeh M, Ghaderi A (2014) Umbelliprenin induced production of IFN-γ and TNF-α, and reduced IL-10, IL-4, Foxp3 and TGF-β in a mouse model of lung cancer. Immunopharmacol Immunotoxicol 36:25–32
16. Sakurai T, Kudo M (2011) Signaling pathways governing tumor angiogenesis. Oncology 81(Suppl 1):24–29
17. Eusebio M, Kuna P, Kraszula L, Kupczyk M, Pietruczuk M (2014) Allergy-related changes in levels of $CD8^+CD25^+$FoxP3 (bright) Treg cells and FoxP3 mRNA expression in peripheral blood: the role of IL-10 or TGF-beta. J Biol Regul Homeost Agents 28:461–470
18. Mason CM, Porretta E, Zhang P, Nelson S (2007) $CD4^+CD25^+$ transforming growth factor-beta-producing T cells are present in the lung in murine tuberculosis and may regulate the host inflammatory response. Clin Exp Immunol 148:537–545
19. Khattri R, Cox T, Yasayko SA, Ramsdell F (2003) An essential role for Scurfin in $CD4^+CD25^+$ T regulatory cells. Nat Immunol 4:337–342
20. Buck-Koehntop BA, Defossez PA (2013) On how mammalian transcription factors recognize methylated DNA. Epigenetics 8:131–137
21. Pan S, Zhang L, Gao L, Gu B, Wang F, Xu J, Shu Y, Yang D, Chen Z (2009) The property of methylated APC gene promotor and its influence on lung cancer cell line. Biomed Pharmacother 63:463–468
22. Hattori N, Ushijima T (2014) Compendium of aberrant DNA methylation and histone modifications in cancer. Biochem Biophys Res Commun 455:3–9
23. Subramaniam D, Thombre R, Dhar A, Anant S (2014) DNA methyltransferases: a novel target for prevention and therapy. Front Oncol 4:80
24. Kar S, Deb M, Sengupta D, Shilpi A, Parbin S, Torrisani J, Pradhan S, Patra S (2012) An insight into the various regulatory mechanisms modulating human DNA methyltransferase 1 stability and function. Epigenetics 7:994–1007
25. Robertson KD (2001) DNA methylation, methyltransferases, and cancer. Oncogene 20:3139–3155
26. Okano M, Bell DW, Haber DA, Li E (1999) DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell 99:247–257
27. Kangaspeska S, Stride B, Métivier R, Polycarpou-Schwarz M, Ibberson D, Carmouche RP, Benes V, Gannon F, Reid G (2008) Transient cyclical methylation of promoter DNA. Nature 452:112–115
28. Mazzio EA, Soliman KF (2012) Basic concepts of epigenetics: impact of environmental signals on gene expression. Epigenetics 7:119–130
29. Reik W (2007) Stability and flexibility of epigenetic gene regulation in mammalian development. Nature 447:425–432
30. Janson PC, Winerdal ME, Marits P, Thörn M, Ohlsson R, Winqvist O (2008) FOXP3 promoter demethylation reveals the committed Treg population in humans. PLoS ONE 3:e1612
31. Lal G, Bromberg JS (2009) Epigenetic mechanisms of regulation of Foxp3 expression. Blood 114:3727–3735
32. Wang F, Chi J, Peng G, Zhou F, Wang J, Li L, Feng D, Xie F, Gu B, Qin J, Chen Y, Yao K (2014) Development of virus-specific $CD4^+$ and $CD8^+$ regulatory T cells induced by human Herpesvirus 6 infection. J Virol 88:1011–1024
33. La Rocca C, Carbone F, Longobardi S, Matarese G (2014) The immunology of pregnancy: regulatory T cells control maternal immune tolerance toward the fetus. Immunol Lett 162:41–48
34. Belkaid Y, Piccirillo CA, Mendez S, Shevach EM, Sacks DL (2002) $CD4^+CD25^+$ regulatory T cells control Leishmania major persistence and immunity. Nature 420:502–507
35. Larmonier N, Marron M, Zeng Y, Cantrell J, Romanoski A, Sepassi M, Thompson S, Chen X, Andreansky S, Katsanis E (2007) Tumor-derived $CD4^+CD25^+$ regulatory T cell suppression of dendritic cell function involves TGF-beta and IL-10. Cancer Immunol Immunother 56:48–59
36. Erfani N, Mehrabadi SM, Ghayumi MA, Haghshenas MR, Mojtahedi Z, Ghaderi A, Amani D (2012) Increase of regulatory T cells in metastatic stage and CTLA-4 over expression in lymphocytes of patients with non-small cell lung cancer (NSCLC). Lung Cancer 77:306–311

37. Zou W (2006) Regulatory T cells, tumour immunity and immunotherapy. Nat Rev Immunol 6:295–307
38. Ju S, Qiu H, Zhou X, Zhu B, Lv X, Huang X, Li J, Zhang Y, Liu L, Ge Y, Johnson DE, Ju S, Shu Y (2009) $CD13^{+}CD4^{+}CD25hi$ regulatory T cells exhibit higher suppressive function and increase with tumor stage in non-small cell lung cancer patients. Cell Cycle 8:2578–2585
39. Schneider T, Kimpfler S, Warth A, Schnabel PA, Dienemann H, Schadendorf D, Hoffmann H, Umansky V (2011) $Foxp3^{+}$ regulatory T cells and natural killer cells distinctly infiltrate primary tumors and draining lymph nodes in pulmonary adenocarcinoma. J Thorac Oncol 6:432–438
40. Yang G, Li H, Yao Y, Xu F, Bao Z, Zhou J (2015) Treg/Th17 imbalance in malignant pleural effusion partially predicts poor prognosis. Oncol Rep 33:478–484
41. Karimi S, Chattopadhyay S, Chakraborty NG (2015) Manipulation of regulatory T cells and antigen-specific cytotoxic T lymphocyte-based tumourimmunotherapy. Immunology 144:186–196
42. Shen X, Du J, Guan W, Zhao Y (2014) The balance of intestinal $Foxp3^{+}$ regulatory T cells and Th17 cells and its biological significance. Expert Rev Clin Immunol 10:353–362
43. Yang R, Qu C, Zhou Y, Konkel JE, Shi S, Liu Y, Chen C, Liu S, Liu D, Chen Y, Zandi E, Chen W, Zhou Y, Shi S (2015) Hydrogen sulfide promotes Tet1- and Tet2-mediated Foxp3 demethylation to drive regulatory T cell differentiation and maintain immune homeostasis. Immunity 43:251–263
44. Anderson MR, Enose-Akahata Y, Massoud R, Ngouth N, Tanaka Y, Oh U, Jacobson S (2014) Epigenetic modification of the FoxP3 TSDR in HAM/TSP decreases the functional suppression of Tregs. J Neuroimmune Pharmacol 9:522–532
45. Li E, Zhang Y (2014) DNA methylation in mammals. Cold Spring Harb Perspect Biol 6:a019133