

Effect of TGF- β 1 on blood CD4⁺CD25^{high} regulatory T cell proliferation and Foxp3 expression during non-small cell lung cancer blood metastasis

YI HU¹, WEIBO QI¹, LI SUN², HUI ZHOU², BILIU ZHOU³ and ZHIPING YANG⁴

¹Department of Cardiothoracic Surgery, Jiaxing No. 1 Hospital;

²Clinical Laboratory, Jiaxing No. 1 Hospital; ³Zhejiang Guojian Biotech Co., Ltd.;

⁴Department of Medical Oncology, Jiaxing No. 1 Hospital, Jiaxing, Zhejiang 314001, P.R. China

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Abstract. Metastatic circulating tumor cells in non-small cell lung cancer (NSCLC) metastasis have been reported to be associated with an immune response. The present study aimed to provide a theoretical basis for the immunomodulatory processes during NSCLC blood metastasis. NSCLC blood and normal peripheral blood mononuclear cells (PBMCs) were collected. The quantity of cluster of differentiation (CD)4⁺CD25^{high} regulatory T (Treg) cells and the intracellular forkhead box protein 3 (Foxp3) expression in CD4⁺CD25^{high} Treg cells were determined by flow cytometry. Furthermore, the effect of transforming growth factor β 1 (TGF- β 1) on NSCLC blood CD4⁺CD25⁺ Treg cell proliferation was explored by activating blood mononuclear cells with an anti-CD3 monoclonal antibody, interleukin-2 and different doses of TGF- β 1. Reverse transcription-quantitative polymerase chain reaction assays were used to detect the mRNA expression of Foxp3. Carboxyfluorescein succinimidyl ester staining was used to analyze the proliferation dynamics of lymphocyte subsets. Results indicate that the proportion of CD4⁺ T cells in the blood of patients with NSCLC was significantly higher compared with normal peripheral blood ($P < 0.01$). Foxp3 expression in NSCLC blood Treg cells was significantly decreased compared with normal peripheral blood ($P < 0.01$). NSCLC blood mononuclear cells treated with TGF- β 1 at 1, 5 and 25 ng/ml significantly induced Foxp3 expression in CD4⁺CD25⁺ Treg cells compared with the control group ($P < 0.05$). The proportion of CD4⁺CD25⁺ Treg and CD8⁺ T cells were elevated in generation 6, 7, 8 after 6 days of TGF- β 1 treatment compared with untreated cells. The proportion of CD4⁺CD25⁺ Treg and

CD8⁺ T cells were elevated in generation 8, 9 and with TGF- β 1 treatment after 8 days compared with untreated cells. These results indicate that CD4⁺CD25⁺ Treg cells proliferate at a greater rate compared with CD8⁺ T cells after 4, 6 or 8 days of treatment. The proportion of CD4⁺CD25^{high} Treg cells in NSCLC blood was significantly higher ($P < 0.05$) compared with normal peripheral blood. The number of Foxp3⁺ T cells was significantly lower ($P < 0.05$) compared with normal peripheral blood. The data presented in this study suggest that NSCLC blood CD4⁺CD25^{high} Treg cells are functionally immature and that TGF- β 1 may promote maturation.

Introduction

A special class of regulatory T (Treg) cells, cluster of differentiation (CD)4⁺CD25⁺ Treg cells, characterized by high expression of interleukin-2 (IL-2) receptor α -chain, exists in the peripheral blood and spleen of normal humans and mice (1,2). Treg cells are divided into two categories: Natural regulatory T cells (nTreg), which are produced in the thymus (3) and induced Treg cells (iTreg), which are induced by inhibitive cytokines, drugs and immature dendritic cells *in vitro* or *in vivo* (4,5). Recently, studies have demonstrated that CD4⁺CD25⁺ Treg cells with low reactivity and immunosuppressive properties may serve an important role in maintaining homeostasis within the internal environment, and inducing transplantation tolerance, autoimmune diseases, the response to infections and tumor immunity (6-8). The proportion of Treg cells in normal peripheral blood, which has immunosuppressive or tumor immunity abilities, is very small, accounting for 1-3% of peripheral blood CD4⁺ T cells (9,10).

Forkhead box protein 3 (Foxp3) belongs to the forkhead/winged-helix transcription factor family and displays a fork-like helical, a C2H2 zinc finger and a leucine zipper structure (11,12). In humans, Foxp3 is located at p11.23-q13.3 on the X chromosome, containing 11 exons and 10 introns. It encodes a 48 kDa protein, Scurfin, which is a key factor in Treg cell development and immunosuppressive function (13,14). Jiang *et al* (15) reported that Foxp3 protein was more specific than CD4, CD25 and other surface markers, serving a pivotal role in the inhibitive function of Treg cells.

Correspondence to: Dr Zhiping Yang, Department of Medical Oncology, Jiaxing No. 1 Hospital, 1882 South Zhonghuan Road, Jiaxing, Zhejiang 314001, P.R. China
E-mail: zkx0925@163.com

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Schoenbrunn *et al* (16) reported that in mice, CD4⁺ cells could convert to Treg cells when Foxp3 was introduced via a retroviral vector. CD4⁺CD25⁺ T cells displayed no immune regulatory function in Foxp3-deficient mice (16). Chauhan *et al* (17) reported that Foxp3 expression determined the regulatory ability of Treg cells and Foxp3 overexpression could lead to a low immune activity status in the body, which illustrated that Foxp3 was the central regulator of Treg cell activity.

Circulating tumor cells (CTCs) are a type of tumor cell that enters the peripheral blood circulation from the primary tumor or metastasis (18). Over the course of a malignancy, tumors may spread from the local site to the blood or lymph circulation. The clinical relevance of CTCs and metastasis has been confirmed in metastatic breast cancer, colorectal cancer and prostate cancer (19). There are numerous reports on the correlation between non-small-cell lung cancer (NSCLC) metastasis and CTCs (18,20). Additionally, the CTCs in NSCLC metastasis were reported to cause immune responses, including both proinflammatory and anti-inflammatory regulation (21,22). However, the molecular mechanism of CD4⁺CD25⁺ Treg cell development, maturation and function in NSCLC development remains unclear.

Duan *et al* (23) reported that NSCLC blood CD4⁺CD25⁺ Treg cells could not inhibit proliferation of reactive T cells activated by auto-antigens. Thus, the authors proposed that functional maturation of human CD4⁺CD25⁺ Treg cells occurred during metastasis (23). Li *et al* (24) reported that NSCLC blood CD4⁺CD25⁺ Treg cells cultured with anti-CD3/CD28 mAb *in vitro* could suppress 95% of allogeneic mixed lymphocyte reaction and overexpress Foxp3 protein. Furthermore, the authors indicated that NSCLC blood Treg cells following treatment have stronger immune suppression ability compared with normal blood Treg cells. Transforming growth factor β 1 (TGF- β 1) serves an important role in the development and maturation of T cells (4). However, the role of TGF- β 1 in proliferation of CD4⁺CD25⁺ Treg cells and Foxp3 expression regulation remains unclear.

To analyze the differences between NSCLC and normal peripheral blood, the immune suppressive ability of CD4⁺CD25⁺ Treg cells, the number of CD4⁺CD25^{high} Treg cells and Foxp3 expression were measured. In addition, due to low immunogenicity and strong regeneration ability, TGF- β 1 was used to produce iTreg cells with tumor inhibitive functions in the process of NSCLC blood mononuclear cell (BMC) culturing. Furthermore, protein and mRNA expression of Foxp3 were monitored by flow cytometry and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay. To explore the role of TGF- β 1 in NSCLC blood iTreg cells, proliferative response of iTreg cells induced by TGF- β 1 was analyzed by carboxyfluorescein succinimidyl ester (CFSE) staining. The present study aimed to provide a potential new route for clinical immunotherapy during NSCLC blood metastasis.

Materials and methods

The present study was approved by the Medical Ethics Committee of Jiaying No.1 Hospital (approval no. JY201513R; Jiaying, China). A total of 231 patients (age, 67 \pm 15 years old; 65% male) who underwent clinical treatment in Jiaying No. 1

Hospital were enrolled from January 2016 to December 2017. All patients provided written informed consent. The procedure of the study strictly followed the Declaration of Helsinki. Blood samples were collected according to normal clinical practice.

Preparation of NSCLC BMCs and normal peripheral BMCs (PBMCs). NSCLC and normal peripheral blood were collected, restored in heparin anticoagulation (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) and diluted with an equal amount of Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The blood was treated with lymphocyte separation liquid (Beyotime Institute of Biotechnology, Haimen, China). Mononuclear cells were isolated by density gradient centrifugation (400 x g, 20 min, 20°C), washed twice with serum-free RPMI-1640 (400 x g, 10 min, 4°C, followed by 300 x g, 10 min, 4°C) and adjusted to 1-2x10⁶ cells/ml with RPMI 1640 containing 10% human autologous serum (Gibco; Thermo Fisher Scientific, Inc.) and 50 ml/l *N*-bromosuccinimide (NBS; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany).

NSCLC BMC labeling by CFSE. Cells were analyzed using a flow cytometer. Briefly, CFSE (Molecular Probes; Thermo Fisher Scientific, Inc.) was dissolved in dimethyl sulfoxide (2 mmol/l) and stored at -80°C following aliquoting. Serum-free RPMI-1640 was used to adjust BMCs to 1x10⁷ cells/ml. Fluorescence intensity of the parent generation cells labeled by CFSE (FL1) at 10⁴-10⁶ cells per 200 μ l well was determined in a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). CFSE stock solution was added to the cell suspension to achieve a final concentration of 1.6 μ mol/l. Following staining at 37°C for 10 min, the cells were washed three times with RPMI-1640 containing 10% human autologous serum and 100 ml/l NBS to remove excess dye and adjusted to 1x10⁶ cells/ml with RPMI 1640 containing 10% human autologous serum and 50 ml/l NBS. The cells were assessed using a flow cytometer and evaluated through an auto-analyze method using ModFit software (version 4.1; Verity Software House, Topsham, ME, USA).

T cell culturing and proliferation. A total of 10⁴-10⁶ BMCs were added to 24-well cell culture plates at 1 ml per well and incubated with anti-CD3 monoclonal antibodies (3 μ g/ml; cat. no. ab11089; Abcam, Cambridge, MA, USA) and IL-2 (100 U/ml; PeproTech, Inc., Rocky Hill, NJ, USA). Experimental groups were defined by the addition of varying concentrations (1, 5 and 25 ng/ml) of recombinant human TGF- β 1 (PeproTech, Inc.). Cells were cultured at 37°C, 5% CO₂ and harvested on day 4. Experiments were performed in triplicate.

Proliferation of T cell subsets analyzed by flow cytometry. Flow cytometry was performed according to a previous study (19). T cell subsets were collected. The following antibodies were applied to stain for CD3, CD4, CD8 on the cell surface with different colored fluorophores, following previous reports (23): Anti-CD3-PE/Cy5.5[®] (cat. no. ab190285) and anti-CD8-PE/Cy7[®] (cat. no. ab39853) or anti-CD3-APC (1:5,000; cat. no. 17-0036-42; Invitrogen;

Thermo Fisher Scientific, Inc), anti-CD4-PerCP/Cy5.5 (cat. no. ab210324) and anti-CD25-PE/Cy7 (cat. no. ab210335; all 1:5,000; Abcam). Proliferation of T cell subsets was analyzed by flow cytometry. CFSE was considered as the first fluorescence (FL1), PE-labeled antibody as the second fluorescence (FL2), PeCy5.5 as the third fluorescence (FL3), APC as the fourth fluorescence (FL4). Flow cytometry was assessed using FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA). Cell Quest software (version 5.1; BD Biosciences) was used for analyzing the data. The cell proliferation ability and the proportions of CD4⁺CD25⁺ and CD8⁺ T cells were calculated with the Cell Quest software.

Analysis of Foxp3 expression in CD4⁺CD25⁺ Treg cells by flow cytometry. Following the collection of 3x10⁵ BMCs and PBMCs cultured at different TGF- β 1 concentration (~300 μ l/tube), cells were washed with eBioscience™ Flow Cytometry Staining Buffer (Invitrogen; Thermo Fisher Scientific, Inc.) and centrifuged at 300 x g for 5 min at 4°C. The supernatant was discarded; cells were re-suspended in PBS and incubated with anti-human CD3-APC, anti-human CD25-PE/Cy7 and anti-human CD4-PerCP/Cy5.5 antibodies for cell surface staining at 4°C for 30 min. Cells were then washed twice with staining buffer, re-suspended, centrifuged at 300 x g for 5 min at 4°C and fixed with paraformaldehyde (PFA; cat. no. A500684; 20 g/l; ~500 μ l/tube; Sangon Biotech Co., Ltd., Shanghai, China) at 4°C for 30 min. The supernatant was discarded after centrifugation (300 x g for 5 min at 4°C) and the cells were washed twice with staining buffer. The cells were incubated with a permeable membrane buffer, Fixation/Permeabilization Solution kit with BD GolgiStop™ (BD Biosciences) at 4°C for 15 min. Cells were centrifuged at 400 x g for 15 min at 4°C and the supernatant was discarded. Staining was performed with anti-human Foxp3-fluorescein isothiocyanate (FITC; 5 μ l (0.5 μ g)/test; cat. no. 11-4776-41; Invitrogen; Thermo Fisher Scientific, Inc.) at 4°C for 30 min. Cells were washed with permeable membrane buffer and with staining buffer. PFA (10 g/l; ~300 μ l/tube) was added and Foxp3 expression in CD4⁺CD25⁺ Treg cells was analyzed by flow cytometry. The control group was prepared without intracellular Foxp3 stain and mouse Immunoglobulin G1-FITC (5 μ l (0.5 μ g)/test; cat. no. 11-4015-80; Invitrogen; Thermo Fisher Scientific, Inc.) intracellular staining was used as isotype control. Anti-CD3-FITC (cat. no. ab34275; Abcam), anti-CD4-PE (cat. no. ab134354; Abcam), anti-CD3-PE/Cy5.5 or anti-CD3-APC antibodies were used for quality control of the flow cytometer prior to the experiments, as recommended by a previous report (23).

mRNA expression of Foxp3 is detected by RT-qPCR. Total RNA from NSCLC BMCs and normal peripheral BMCs was isolated using TRIzol® reagent (Invitrogen) according to the manufacturer's protocol. NanoDrop-1000 (Thermo Fisher Scientific, Inc.) was used to determine the concentration and purity of total RNA. A GoScript™ reverse transcription kit (Promega, Madison, WI, USA) was used to synthesize the first strand of cDNA with 1 μ g total RNA. RT-qPCR assay was used to detect the mRNA expression of transcription factor Foxp3 (forward primer: 5'-CTGGCGAAGGGCTCG GTAGTCCT-3', reverse primer: 5'-CTCCCAGAGCCCATG

GCAGAAAGT-3') using a PowerUp™ SYBR® Green kit (cat. no. A25778; Thermo Fisher Scientific, Inc). GAPDH (cat. no. B661104-0001; Sangon Biotech Co., Ltd.) mRNA served as a loading controls (forward primer: 5'-AATGCATCCTGC ACCACCAA-3' and reverse primer: 5'-GTAGCCATATTC ATTGTCATA-3'). The thermocycling protocol was performed as previously described (25).

Statistical analysis. SPSS (version 22.0; IBM Corp., Armonk, NY, USA) statistical software was used for data processing. Data are presented as the mean \pm standard deviation. Student's t-test was applied to compare the CD4⁺ T cell subpopulation ratio and Foxp3 expression in NSCLC blood and normal peripheral blood. A one-way analysis of variance was performed, followed by a Tukey's post hoc test, to analyze the effects of different concentrations of TGF- β 1 on Foxp3 expression in CD4⁺CD25⁺ T cells. P<0.05 was considered to indicate a statistically significant difference.

Results

Proportion of CD4⁺CD25^{high} Treg cells in NSCLC blood is significantly higher compared with normal peripheral blood. The present study demonstrated that CD25⁺ and CD25⁻ cells could form two different subsets with different proportions in NSCLC blood CD4⁺ T cells (Fig. 1A). No boundary between CD25⁺ and CD25⁻ cells in normal peripheral blood was observed, displaying a transitional distribution. CD25⁺ T cells may be divided into high expression (CD25^{high}) and low/moderate expression (CD25^{int}), according to the expression intensity of CD25 analyzed by flow cytometry. In general, CD4⁺CD25^{high} T cells are regarded as Treg cells (24). In the current study, the data demonstrated that the proportion of CD4⁺CD25^{high} T cells in CD4⁺ T cells in NSCLC blood (6.89 \pm 1.78%) was significantly increased compared with normal peripheral blood (2.75 \pm 1.34%; P<0.05; Fig. 1A).

Foxp3 expression in CD4⁺ T cells in NSCLC blood is significantly decreased compared with normal peripheral blood. To detect the Foxp3 expression in CD4⁺CD25^{high} Treg cells, flow cytometry was used. Data revealed that the amount of CD4⁺CD25^{high} Foxp3⁺ Treg cells and CD4⁺CD25^{int} T cells in NSCLC blood (23.21 \pm 3.56 and 9.14 \pm 2.89%, respectively) was significantly lower compared with normal peripheral blood (72.3 \pm 7.89 and 17.11 \pm 4.28%, respectively; both P<0.05; Figs. 1B). The expression of Foxp3 in CD4⁺CD25^{high} Treg cells in NSCLC blood (32.11 \pm 3.21%) was significantly lower compared with normal peripheral blood (68.02 \pm 5.59%; P<0.05; Fig. 1C). These results indicate that CD4⁺CD25^{high} Treg cells in NSCLC blood were functionally immature.

Proliferation ability of CD4⁺CD25⁺ T cells and CD8⁺ T cells is significantly enhanced following TGF- β 1 treatment. To explore changes to the proliferative dynamics of T cells treated with TGF- β 1, the proliferation changes of CD4⁺CD25⁺ T cells and CD8⁺ T cells following TGF- β 1 treatment were measured by flow cytometry. Results demonstrated that on day 4 following treatment, the first four generations [from parental cells (G0) to the 4th generation (G4)] of CD4⁺CD25⁺ Treg cells accounted for 73.77% (treated with 5 ng/ml TGF- β 1) and

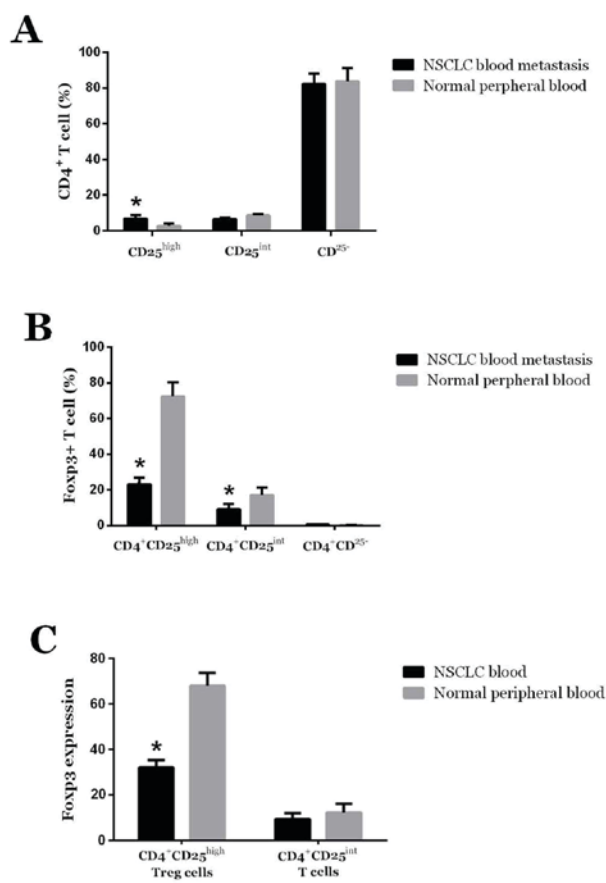


Figure 1. CD4⁺ T cell subsets and Foxp3⁺ T cell subsets in NSCLC blood and normal peripheral blood. (A) Percentage of CD4⁺ T cell subsets in NSCLC blood and normal peripheral blood; (B) Percentage of Foxp3⁺ T cell subsets in NSCLC blood and normal peripheral blood. (C) Relative Foxp3 expression in CD4⁺CD25^{high} Treg and CD4⁺CD25^{int} T cells in NSCLC blood and normal peripheral blood by flow cytometry *P<0.05 vs. normal peripheral blood. Foxp3, forkhead box protein 3; NSCLC, non-small cell lung cancer; CD, cluster of differentiation; CD25^{high}, CD25⁺ cells with high expression; CD25^{int}, CD25⁺ cells with low/moderate expression; Treg, T regulatory.

83.70% (untreated), while the proportion G5-6 accounted for 25.28% (treated) and 13.70% (untreated) of all CD4⁺CD25⁺ Treg cells at that time point (Fig. 2A and B). For CD8⁺ T cells, G0-4 accounted for 94.18% (treated) and 95.62% (untreated), and the G5-6 accounted for 5.70% (treated) and 4.83% (untreated) on day 4 of all CD8⁺ T cells at that time point (Fig. 2A and B). On day 6, the amount of CD4⁺CD25⁺ Treg cells from G0-4 was 18.65% (treated) and 45.01% (untreated) of all CD4⁺CD25⁺ Treg cells at that time point, and for G5-8, the proportion of CD4⁺CD25⁺ Treg cells was determined to be 81.29% (treated) and 55.20% (untreated) (Fig. 2C and D). For CD8⁺ T cells on day 6, the proportion from G0-4 were 55.92% (treated) and 77.73% (untreated), while the proportion for G5-8 were 44.04% (treated) and 23.20% (untreated), respectively (Fig. 2C and D). On day 8, CD4⁺CD25⁺ Treg cells for G0-4 accounted for 8.54% (treated) and 9.54% (untreated), and for G8-10 accounted for 54.35% (treated) and 34.79% (untreated) of all CD4⁺CD25⁺ Treg cells at that time point (Fig. 2E and F). For CD8⁺ T cells, the proportion for G0-4 was 42.43% (treated) and 66.20% (untreated), while the proportion for G8-10 and 13.86% (treated) and 7.46% (untreated) were determined, respectively (Fig. 2E and F). Blue and orange

arrows reveal increased proliferation (or proliferation trend) of CD4⁺CD25⁺ Treg and CD8⁺ T cells. The arrows shift from the prior generations to later generations in Fig. 2A, C and E. In addition, proportions of CD4⁺CD25⁺ Treg and CD8⁺ T cells were significantly increased following treatment with TGF- β 1 on G6-8 at day 6 and G8-9 at day 8 (Fig. 2C and E) compared with untreated cells (Fig. 2D and F; Student's t-test; all P<0.05). In summary, TGF- β 1 treatment enhanced the proliferation ability of CD4⁺CD25⁺ Treg and CD8⁺ T cells.

Foxp3 mRNA expression in CD4⁺CD25⁺ T cells treated with TGF- β 1. To examine the effect of TGF- β 1 on Foxp3 expression, varying concentrations of TGF- β 1 were used to treat CD4⁺CD25⁺ T cells and Foxp3 mRNA expression was detected by RT-qPCR. Data indicated that Foxp3 mRNA expression was significantly increased in cells treated with TGF- β 1 (5 ng/ml) compared with the control group on day 4 and 6 of culturing (P<0.05; Fig. 3A). In addition, Foxp3 mRNA expression was examined in TGF- β 1 treated CD4⁺, CD4⁺CD25⁺, CD4⁺CD25^{high} CD3⁺ T cells, CD4⁺CD25⁺ and CD4⁺CD25^{high} Foxp3⁺ T cells. The results indicated that Foxp3 mRNA expression in CD4⁺CD25⁺ and CD4⁺CD25^{high} T cells was not significantly different in CD3⁺ T cells following TGF- β 1 treatment, while Foxp3 mRNA was significantly upregulated in CD4⁺CD25^{high} Foxp3⁺ T cells compared with CD4⁺CD25⁺ Foxp3⁺ T cells (P<0.05; Fig. 3B), suggesting that these cells may have transformed into CD4⁺CD25⁺ Treg cells.

Proportion of CD4⁺CD25^{high} Foxp3⁺ T cells is significantly increased following TGF- β 1 treatment. The effect of TGF- β 1 treatment on the induction of CD4⁺CD25⁺ T cell proliferation and Foxp3 expression regulation was determined by analyzing the proportions of CD4⁺CD25⁺, CD4⁺CD25^{high} and CD4⁺CD25^{high} Foxp3⁺ T cells by flow cytometry following TGF- β 1 treatment at varying concentrations. Data revealed that on day 4 following TGF- β 1 treatment, the proportion of CD4⁺CD25⁺ and CD4⁺CD25^{high} T cells was not significantly different among the different TGF- β 1-treated groups (Fig. 4A). However, the proportion of CD4⁺CD25^{high} Foxp3⁺ T cells was significantly increased in the treatment groups compared with control group (all P<0.05; Fig. 4A and B). Foxp3 expression in CD4⁺CD25^{high} T cells treated with 1, 5 and 25 ng/ml TGF- β 1 was 69.24±4.91%, 81.99±5.29% and 72.34±3.41%, respectively; it was significantly increased compared with the control group (42.38±2.13%; P<0.05; Fig. 4C and D). It was demonstrated that CD4⁺CD25⁺ T cells displayed negligible Foxp3 expression (data not shown).

Discussion

CD4⁺CD25⁺ Treg cells are a subset of T cells serving a central role in maintaining the body's peripheral immune tolerance, including tumor surveillance progress (6-8). Previous reports have demonstrated that abnormal numbers of CD4⁺CD25⁺ Treg cells or CD4⁺CD25⁺ Treg cell dysfunction are closely associated with autoimmune diseases, cancer, infectious diseases, organ transplant rejection, allergic diseases and other diseases (25,26). CD4⁺CD25⁺ Treg cells overexpress the IL-2 receptor α chain, the cytotoxic T lymphocyte associated antigen-4, the IL-2 receptor β chain, neuropilin-1, the

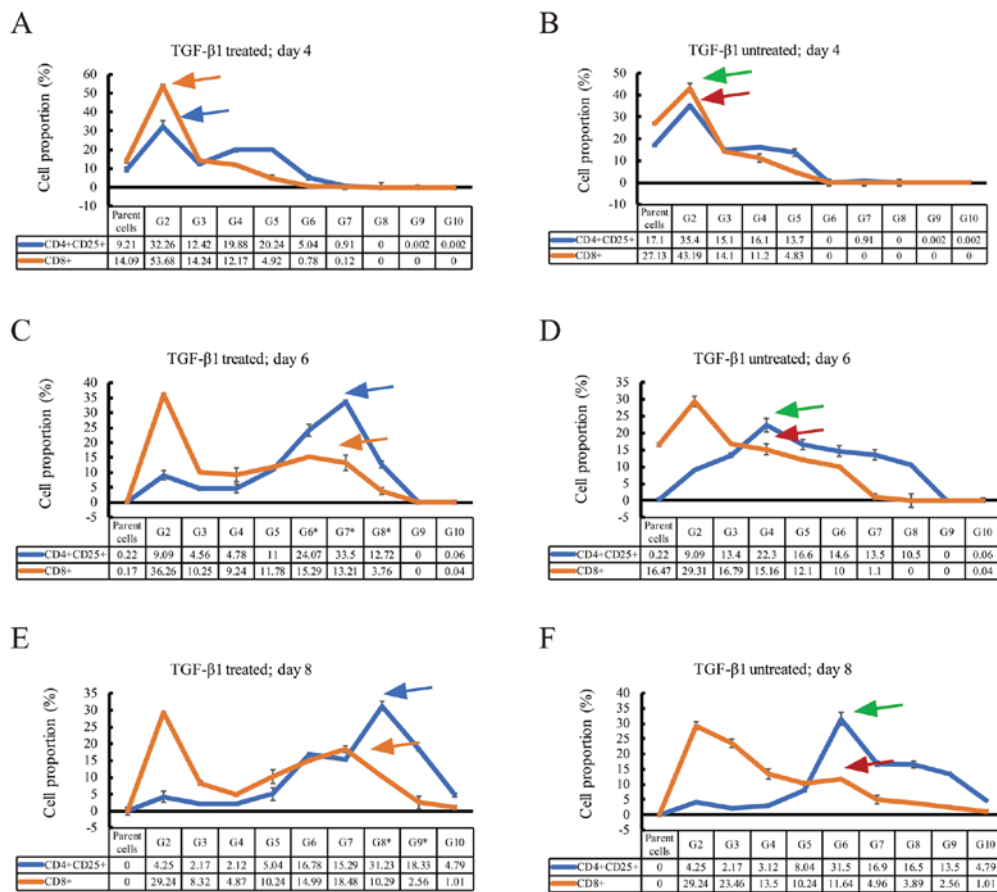


Figure 2. Proportion of CD4⁺CD25⁺ T cells and CD8⁺ T cells at given generations following TGF-β1 (5 ng/ml) induction at different time points. (A) TGF-β1 treated and (B) untreated groups at day 4. (C) TGF-β1 treated and (D) untreated groups at day 6. (E) TGF-β1 treated and (F) untreated groups at day 8. Blue and orange arrows represent the peaks or peaks of trends of the proliferation curves in the TGF-β1 treated groups, while green and red arrows represent this in the TGF-β1 untreated groups; the predominant generation is indicated in each group. *P<0.05 vs. untreated of the corresponding generation on each day. TGF-β1, transforming growth factor β1; CD, cluster of differentiation; G, generation.

glucocorticoid-induced tumor necrosis factor receptor, integrin αE, the chemokine receptor (CCR)4 and CCR8 (27,28). However, these molecules are all expressed on activated CD4⁺ effector T cells, with a lack of specificity. In addition to these molecules, Guo *et al* (29) reported increased Foxp3 expression on CD4⁺CD25⁺ Treg cells, but decreased expression on CD8⁺ T and CD4⁺CD25⁻ T cells. Although the proposal that Foxp3 is a specific marker for Treg cells remains controversial, numerous studies have confirmed that Foxp3 serves a crucial role in CD4⁺CD25⁺ T cell development and its immune suppression (30). In the current study, the results indicated high Foxp3 expression in CD25^{high} T cells, low expression in CD25^{int} T cells and further decreased expression in CD25⁻ T cells. Therefore, it was postulated that CD25^{high} T cells may be leading contributors in the immune regulation of the CD4⁺ nTreg cell cluster.

Additionally, the data revealed that CD25 positive and negative cells formed two distinct groups in NSCLC blood CD4⁺ T cell clusters, while CD25 positive and negative cells formed a continuous cell population in normal peripheral blood CD4⁺ T cell clusters. Furthermore, it was demonstrated that NSCLC blood CD4⁺CD25^{high} T cells have clear distinctions from CD4⁺CD25^{int} T cells. The number of CD4⁺CD25^{high} T cells was significantly increased in NSCLC blood compared with normal peripheral blood (6 vs. 3%), which is consistent

with previous reports (31). The level of intracellular Foxp3 expression in NSCLC blood CD4⁺CD25^{high} T cells was significantly decreased compared with normal peripheral blood CD4⁺CD25^{high} T cells. This phenomenon suggests that CD4⁺CD25^{high} T cells in NSCLC blood were rarely stimulated by antigens, therefore only a small number of CD4⁺CD25^{high} T cells became mature. Therefore, it is considered that Foxp3 may be a specific marker of CD4⁺ Treg cell functional maturation, where low expression of Foxp3 in NSCLC blood CD4⁺CD25^{high} Treg cells suggests its immaturity.

CD4⁺CD25⁺ Treg cells can be divided into two categories, nTreg and iTreg cells. iTreg cells used as specific immunotherapy have become a hotpot in immunologists (32). Either immature dendritic cells (33) or drugs (34) have been used to induce Treg cells *in vitro*. Baecher-Allan *et al* (35) established that CD4⁺CD25⁻ T cells could be converted to CD4⁺CD25⁺ Treg cells with low suppressive ability, when dexamethasone and IL-7 were added to purified CD4⁺CD25⁻ T cells. Furthermore, CD4⁺CD25⁺ Treg cells may be induced by IL-10, IL-2, TGF-β and other cytokines (36). In the current study, the number, proliferation effect and Foxp3 expression of NSCLC blood CD4⁺CD25⁺ Treg cells were detected following TGF-β1 treatment. The results demonstrated that the proportion of CD4⁺ and CD4⁺CD25⁺ T cells was increased in TGF-β1 treated cells compared with control groups. Foxp3 protein and

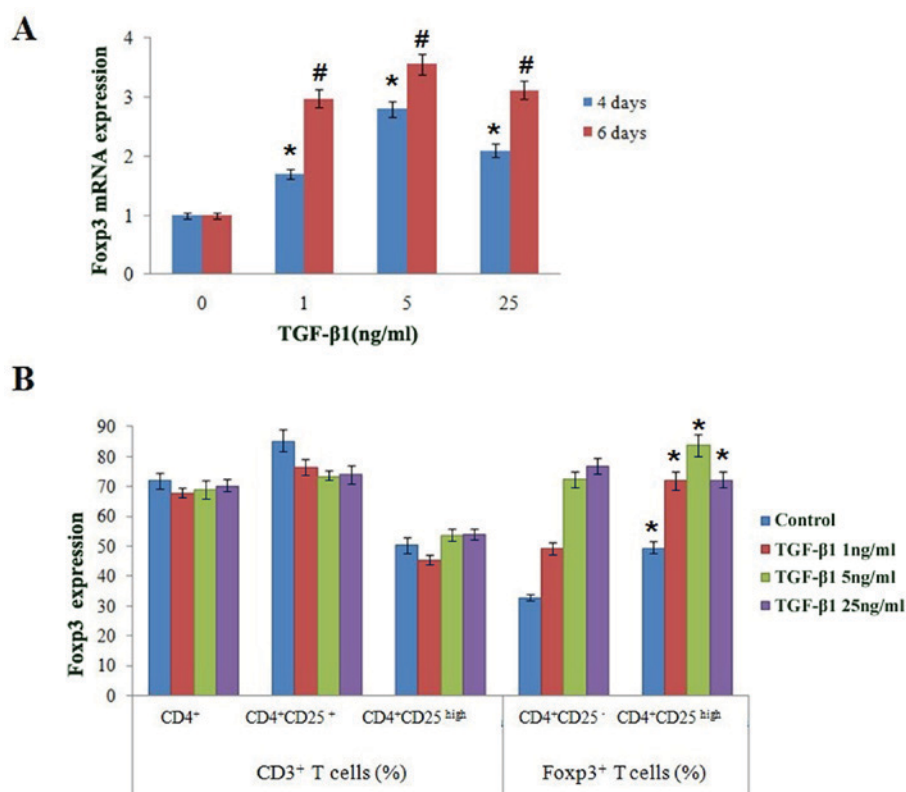


Figure 3. Foxp3 expression in CD4⁺CD25⁺ T cells induced by TGF- β 1. (A) Foxp3 expression in CD4⁺CD25⁺ T cells treated with 1, 5 and 25 ng/ml TGF- β 1 for 4 days and 6 days determined using reverse transcription-quantitative polymerase chain reaction assays. *P<0.05 vs. untreated control on day 4; #P<0.05 vs. untreated control on day 6. (B) Foxp3 expression in CD3⁺ T cells and Foxp3⁺ T cells on day 4 following TGF- β 1 treatment (1, 5 and 25 ng/ml) compared with an untreated control. *P<0.05 vs. corresponding groups in CD4⁺CD25⁺ cells. Foxp3, forkhead box protein 3; TGF- β 1, transforming growth factor β 1; CD, cluster of differentiation; CD4⁺CD25^{high}, CD4⁺CD25⁺ cells with high CD25 expression.

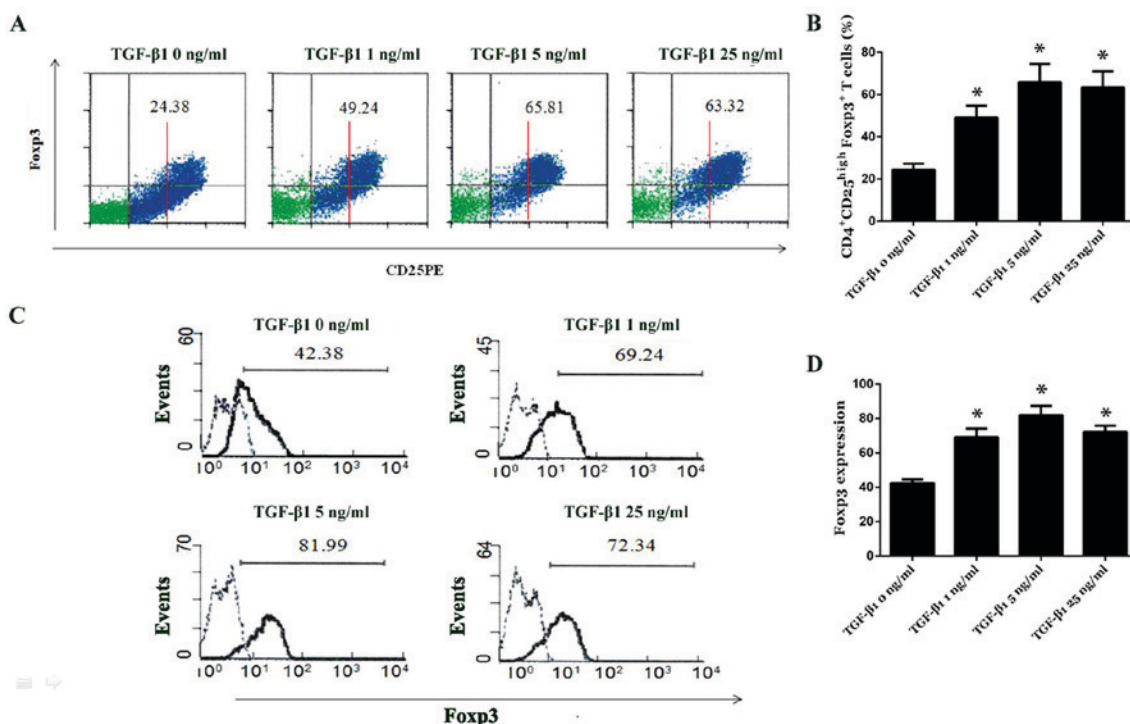


Figure 4. Foxp3 expression in CD4⁺CD25⁺ T cells and CD4⁺CD25^{high} T cells treated with varying amounts of TGF- β 1 detected by flow cytometry. (A) Representative images and (B) quantified results of Foxp3⁺CD4⁺CD25^{high} T cells following TGF- β 1 treatment (1, 5 and 25 ng/ml) compared with an untreated control on day 4. Cells beyond the red line were considered CD25^{high}. (C) Representative images and (D) quantified results of Foxp3 expression in CD4⁺CD25^{high} T cells following TGF- β 1 treatment (1, 5 and 25 ng/ml) compared with an untreated control on day 4. *P<0.05 vs. untreated control. Foxp3, forkhead box protein 3; CD, cluster of differentiation; CD25PE, cells expressing CD25 marker; CD4⁺CD25^{high}, CD4⁺CD25⁺ cells with high CD25 expression; TGF- β 1, transforming growth factor β 1.

mRNA expression was significantly increased in the control group in NSCLC blood CD4⁺CD25⁺T cells following TGF-β1 treatment, suggesting that TGF-β1 may promote proliferation of CD4⁺ Foxp3⁺ Treg cells. The proliferation of CD4⁺CD25⁺ Treg cells induced by TGF-β1 surpassed that of CD8⁺ T cells. TGF-β1 inducing CD4⁺CD25^{high} Treg cells has been reported previously (37,38). Fu *et al* (37) reported that TGF-β1 induced Foxp3⁺ Treg cells from CD4⁺CD25⁻ precursors during mice transplantation. Ethan *et al* (38) reviewed studies of TGF-β1 induced Foxp3⁺ Treg cells in both mouse and human. However, the molecular mechanism underlying the upregulation of Foxp3 expression induced by TGF-β1 in CD4⁺CD25⁺ T cells requires further exploration.

Immune regulation in tumor metastasis demands a complex balance (39). Enhancing or suppressing immunity may cause totally different effects. This preliminary study indicates that NSCLC blood CD4⁺CD25^{high} Treg cells may be functionally immature. The induction of the immune naive precursor cells in NSCLC blood by Treg cells with strong immunosuppressive ability may provide a new basis for the prognosis and treatment of NSCLC blood metastasis in the future.

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Availability of data and materials

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Authors' contributions

YH analyzed and interpreted the patient data. LS and HZ performed the experiments, including the flow cytometry. WQ and BZ were responsible for the patient sample collection. YH performed the statistical analysis and drafted the manuscript. ZY designed this study and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All patients provided written informed consent. The present study was approved by the Medical Ethics Committee of the Jiaying No. 1 Hospital (Zhejiang, China; approval no. JY201513R), and experiments were performed in accordance with institutional guidelines and strictly followed the Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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