SPANDIDOS PUBLICATIONS

ONCOLOGY REPORTS 28: 615-621, 2012

TGF-β of lung cancer microenvironment upregulates B7H1 and GITRL expression in dendritic cells and is associated with regulatory T cell generation

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Received February 9, 2012; Accepted April 11, 2012

DOI: 10.3892/or.2012.1822

Abstract. The effects of TGF-β on dendritic cells (DCs) on the tumor microenvironment are not well understood. We report, here, the establishment of an *in vitro* lung cancer microenvironment by co-incubation of seminaphtharhodafluor (SNARF) labeled Lewis lung cancer (LLC) cells, carboxyfluorescein succinimidyl ester (CFSE) labeled fibroblasts and 4-chloromethyl-7-hydroxycoumarin (CMHC) labeled DCs. Raw 264.7, EL4 and NCI-H446 cells were able to synthesize TGF-β which was determined by flow cytometry and western blotting, respectively. Furthermore, TGF-β efficiently increased regulatory T-cell (Treg) expansion and upregulated DC B7H1 and GITRL expression. TGF-β and the co-incubation of LLC cells, fibroblasts with DCs could augment the expression of B7H1 and GITRL molecules of DCs. The data presented here indicate that the B7H1 and GITRL molecules may play an important role in TGF-β-induced Treg expansion of lung cancer microenvironment.

Introduction

Tumor microenvironment is recognized as the product of a developing crosstalk between different cells types. In addition to tumor cells, tumor microenvironment is comprised of immune cells, fibroblasts, stromal cells and extracellular matrix (1). Fibroblasts within the tumor stroma have been termed tumor-associated fibroblasts (TAFs) (2,3) and play important roles in the initiation, progression, and metastasis of cancer (4).

Dendritic cells (DCs) were able to recognize tumor antigens and activate tumor-specific T-cell response (5). However, this response does not usually occur in most types of human cancer or in animal models, indicating that the dysfunction or immuno-suppression of host immune system might be the main mechanisms by which tumors escape from host immune control (6-8). Previously, we co-incubated DCs with different tumor cells and found that co-incubation of tumor cell-DCs down-regulated the abilities of DCs in T cell proliferation, IL-12 secretion and tumor antigenic-specific CTL priming (9). Also, Yuan *et al* established an *in vitro* incubation system including gastric cancer cell line, with sorting $CD4^+$ T cells and found that gastric cancer cells induced human $CD4^+Foxp3^+$ regulatory T cells (Tregs) through the production of transforming growth factor β1 (TGF-β1) (10). All these co-incubation systems only included relative tumor immune cells to observe the direct effect of tumor cells on immune cells. Although a novel 3-dimensional (3D) culturing system was employed to recapitulate stromal and extracellular matrix interactions (11), little is known about the effects of tumor cells and TAFs on immune cells. Therefore, an *in vitro* tumor micro-environmental model established of tumor cells, fibroblasts and immune cells is urgently needed to explore the effects of tumor cells and TAFs on DCs.

$Foxp3^+$ Tregs, including thymic-derived natural Treg and conventional T-derived adaptive Treg cells, are proposed to play important roles in tumor associated immunosuppression. However, the mechanisms of Treg expansion in tumor microenvironment remain unclear. Programmed cell death 1 ligand 1 (PD-L1) known as B7 homolog 1 (B7H1) and glucocorticoid-induced TNFR-related protein ligand (GITRL), expressing DCs, were reported to be candidate immune modulation molecules (12-15). Yi *et al* reported that host APCs augment *in vivo* expansion of donor natural regulatory T cells via B7H1/B7.1 in allogeneic recipients (12), which was further confirmed by high expression of B7H1 molecules on keratinocytes suppressing xeno- and allo-reactions by inducing Tregs (13). Although B7H1 had the characteristics of co-inhibitor molecules of modulating inflammatory response, the role of B7H1 in tumor associated

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Abbreviations: CMHC, 4-chloromethyl-7-hydroxycoumarin; CFSE, carboxyfluorescein succinimidyl ester; LLC, Lewis lung cancer; SNARF, seminaphtharhodafluor; GITRL, glucocorticoid-induced TNFR-related protein ligand; B7H1, B7 homolog 1; PD-L1, programmed cell death 1 ligand 1; TGF-β, transforming growth factor β

Key words: regulatory T cells, dendritic cells, B7H1, GITRL, tumor microenvironment

immuno-suppression is still to be determined. GITRL-dependent expansion of Tregs underlying immune privilege in corneal allograft was recently described (14), but blockade of GITR-GITRL interaction was necessary for maintaining Treg function and prolonging allograft survival was also reported (15). So, the exact functions of GITRL in inducing tumor associated Treg are still to be clarified.

In the present study, we firstly characterized that TGF-β were synthesized by macrophage cell line (Raw 264.7), lymphoma cell line (EL4) and small cell lung cancer cell line (NCI-H446) by intracellular flow cytometry analysis and Western blotting respectively, and further confirmed that TGF-β could efficiently induce Treg generation *in vitro*; secondly, TGF-β treatment clearly up-regulated B7H1 and GITRL expressions of DCs; thirdly and importantly, a tumor microenvironment co-incubation system was established by co-incubation of semi-naphtharhodafluor (SNARF) labeled Lewis lung cancer (LLC) cells, carboxyfluorescein succinimidyl ester (CFSE) labeled fibroblasts and 4-chloromethyl-7-hydroxycoumarin (CMHC) labeled DCs; fourthly and consistent with TGF-β treatment, the co-incubation of LLC cells, fibroblasts with DCs could augment the expressions of B7H1 and GITRL molecules of DCs. The data presented here indicate that B7H1 and GITRL molecules might play an important role in TGF-β-induced Treg expansion of lung cancer microenvironment.

Materials and methods

Reagents. Mouse recombinant GM-CSF, TGF-β and IL-4 were obtained from R&D (Minneapolis, MN, USA). Fluorescence conjugated B7H1, GITRL, CD4, CD25, Foxp3 antibodies, CD16/CD32 antibody and isotype control were from eBioscience (San Diego, CA, USA). Murine TGF-β and secondary HRP-conjugated goat anti-mouse antibody were bought from Santa Cruz Biotechnology. Murine anti-CD3, anti-CD28, anti-IL-4, anti-IFN-γ, IL-2, PMA, ionomycin were obtained from eBioscience. RPMI-1640 medium, Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from Hyclone (Logan, UT, USA). Murine CD11c and CD4 beads were from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany). CellTracker Blue CMHC, Carboxy SNARF-1, CellTrace CFSE were obtained from Molecular Probes (Eugene, USA).

Animals. Pathogen-free Balb/c mice (female, 6 to 8-week-old, or newborn) were bought from Shanghai Laboratory Animal Center of Chinese Academy of Sciences (China) and kept at the Animal Center of Xiamen University. All animal studies were approved by the Review Board, Medical College, Xiamen University.

Cell lines. LLC cell line, established from the lung of a mouse bearing tumor resulting from implantation of primary Lewis lung carcinoma, was obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (ATCC CRL-1642). LLC cells, NCI-H446 were cultured in DMEM medium (Dulbecco's modified Eagle's medium, 4 mM L-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10% FBS). Raw 264.7 and EL4 cells were cultured in RPMI-1640 medium with 10% FBS.

Fibroblasts separation from murine skin. Fibroblasts were separated from murine skin as previously described (16). Briefly, skin was firstly taken from newborn Balb/c mice and cut to 1 mm^2 size with sterile scissors. Then, tissues were explanted into culture dishes in equil amounts with DMEM (including 10% FBS) medium. Thirdly, the purification of fibroblasts was completed by digesting cells with trypsin and re-explanting cells into dishes.

Bone marrow-derived murine DCs. Bone marrow-derived DCs were prepared as previously described (17). Briefly, bone marrow mononuclear cells were prepared from bone marrow suspensions by depletion of red cells and then were cultured at a density of $1x10^6$ cells/ml in RPMI-1640 medium with 10 ng/ml of GM-CSF and 1 ng/ml of IL-4. Non-adherent cells were gently washed out on day 4 of culture; the remaining loosely adherent clusters were used as DCs.

Labeling cells for analysis with fluorescence. Cells were labeled according to the product description. Briefly, cells were re-suspended in pre-warmed PBS/0.1% BSA at a final concentration of $1x10^6$ cells/ml. Then, CellTracker Blue CMHC, Carboxy SNARF-1 or CellTrace CFSE was added at the final concentration of 10 μM, respectively, and incubated at 37˚C. For labeling DCs, the incubation time was 30, 15, 30 min for CMHC, CFSE and SNARF, respectively; for labeling fibroblasts, the incubation time was 15, 30 min for CFSE, SNARF, respectively; for labeling LLC cells, the incubation time was 30 min for SNARF. After labeling, the dyes were removed from cells with pre-warmed PBS and the cells were added with fresh, pre-warmed medium for further culture.

Co-incubation of DCs and fibroblasts. To establish the DC-fibroblast co-incubation system, $1x10^6$ cells/ml DCs were labeled with 10 μM SNARF for 30 min and fibroblasts were labeled with 10 μM CFSE for 15 min. Then, DCs and fibroblasts were removed from dishes with 0.05% trypsin and mixed at a ratio of 1:1 for further co-incubation. After 12-h co-incubation, the cells were observed and images were recorded by fluorescence microscope at the wavelength of 488 nm.

Co-incubation of LLC, DCs and fibroblasts. To establish the LLC-DC-fibroblast co-incubation system, $1x10^6$ cells/ml LLC cells, DCs and fibroblasts were labeled with SNARF for 30 min, CMHC for 30 min and CFSE for 15 min, respectively, at the final concentration of 10 μM. Then, DCs, fibroblasts and LLC cells were removed from dishes with 0.05% trypsin and mixed at a ratio of 2:2:1 for further co-incubation. After 12 h co-incubation, the cells were observed and images were recorded by fluorescence microscope at the wavelength of 488 nm.

MACS separation. DCs of $CD4^+$ T cells were separated from the LLC-fibroblast-DC co-incubation system and mouse splenocytes according to the methods previously described (18). Briefly, all cells collected from co-incubation system or splenocytes were counted and re-suspended in 400 μl buffer per 10^8 total cells. Then, 100 μl of CD11c/CD4 beads was mixed with cells and incubated for 15 min at 4-8˚C. After incubation, the cells were washed, and re-suspended in 500 μl buffer. The column was prepared by rinsing with appropriate amount of buffer and the

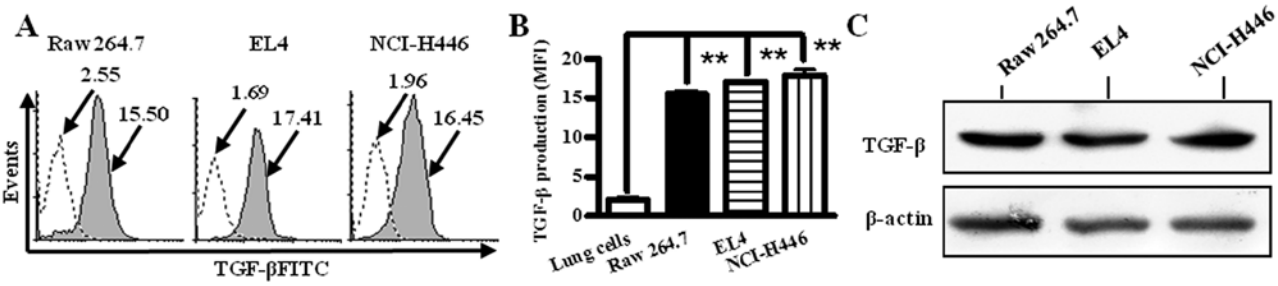

Figure 1. TGF-β is expressed in RAW 264.7, EL4 and NCI-H446 cells. RAW264.7, EL4 and NCI-H446 cells were collected from the culture medium and fixed with IC fixation buffer. After washes, the cells were labeled with TGF-β and fluorescence-conjugated secondary antibodies. Then, the cells were washed with permeabilization buffer and flow cytometry staining buffer, respectively, and data were acquired on flow cytometry. Primary murine lung cells were used as TGF-β negative control. Numbers in the histogram indicate geometric mean fluorescence of test samples. (A) Histogram presentation of TGF-β production of RAW264.7, EL4 and NCI-H446 cells. (B) Histographic presentation of MFI on expression of TGF-β. Data are given as mean ± SEM, out of 3 experiments. $^{**}p<0.001$, one-way ANOVA with post Newman-Keuls test. (C) TGF-β expression of RAW264.7, EL4 and NCI-H446 cells was assessed by Western blotting. Data are shown of representative Western blot analyses (n=3). β-actin was used as loading control.

cell suspension was applied onto the column. After three washes of column, the labeled cells were collected by removing the column from the separator and pipetting appropriate buffer and firmly applying the plunger of the column. The labeled cells were washed and the assay performed.

Flow cytometric measurement. The expressions of surface molecules on DCs were determined by flow cytometry according to the methods described previously (17). Briefly, DCs separated from the LLC-DC-fibroblast co-incubation system or DCs treated with TGF-β were incubated for 15 min at 4˚C with antibody to CD16/CD32 at a concentration of 1 μg per $1x10^6$ cells for blockade of Fc receptors. Cell staining was performed on ice for 30 min with related fluorescence conjugated antibodies and then cells were washed with ice-cold PBS, containing 0.1% NaN_3 and 0.5% BSA. Flow cytometry was done with FACSCalibur and data were analyzed with CellQuest software.

TGF-β quantification of tumor cell lines. TGF-β of tumor cell lines was determined by the method of staining intracellular antigens for flow cytometry (18). Briefly, RAW264.7, EL4 and NCI-H446 cells were collected from the culture medium and washed with PBS. Then cells were fixed with IC fixation buffer in the dark at room temperature for 20 min and further washed twice with permeabilization buffer. TGF-β antibody and secondary antibody were added to the cells and incubated in the dark at room temperature for 20 min to detect intracellular TGF-β expression. The cells were washed with permeabilization buffer and flow cytometry staining buffer, respectively, and data were acquired by flow cytometry.

Western blot analysis. For analysis of TGF-β expression of RAW264.7, EL4 and NCI-H446 cells, proteins were obtained in lysis buffer as previously described (18). Protein lysates (30 μg/ml) were electrophoresed on 12% SDS-PAGE gels, transferred to PVDF membranes, and blotted with polyclonal TGF-β antibodies, followed by anti-mouse horseradish peroxidase and detected by chemiluminescence ECL. As loading controls, antibodies against β-actin were used.

Treg expansion induced by TGF-β in vitro. The expansion of TGF-β induced by Tregs was performed according to previous description (19). Briefly, $CD4^+$ T lymphocytes were firstly separated from splenocytes by $CD4^+$ MACS beads. Then, $5x10^5$ $CD4^+$ T cells were cultured in a 24-well plate in RPMI-1640 medium in the presence of anti-CD3 (5 μg/ml), anti-CD28 (1 μg/ml), anti-IL-4 (1 μg/ml), anti-IFN-γ (1 μg/ml), IL-2 (50 U/ml), with or without TGF-β (5 ng/ml) for 3 days. PMA (50 ng/ml), ionomycin (1 μM) was used to stimulate cells for 4-6 h, then the cells were labeled with CD4, CD25 and Foxp3 antibodies. Flow cytometry was done with FACSCalibur and data were analyzed with CellQuest software.

Statistical analysis. The data are expressed as average of experimental data points, and standard error of means (SEM) were determined using the calculated standard deviation of a data set. Statistical significance was tested using Student's t-test and one-way ANOVA test by Prism software. Statistical differences were considered to be significant at $p<0.05$.

Results

TGF-β was expressed by RAW 264.7, EL4 and NCI-H446 cells. It was reported that TGF-β plays an important role in mediating tumor-associated immuno-suppression (20). To investigate TGF-β generation of tumor microenvironment, macrophage cell line (Raw 264.7), lymphoma cell line (EL4) and small cell lung cancer cell line (NCI-H446) were stained with TGF-β antibody and TGF-β production was determined by intracellular staining for flow cytometry and western blotting, respectively. The results showed that in contrast to primary lung cells control, Raw 264.7, EL4 and NCI-H446 cells have high values of geometric mean fluorescence (MFI). The MFI values of Raw 264.7, EL4 and NCI-H446 cells were 15.50, 17.41 and 16.45, respectively (Fig. 1A), which indicated that all these cells could generate TGF-β (Fig. 1B). The results of western blotting also confirmed that Raw 264.7, EL4 and NCI-H446 cells produced TGF-β (Fig. 1C).

TGF-β is able to efficiently induce Treg generation in vitro. Tregs play an important role in tumor-associated immunosuppression (20-23). As TGF-β could be produced by Raw 264.7, EL4 and NCI-H446 cells (Fig. 1), $CD4^+$ T cells separated from splenocytes by $CD4^+$ MACS beads were treated with

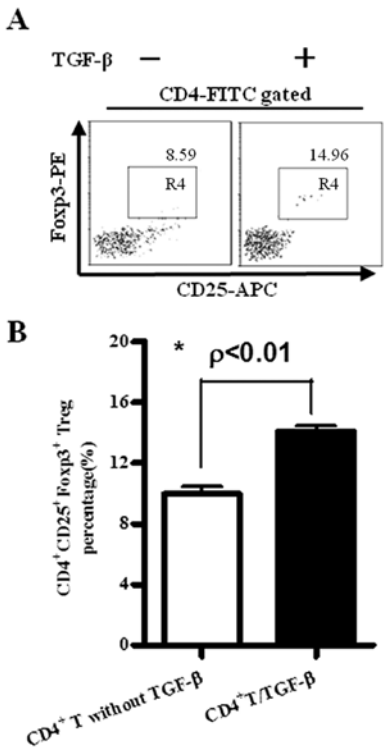

Figure 2. TGF-β efficiently induces Treg expansion *in vitro*. $CD4^+$ splenic T cells were purified by $CD4^+$ MACS beads and cultured for 3 days in the presence of anti-CD3 (5 μg/ml), soluble anti-CD28 (1 μg/ml), IL-2 (50 U/ml), anti-IL-4 (1 μg/ml), and anti-IFN-γ (1 μg/ml), with or without exogenous TGF-β (5 ng/ml). The cells re-stimulated with PMA (50 ng/ml) and ionomycin (1 μM) for 4-6 h were then stained with antibodies to CD4, CD25 and Foxp3 and analyzed by flow cytometry. (A) Dot blot presentation of Treg generation induced by TGF-β *in vitro*. A representative flow cytometry analysis out of 3 is shown. (B) Histogram presentation of $CD4^+CD25^+Foxp3^+$ Treg percentages induced by TGF-β *in vitro*. Data are given as the mean ± SEM, out of 3 experiments. $p<0.01$, Student's t-test.

TGF-β and Treg generation was investigated by flow cytometry. The results showed that with TGF-β treatment, the percentage of Treg generation was increased from 8.59 to 14.96% (Fig. 2A), ~74% higher than that of cells without TGF-β treatment, which indicated that TGF-β could efficiently transform $CD4^+CD25^-Foxp3^-$ cells into $CD4^+CD25^+Foxp3^+$ cells (Fig. 2B).

The expressions of B7H1 and GITRL on DCs were up-regulated by TGF-β treatment. B7H1, GITRL were reported to be candidate immune modulation molecules (12-15). To investigate the roles of B7H1 and GITRL in TGF-β induced Treg generation, DCs were treated with TGF-β, and B7H1 and GITRL expression of DCs were determined by flow cytometry. The results showed that TGF-β 1 ng/ml treatment could increase B7H1 and GITRL expression 141.6 and 149.5%, respectively (Fig. 3).

The establishment of the tumor-DC-fibroblast co-incubation system in vitro. Tumor cells, fibroblasts and immune cells are the main components of tumor microenvironment (4). To explore the role of immune cells in the immune depression induced by tumor cells, fibroblasts separated from murine skin and DCs induced from bone marrow were labeled with CFSE and SNARF, respectively, and mixed for further co-incubation. The results showed that: DCs and fibroblasts could be successfully labeled with 10 μM SNARF and CFSE, respectively. To establish lung cancer microenvironment *in vitro*, LLC, DCs and fibroblasts were labeled with SNARF, CMHC and CFSE, respectively, and mixed for further co-incubation. LLC, DCs and fibroblasts were visible as red, blue and green respectively when co-incubation system was observed by the fluorescence microscope, indicating that co-incubation system could be useful for study of DC function in tumor microenvironment-associated Treg expansion.

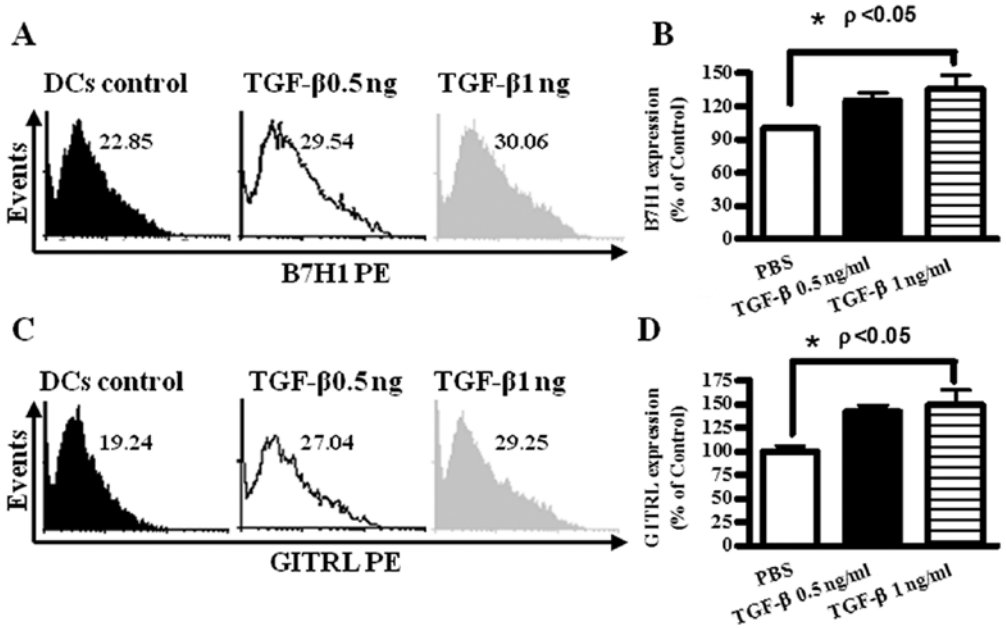

Figure 3. The B7H1 and GITRL expressions of DCs are up-regulated by TGF-β treatment. DCs of day 4 induced from murine bone marrow were treated with TGF-β at 0.5 and 1 ng/ml for 12 h, respectively, then, the expressions of B7H1 and GITRL on DCs were determined by flow cytometry. (A) Histographic presentation of B7H1 expression of DCs with TGF-β treatment. (B) B7H1 on DCs was up-regulated by TGF-β treatment. (C) Presentation of GITRL expression on DCs with TGF-β treatment. (D) GITRL on DCs was up-regulated by TGF-β treatment. A representative flow cytometry analysis out of 3 is shown, numbers in the histogram indicate geometric mean fluorescence of test samples (A and C). Data are given as the mean ± SEM, out of 3 experiments, $^*p<0.05$, one-way ANOVA with post Newman-Keuls test (B and D).

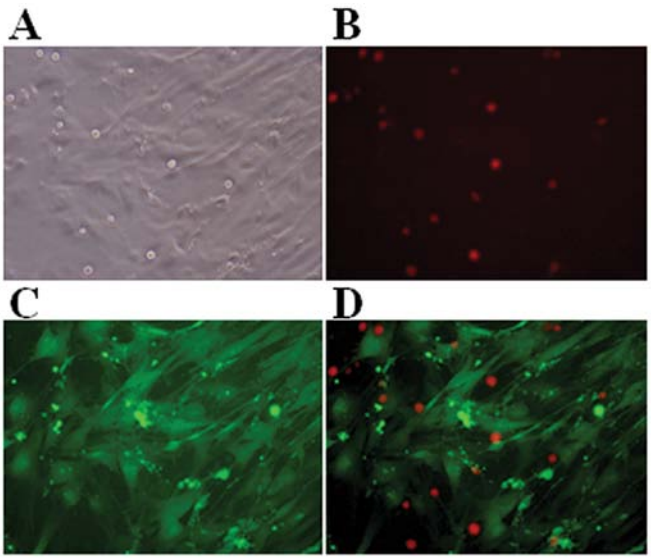

Figure 4. The images of co-incubation of fibroblasts and DCs. Fibroblasts were separated from newborn Balb/c mouse skin by tissue explanting methods and DCs were induced from bone marrow by 10 ng/ml GM-CSF and 1 ng/ml IL-4. Then, 1x10^6 cells/ml fibroblasts and DCs were labeled with 10 μM CFSE and SNARF, respectively. DCs and fibroblasts removed from dishes were mixed for further co-incubation. After 12 h co-culture, the cells were observed and images were recorded by fluorescence microscopy at the wavelength of 488 nm. A representative image out of 3 is shown. (A) Image of fibroblast-DC co-incubation taken by an optical microscope. (B) Image of SNARF labeled DCs (red) taken by fluorescence microscopy. (C) Image of CFSE labeled fibroblasts (green) taken by using fluorescence microscopy. (D) Merged image of SNARF labeled DCs (red) and CFSE labeled fibroblasts. Magnification x200.

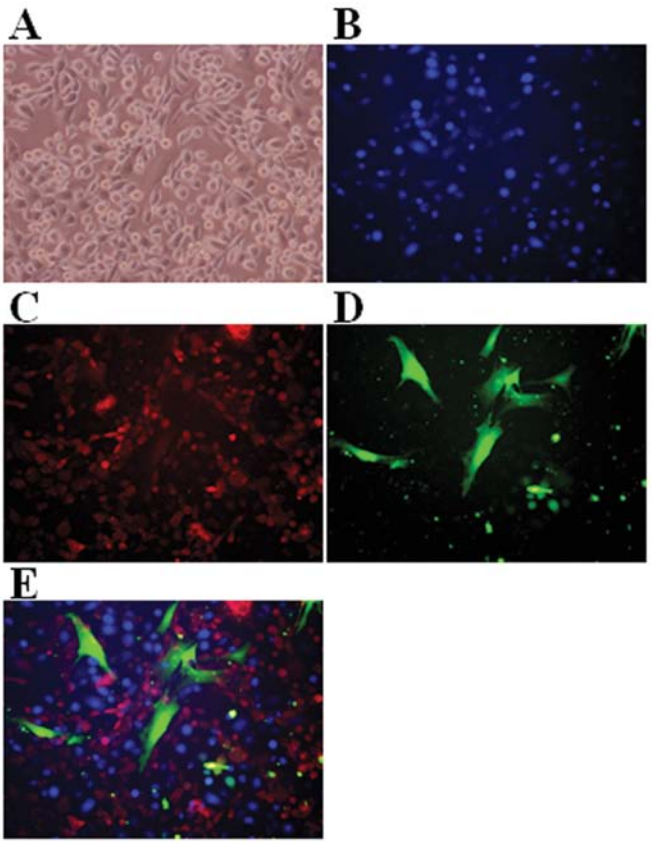

Figure 5. The images of the LLC-fibroblast-DC co-incubation. Fibroblasts separated from newborn Balb/c mouse skin and DCs induced from bone marrow by 10 ng/ml GM-CSF and 1 ng/ml IL-4 were labeled with 10 μM CFSE and CMHC, respectively. LLC were labeled with 10 μM SNARF. DCs, fibroblasts and LLC cells were removed from dishes and mixed for further co-incubation. The cells were observed and images were recorded by fluorescence microscope at the wavelength of 488 nm. A representative image out of 3 is shown. (A) Images of the LLC-fibroblast-DC co-incubation taken by an optical microscope. (B) Image of CMHC labeled DCs (blue) taken by fluorescence microscopy. (C) Image of SNARF labeled LLC (red) taken by fluorescence microscopy. (D) Image of CFSE labeled fibroblasts (green) taken by fluorescence microscopy. (E) Merged image of CMHC labeled DCs (blue), SNARF labeled LLC (red) and CFSE labeled fibroblasts. Magnification x200.

The B7H1 and GITRL expressions on DCs were up-regulated by the LLC-fibroblast-DC co-incubation. Several reports have described the effects of co-incubation of tumor cells and DCs on molecules expression of DCs (7-9), but only DCs and tumor cells were included in these co-incubation systems. The effects of the tumor-fibroblast-DC co-incubation are still unknown. To investigate the effects of tumor microenvironment on B7H1 and GITRL expressions of DCs, DCs were separated from the LLC-fibroblast-DC co-incubation system and B7H1 and GITRL expressions were determined by flow cytometry. The results showed that in contrast to DCs pulsed with the same amount of LLC lysate, the LLC-fibroblast-DC co-incubation could increase B7H1 expression from 68.51 to 168.51 and up-regulated GITRL expression from 26.61 to 46.00, respectively, which indicated that the LLC-fibroblast-DC co-incubation obviously augmented B7H1 and GITRL expressions of DCs (Fig. 6A, $p<0.001$, DCs versus DCs-Tm; Fig. 6B, $p<0.001$, DCs versus DCs-Tm).

Discussion

Cell biology studies indicate that tumor growth is not just determined by malignant cancer cells themselves, but also by the tumor microenvironment. In addition to tumor cells, tumor microenvironment is comprised of immune cells, fibroblasts, stromal cells and extracellular matrix (1). Previous studies showed that co-incubation of DCs with different tumor cells could down-regulate the abilities of DCs mediating tumor antigenic-specific CTL priming (9) or inducing human Treg expansion (6). Suppressed DCs functions have been reported in various tumor microenvironment models, in tumor-bearing animals, as well as in cancer patients (24-26). However, these studies explored immune cell dysfunction by tumor cell and immune cell co-incubation *in vitro* or direct cell separation *in vivo*. Hence, previously little was known about the effects of tumor microenvironment on DCs by direct tumor cell-fibroblast-DC contact.

In the present study, we successfully established lung cancer microenvironment system by LLC, fibroblast and DC co-incubation *in vitro*. DCs separated from co-incubation system were further evaluated by flow cytometry analysis and the results showed that B7H1, and GITRL expressions were up-regulated by LLC microenvironment. As TGF-β, an efficient inducer of Treg expansion, could be generated by Raw 264.7, EL4 and NCI-H446 cells, the effects of TGF-β on B7H1 and GITRL expressions of DCs were explored. Our data demonstrated that

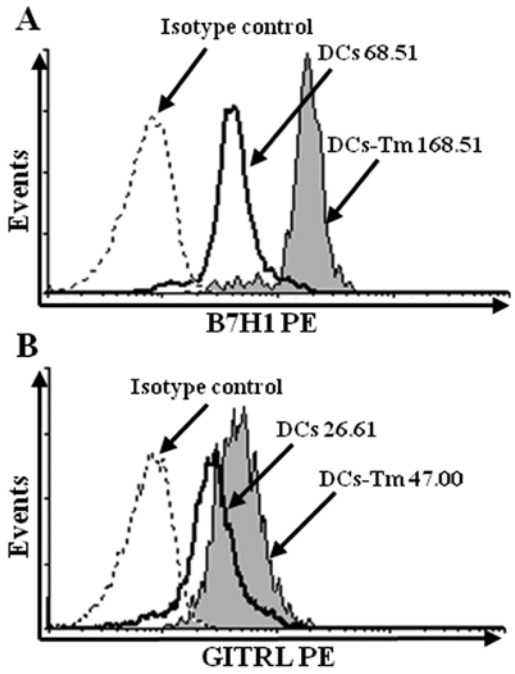

Figure 6. The expressions of B7-H1 and GITRL on DCs were up-regulated by the LLC-fibroblast-DC co-incubation *in vitro*. DCs of LLC-fibroblast-DC system were separated by CD11c MACS beads and investigated further with PE-B7-H1, PE-GITRL antibodies labeling on ice. The expressions of B7H1 and GITRL were determined by flow cytometry. Numbers in the histogram indicate geometric mean fluorescence of test samples. A representative flow cytometry analysis of 3 is shown. (A) Histogram presentation of B7H1 expression on DCs. (B) Histogram presentation of GITRL expression on DCs. Isotype control, DCs labeled with isotype antibody (dash line); DCs, DCs induced from bone marrow pulsed with the same tumor cell lysate (solid line); and DCs-Tm, DCs separated from co-incubation system by CD11c MACS beads (neutral).

TGF-β treatment could up-regulate B7H1, GITRL expressions of DCs, indicating that B7H1 and GITRL might play important roles in TGF-β induced Treg expansion of lung cancer microenvironment.

Our results showed that Raw 264.7, EL4 and NCI-H446 cells produced TGF-β (Fig. 1), which is known to shape the tumor microenvironment, exerting both positive and negative effects on cancer (20-22). Other sources of TGF-β included inflammatory cells and stromal fibroblasts (27). At later stages of tumor progression, cancer cells acquired increasing resistance to TGF-β growth inhibitory signals (22), which was frequently accompanied by increased expression of TGF-β by the same cells. Subsequently, cancer cells started to secrete non-physiological levels of TGF-β (28). As TGF-β was necessary for expansion of Treg (10, 29), the increase of Treg percentage from 8.59 to 14.96% with TGF-β treatment (Fig. 2) indicated that the presence of TGF-β in the tumor microenvironment might impair immune surveillance by inducing TGF-β expansion.

There is mounting evidence that $Foxp3^+$ Tregs can develop extrathymically under certain conditions (13,29-31). Although literature show a potential role for Tregs in the control of autoimmune or inflammatory diseases (32), the nature of APCs involved in Treg expansion remains poorly understood. Our previous report documented that the tumor cell-DC co-incubation decreased CD80, and CD86 expressions of DCs (9). DCs, especially deficient in CD80 and CD86, appeared to be more efficient than other APCs in inducing TGF-β expansion (33,34). Hori *et al* reported that GITRL-dependent Treg expansion underlined immune privilege in corneal allografts (14), but that the blockade of GITR-GITRL interaction was necessary for maintaining Treg function was also reported (15). In the present study, DCs were used in lung cancer microenvironment co-incubation system and its function in Treg expansion was explored. Our data showed that both LLC microenvironment co-incubation and TGF-β treatment could augment B7H1 and GITRL expressions on DCs (Figs. 3 and 6), indicating that Treg expansion in tumor microenvironment might depend on TGF-β inducing B7H1 and GITRL up-regulation.

The data presented here offered new information on the immunological alterations associated with tumor microenvironment, especially the role of TGF-β mediating B7H1 and GITRL up-regulation in lung cancer microenvironment associated Treg expansion. This finding is important because it provides a rationale for further investigation of the mechanisms by which tumor and tumor associated fibroblasts induce immunosuppression *in vivo*.

Acknowledgements

This study was supported by grants from the Natural Science Foundation of Xiamen (no. 3502Z20104002), the Xiamen Science and Technology Key program grant (no. 3502Z20100006) and the grant from the National Laboratory for Oncogenes and Related Genes of China (90-08-02). We thank Jin Hua Su and Fu Chen for excellent animal care.

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