

RESEARCH ARTICLE

Tumor-Derived Transforming Growth Factor- β is Critical for Tumor Progression and Evasion from Immune Surveillance

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Abstract

Tumors have evolved numerous mechanisms by which they can escape from immune surveillance. One of these is to produce immunosuppressive cytokines. Transforming growth factor- β (TGF- β) is a pleiotropic cytokine with a crucial function in mediating immune suppression, especially in the tumor microenvironment. TGF- β produced by T cells has been demonstrated as an important factor for suppressing antitumor immune responses, but the role of tumor-derived TGF- β in this process is poorly understood. In this study, we demonstrated that knockdown of tumor-derived TGF- β using shRNA resulted in dramatically reduced tumor size, slowing tumor formation, prolonging survival rate of tumor-bearing mice and inhibiting metastasis. We revealed possible underlying mechanisms as reducing the number of myeloid-derived suppressor cells (MDSC) and CD4⁺Foxp3⁺ Treg cells, and consequently enhanced IFN- γ production by CTLs. Knockdown of tumor-derived TGF- β also significantly reduced the conversion of naïve CD4⁺ T cells into Treg cells *in vitro*. Finally, we found that knockdown of TGF- β suppressed cell migration, but did not change the proliferation and apoptosis of tumor cells *in vitro*. In summary, our study provided evidence that tumor-derived TGF- β is a critical factor for tumor progression and evasion of immune surveillance, and blocking tumor-derived TGF- β may serve as a potential therapeutic approach for cancer.

Keywords: Tumor-derived TGF- β - shRNA - immune surveillance - Treg - MDSC

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Introduction

Intact immune responses, such as immune surveillance, are required for suppressing tumor development and progression, and tumors often create an immunosuppressive microenvironment that can facilitate tumor growth and block antitumor immune responses through producing soluble factors, such as cytokines, which in turn recruiting other regulatory cells. The promotion of MDSCs and Treg cells by tumor cells plays an crucial role in tumor immune evasion (Zou, 2006; Marigo et al., 2008). Blocking these negative pathways might serve as a potential therapeutic approach. Indeed, antibodies against T cell suppressing molecules, such as CTLA-4 and PD-1 have shown positive effects on tumor patients (Li et al., 2013). However, tumor-derived suppressing molecules have not been well defined yet.

The TGF- β family are pleiotropic cytokines which play important roles in cancer and immunoregulation. The canonical TGF- β signaling pathways involves the binding of TGF- β ligands to the type I and type II TGF- β receptors, followed by the phosphorylation of Smad2 and Smad3, which then bind to Smad4 and enter the nucleus to regulate downstream gene transcriptions (Li et al., 2008). Three

isoforms have been observed in mammalian animals, including TGF- β 1, TGF- β 2 and TGF- β 3, and TGF- β 1 is the most highly expressed by cells of the immune system. Alterations in TGF- β signaling have significant effects on tumor progression, and TGF- β is known to work as both a tumor suppressor and a tumor promoter (Bierie et al., 2006). TGF- β inhibits tumor progression through increased apoptosis, decreased cell cycle progression and reduced expression of growth factors. During the process of cancer progression, tumor cells frequently acquire the capability to resist the growth inhibitory action of TGF- β , and production of TGF- β often leads to tumor evasion from immunosurveillance (Akhurst et al., 2001; Siegel et al., 2003). Increased production of TGF- β occurs in both human cancer patients and animal models (Hu et al., 2013), which is usually considered as a negative prognostic indicator (Buck et al., 2004; Tripsianis et al., 2013).

TGF- β has an inhibitory effect on the immune system. TGF- β 1 deficient mice manifest spontaneously activated T cells and severe autoimmune pathology in vital organs (Kulkarni et al., 1993). Similarly, Smad3-deficient mice also present activated T cells that are resistant to the growth inhibitory action mediated by TGF- β (McKarns

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et al., 2004). Studies with dn-TGF- β RII transgenic mice showed that abrogation of TGF- β signaling in T cells suppressed growth and metastasis when these mice were challenged with lymphoma and melanoma (Gorelik et al., 2001). A recent study suggested that TGF- β 1 produced by activated CD4⁺ T cells antagonized T cell surveillance of tumor development (Donkor et al., 2012). Systemic neutralization of TGF- β *in vivo* enhances immune-mediated recognition and clearance of malignant cells (Thomas et al., 2005).

Actually almost all nucleated cells produce TGF- β , and the sources of TGF- β in tumors include cancer cells themselves as well as various stromal cells in the tumor microenvironment, such as leukocytes, macrophages, bone marrow-derived endothelial cells, and myeloid derived suppressor cells (Massague, 2008). However, the role of tumor cell-derived TGF- β in tumor immune responses as well as the tumor growth is unclear. In this study, we investigated the effect of tumor cell-derived TGF- β in antitumor immune responses by establishing a stable cell line of B16 transfected with TGF- β shRNA and studied the underlying mechanisms. Our study provide evidence that tumor-derived TGF- β is a critical factor for tumor progression and evasion of the immune system, and blocking tumor-derived TGF- β may serve as a potential therapeutic approach.

Materials and Methods

Mice and tumor cell line

C57BL/6 WT male mice were purchased from Beijing Huafukang Bioscience Co., Ltd. All experimental animals were used at 6-8 weeks of age, and maintained in specific pathogen-free conditions at the animal center of Nankai University, Tianjin, China. All animal study protocols were reviewed and approved by the animal care and use committee of Nankai University. The B16F0 cell line was cultured in DMEM-high glucose (Hyclone) supplemented with 10% FBS (Gibco) and 1% penicillin/streptomycin (Gibco) at 37°C under an atmosphere of 5% CO₂.

Establishment of TGF- β 1 knockdown cell line

Four shRNA targeting TGF- β 1 (NM 011577.1) were designed as follows: (1)5'-TAT CTT TGC TGT CAC AAG AGC-3'; (2) 5'-AAT AGT TGG TAT CCA GGG CTC-3'; (3)5'-ATT TCT GGT AGA GTT CCA CAT-3'; (4)5'-AGG GCA AGG ACC TTG CTG TAC-3'. These sequences were cloned into pcDNATM6.2-GW/EmGFP expression vector (Invitrogen). These four vectors and the negative control plasmid were transfected into B16 cells respectively by Lipofectamine 2000 (Invitrogen). The expression of Tgf- β 1 mRNA was analyzed 48 h later by real-time PCR and the most efficient sequence was selected. This sequence was cloned into pLenti6/V5-DEST lentiviral vector. The lentiviral vector was co-transfected into 293FT cells with the Packaging Mix, and the viral supernatant was harvested 48 and 72 h after transfection. B16 cells were infected with the TGF- β 1 specific or negative control lentivirus. After selection by blasticidin (5 μ g/ml) for two weeks, the EmGFP^{high} cells were sorted out by FACS.

Real-time PCR

Real-time PCR was performed by SYBR Premix Ex TaqTM Kit (TaKaRa). Specific primers for mouse TGF- β 1 and GAPDH were as follows: TGF- β 1 forward: 5'-GGC ACC ATC CAT GAC ATG AA-3', reverse: 5'-CCA GAC AGA AGT TGG CAT GGT-3'; GAPDH forward: 5'-AAG GCT GTG GGC AAG GTC A-3', reverse: 5'-TGC TTC ACC ACC TTC TTG ATG T-3'. Data analysis was done by the ΔC_T method for relative quantification.

ELISA assays

Cytokine assay for TGF- β production by B16 cells was done using a ELISA kit (R&D Systems). B16 cells were plated at a density of 1 \times 10⁶/T-25 flask and cultured for 48 h in serum-free medium. The supernatant was collected and TGF- β level was measured according to the manufacturer's instructions.

Tumor models

For subcutaneous tumor model, 2 \times 10⁵ B16 cells were injected subcutaneously into the right flank of B6 WT mice and the tumor growth was measured with caliper every other day for up to 3 weeks. For metastasis assay, B6 WT mice were injected intravenously with 5 \times 10⁵ B16 cells. Eighteen days later, mice were euthanized and lungs were excised, fixed with 2% Formaldehyde solution for gross inspection. To observe survival time, mice were inoculated in the same way as above and monitored every other day.

Preparation of tumor-infiltrating lymphocytes (TILs)

Freshly excised tumor tissues were minced and digested with Collagenase IV and DNase I for 1 hour at 37°C. Cell suspensions were filtered through a 70 μ m cell strainer. TILs were isolated by density-gradient centrifugation at 1500 rpm for 20 min at 25°C over the Mouse Lymphocyte Separation Medium (Solarbio). TILs were collected from the interphase and washed twice in PBS.

Flow cytometry

APC-conjugated anti-mouse CD4 (clone GK1.5), PE-conjugated anti-mouse CD8 α (clone 53-6.7), FITC-conjugated anti-mouse CD11b (clone M1/70), anti-mouse Gr-1 (clone RB6-8C5) and APC-conjugated anti-mouse IFN- γ (clone XMG1.2) were purchased from Sungene. PE-conjugated anti-mouse Foxp3 (clone MF-14) were purchased from Biolegend. For analysis of surface markers, cells were incubated with antibodies for 20 min on ice. Foxp3 staining was performed using Foxp3 Staining Buffer Set (eBioscience). For IFN- γ staining, splenocytes or TILs were stimulated with PMA (50 ng/ml; Sigma, St. Louis, MO) and ionomycin (1 mg/ml; Sigma) in the presence of GolgiPlug (BD Biosciences) for 5.5 h. Data were acquired using BD FACSCalibur flow cytometer and analyzed with FlowJo software.

Culture of CD4⁺CD25⁻ T cells with conditioned medium (CM)

After surface staining with FITC-conjugated anti-mouse CD4 (clone GK1.5), PE-conjugated anti-mouse CD25 (clone PC-61.5.3) and APC-conjugated anti-mouse

CD44 (clone IM7), CD4⁺CD25⁻CD44^{low} T cells were sorted from adult mice spleens on BD FACSAria II. The purity of these T cells was found to be over 90%, as determined by FACS. CM were collected from a 3-day culture of B16 TGF- β knockdown cells and negative control cells respectively, and then diluted 1:1 with complete T cell medium. Isolated CD4⁺CD25⁻CD44^{low} T cells (4×10^5 /well in a 48-well plate) were cultured in complete T cell medium or different CM in the presence of plate-bound anti-CD3, soluble anti-CD28 and IL-2 for 3 days. Three days later, cells were harvested and stained for CD4 and Foxp3. Data were acquired using BD FACSCalibur flow cytometer and analyzed with FlowJo software.

Wound healing assay

Confluent monolayer of B16 cells were scraped with pipette tips, washed with PBS, and incubated in DMEM supplemented with 2% FBS for 24 h.

MTT assay for cell growth inhibition

MTT (Sigma) was added to the culture medium 68 h post transfection. After incubation for 4 h, the culture medium was removed and 200 μ L of DMSO was added to resolve the crystal. Absorbance was measured at 490 nm.

Detection of apoptotic cells by FACS

Cells were harvested by trypsinisation, washed in cold PBS and re-suspended in 1 \times Binding Buffer at a concentration of approximately 1×10^6 cells/mL. 5 μ L of Annexin V-APC and 5 μ L of 7-AAD solution were added to each cell suspension and incubated at RT for 10 min in the dark. The cells were analyzed within 1 hour by FACS.

Statistics

Two-way analysis of variance test (ANOVA) was used for comparing and analyzing the tumor growth curve. The survival curve was compared and analyzed using the Log-rank test. Two-tailed unpaired Student's t test was performed to analyze other data. All values were expressed

as mean \pm SD. All analysis was performed using Graphpad Prism 5.01 for Windows. Throughout the text, figures, and legends, the following terminology was used to denote statistical significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Results

Establishment of TGF- β knockdown B16 cells

To knockdown tumor-derived TGF- β in B16 cells, four plasmids designed to express shRNA targeting mouse TGF- β 1 and negative control plasmid coding for a scramble shRNA were transfected into B16 cells respectively, and the transfection efficiency was monitored by the Emerald green fluorescent protein (EmGFP) reporter within these plasmids (Figure 1a). Cells were collected 48 h post transfection and then analyzed for expression of Tgf- β 1 by real-time PCR. Figure 1b showed that the shRNA4 was the most effective one among the four sequences. This sequence was cloned into pLenti6/V5-DEST lentiviral vector. B16 cells were infected with the TGF- β 1 specific or negative control lentivirus and then cultured for 2 weeks in the presence of blasticidin. The EmGFP^{high} cells were sorted out by FACS and over 99% of sorted B16 cells were EmGFP positive (data not shown). The TGF- β knockdown cell line that we finally obtained had a 79% reduction in the expression of Tgf- β 1 mRNA when compared with negative control B16 cells (Figure 1c). The knockdown efficiency was further confirmed by ELISA (Figure 1d).

Tumor-derived TGF- β is required for tumor formation, progression and metastasis in vivo

To define the role of tumor-derived TGF- β in tumor growth *in vivo*, B6 WT mice were subcutaneously challenged with either negative control or TGF- β knockdown B16 cells, animals bearing TGF- β knockdown B16 cells showed a significant delay in tumor formation as well as a smaller tumor burden, when compared to those bearing negative control B16 cells (Figure 2a and 2b). To further study the effect on metastasis, B6 WT mice were intravenously injected with these two kinds of B16

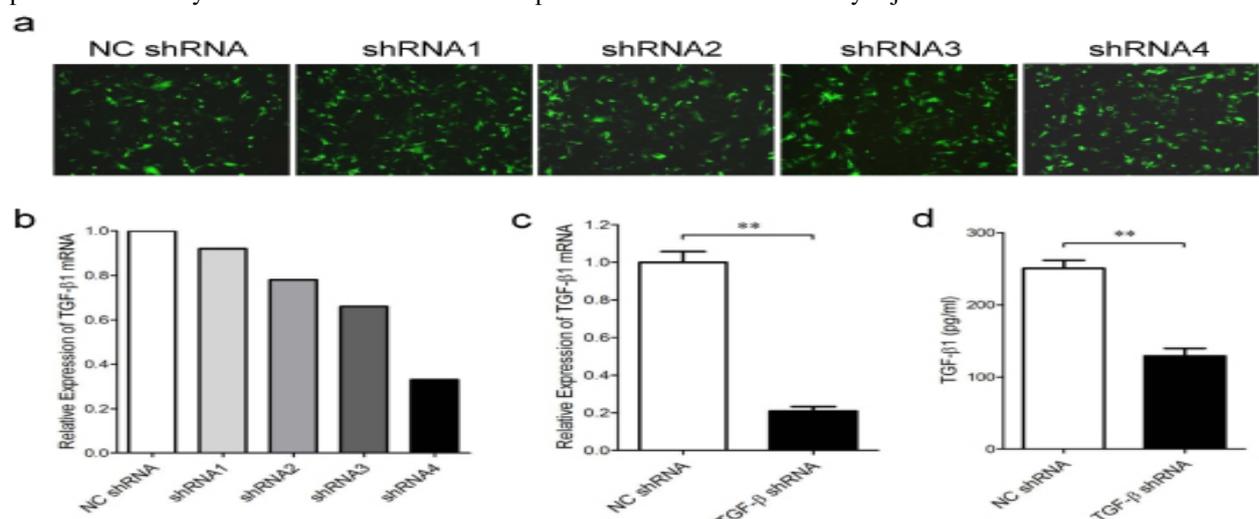


Figure 1. Establishment of TGF- β Knockdown B16 Cell Line. A) B16 cells were transfected with negative control plasmid and four shRNA plasmids. The transfection efficiency was monitored by fluorescence microscopy ($\times 100$) and the expression of mRNA was examined by Real-time PCR B). The effects of TGF- β knockdown in the finally obtained B16 TGF- β knockdown cells were determined by Real-time PCR C) and ELISA D). The data shown are representative of three independent experiments

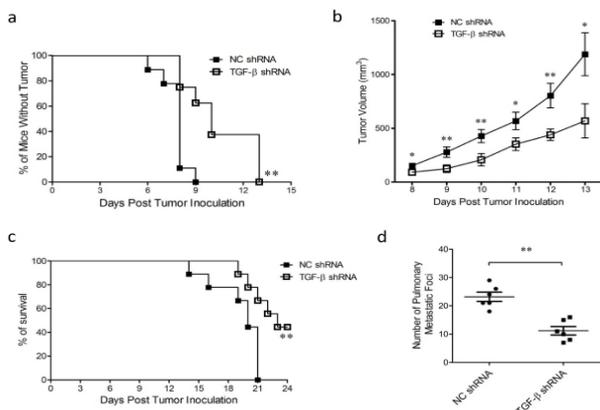


Figure 2. Tumor-derived TGF-β is Required for tumor Formation, Progression and Metastasis *in vivo*. A) and B) B6 WT mice (n=10) were subcutaneously inoculated with B16 negative control and TGF-β knockdown cells (2×10^5) respectively. Tumor size > 4x4 mm was considered positive. C) and D) B16 cells (5×10^5) were injected i.v. into male B6 WT mice (n=10). Eighteen days after inoculation, mice were sacrificed for examining lung metastasis. Data represent three independent experiments

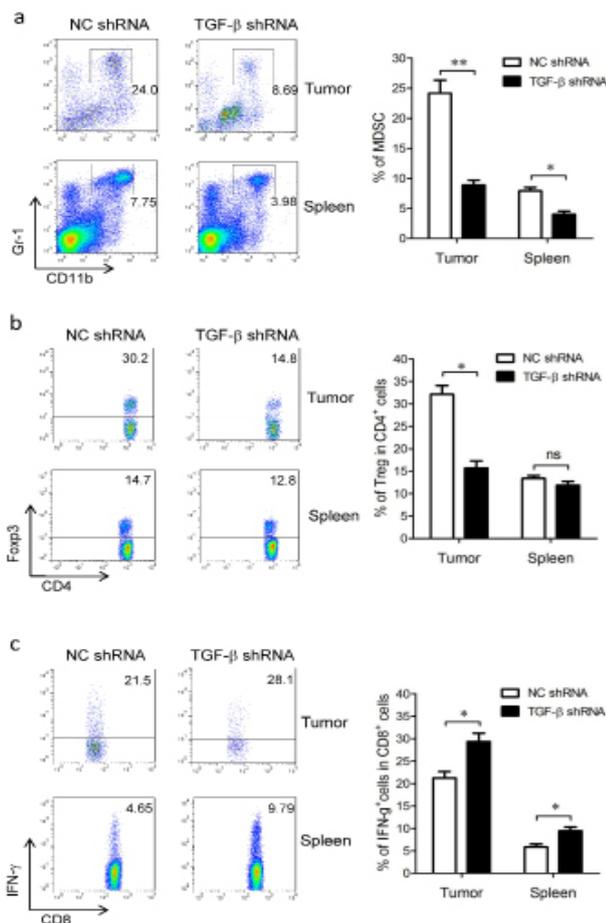


Figure 3. Knockdown of Tumor-Derived TGF-β results in Decreased numbers of Treg Cells and MDSCs, Increased CTL Function, and Partially Blocked Conversion of Naïve CD4⁺ T Cells into Treg Cells. B6 WT mice were subcutaneously inoculated with negative control or TGF-β knockdown B16 cells (2×10^5). Two weeks later, TILs and splenocytes were isolated for A) MDSCs (Gr-1⁺CD11b⁺) and B) Treg cells (CD4⁺Foxp3⁺) analysis by FACS. C) TILs and splenocytes were isolated for IFN-γ analysis by FACS

cells. A significantly increased survival rate was realized in the knockdown group (Figure 2c). It was obvious that the mice challenged with TGF-β knockdown B16 cells bore less pulmonary metastatic nodules than mice in the control group (Figure 2d). These observations indicated that tumor-derived TGF-β plays an important role in tumor formation, progression and metastasis *in vivo*.

Tumor-derived TGF-β regulates the number of CD4⁺Foxp3⁺ Treg cells and MDSCs in tumor microenvironment and CD8⁺ T cell function in spleen and tumor

To define the underline mechanisms by which tumor-derived TGF-β mediates the immune suppression, tumor tissues as well as spleens from mice subcutaneously injected with two kinds of B16 cells were collected for the analysis of the percentage of CD4⁺Foxp3⁺ Treg cells and MDSCs. As shown in Figure 3a, about 24% of TILs were found to be MDSCs in the mice bearing negative control B16 cells, whereas only approximately 9% in the mice bearing TGF-β knockdown B16 cells. Similar reduction was also observed in the spleens. The proportion of Treg cells in CD4⁺ T cells in the TILs was found to be 30% in the control group, but only 14% in the TGF-β knockdown group (Figure 3b). Interestingly, there was no significant difference of Treg cells in the spleen between the two groups. Adding to this, we also observed an increase in the CTL function in the mice challenged with TGF-β knockdown B16 cells. As indicated in Figure 3c, CD8⁺ T cells in both the spleen and tumor tissue of mice challenged with TGF-β knockdown cells produced more IFN-γ than the negative control group. In our preliminary studies, inoculation of these two different B16 cells did not change the total number of TILs and splenocytes (data not shown).

Tumor-derived TGF-β mediates conversion of naïve CD4⁺ T cells into Treg cells in vitro

To study the potential role of tumor-derived TGF-β in Treg conversion, CD4⁺CD25⁻CD44^{low} T cells were sorted from the spleens of adult mice and then cultured in CM in the presence of plate-bound anti-CD3, soluble anti-CD28 and IL-2 for 3 days. Figure 4 shows that Foxp3 expression was significantly higher in naïve T cells cultured with negative control B16 CM, as compared to those cultured with TGF-β knockdown B16 CM. Foxp3 expression was absent in T cells cultured in T cell medium. These results indicate that tumor-derived TGF-β is required in Treg conversion *in vitro*.

Tumor-derived TGF-β promotes cell migration, but does not change the proliferation and apoptosis of tumor cells in vitro

To evaluate whether the knockdown of tumor-derived TGF-β has changed the cell biology characteristics effect of B16 cells, we did the following experiments. Figure 5a showed that knockdown of TGF-β in B16 cells inhibited cell migration *in vitro*. MTT analysis result indicated that there was no significant difference between negative control and TGF-β knockdown B16 cells, suggesting that tumor-derived TGF-β has no inhibitory action on cell

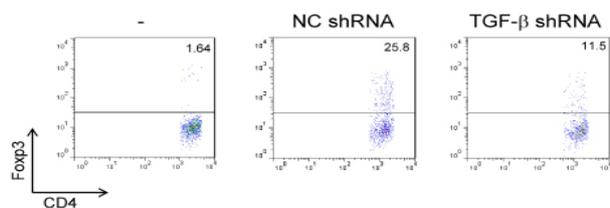


Figure 4. Tumor-Derived TGF- β Mediates the conversion of naïve CD4⁺ T cells into Treg Cells. CD4⁺CD25⁻CD44^{low} T cells were sorted from spleen of adult mouse and cultured in different CM. After culture for 3 days, cells were harvested and stained for CD4 and Foxp3 expression. Data shown are represent of three independent experiments

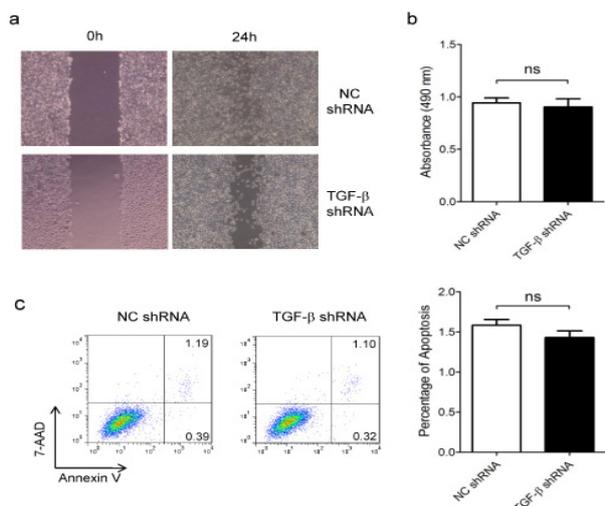


Figure 5. Knockdown of Tumor-Derived TGF- β Leads to Inhibited Cell Migration but not Decreased Cell Proliferation and Increased Apoptosis. Wound-healing assay A) and MTT assay B) were performed. C) Cell apoptosis was evaluated by FACS. Data shown are represent of three independent experiments

proliferation (Figure 5b). The apoptosis rates between the two groups also had no significant difference (Figure 5c).

Discussion

The role of TGF- β in tumor progression and inducing immunosuppression has been extensively studied. TGF- β produced by T cells has been demonstrated as an important factor for suppressing antitumor immune response. Many types of tumors are rich sources of TGF- β ; however, the effects of tumor cell-derived TGF- β on the tumor immune suppression and the underlying mechanisms were not fully elucidated. Melanoma is a type of highly immunogenic cancer that causes almost 75% of deaths related to skin cancer (Jerant et al., 2000). B16 cells produce substantial amounts of TGF- β , and B16 cells implanted in C57Bl/6 mice serves as a popular animal model system to study the anti-tumor immune responses.

In our results, mice challenged with TGF- β knockdown B16 cells showed delayed tumor formation, smaller tumor size, improved survival rate and decreased metastases. No distinct changes in cell proliferation and apoptosis were observed *in vitro*. Only inhibited migration ability was found after the knockdown of TGF- β , which to some extent was responsible for the decreased metastases. Next

we focused on the impaired antitumor immune responses and subsequent tumor immune evasion.

It has long been known that exogenous TGF- β could induce the conversion of CD4⁺CD25⁻ T cells into Treg cells (iTreg). One mechanism through which tumor evades antitumor immunity is the induction of Treg cells. Our results showed that TGF- β produced by B16 tumor cells mediated Treg cells conversion *in vitro*. Moreover, it was further indicated by the *in vivo* observations that the increased prevalence of Treg cells in the tumor microenvironment seemed to be dependent on tumor-derived TGF- β . Published works have elucidated at least three different sources of Treg cells in the tumor microenvironment. First is recruitment. In an ovarian cancer model, tumor-released chemokine, CCL22, can specifically recruit circulating Treg cells to tumor sites (Curiel et al., 2004). Second is proliferation. Antigen-loaded dendritic cells can directly stimulate proliferation of Treg cells in the absence of added cytokines (Yamazaki et al., 2003). Adding to this, TGF- β secreted by tumor cells can convert DCs into regulatory cells, which then in turn stimulate Treg cells proliferation (Ghiringhelli et al., 2005). This way of proliferation is dependent on TGF- β indirectly. Thirdly, naive CD4⁺ T cells can be converted into Treg cells in the presence of TGF- β (Chen et al., 2003). Collectively, both the recruitment and direct proliferation processes seem independent of tumor-derived TGF- β . Our results suggested that Treg cells present in the tumor microenvironment, at least in part, were converted from naive CD4⁺ T cells in the presence of tumor-derived TGF- β . And the TGF- β -rich environment was necessary to the conversion of Treg cells in the tumor microenvironment, which confer cancer cells the advantage of evading the host immune surveillance. The effect of tumor-derived TGF- β on the prevalence of Treg cells was restricted to local tumor sites, and tumor cells themselves seemed to be one of the main sources of TGF- β in tumor microenvironment. Due to the ubiquitous expression of TGF- β receptors (Massague, 2008), we speculated that the TGF- β produced by tumor cells may be consumed by cells in the vicinity of the tumor sites before it reaches the peripheral blood and spleen. Other mechanism besides conversion by TGF- β leads to the prevalence of Treg cells in spleen. Several studies have reported that TGF- β was shown to act on CTLs to specifically inhibit the cytolytic activities of CTL, and CTL dysfunction is also associated with elevated serum levels of TGF- β (Willimsky et al., 2005). Our experiments showed that knockdown of tumor-derived TGF- β resulted in the increased production of IFN- γ by CTLs in both the spleen and tumor tissue, which also facilitated the clearance of malignant cells.

In addition to Treg cells, MDSCs also play an essential role in the immunosuppressive networks that lead to tumor immune evasion (Zou, 2006; Marigo et al., 2008). They expand significantly in the bone marrow, spleen, blood, liver and tumor sites of numerous patients and experimental mice with cancers (Liu et al., 2010). MDSCs can be recruited into mammary carcinomas with TGF- β type II receptor gene deletion and directly promote tumor metastasis (Yang et al., 2008). Our results showed a

reduction in the percentage of MDSCs both in spleen and tumor sites in tumor-bearing mice challenged by TGF- β knockdown B16 cells. Publications from many groups have shown that MDSCs are also one of the major sources of TGF- β in the tumor-bearing host (Ghiringhelli et al., 2005; Yang et al., 2008). So we speculate that reduction of MDSCs in the tumor sites was a possible consequence of the knockdown of tumor-derived TGF- β , and the reduction of MDSCs further resulted in a lower level of TGF- β in the tumor microenvironment. The conversion from naive CD4⁺ T cells into Treg cells was therefore partially blocked. It is necessary to point out that the interaction between these two immunosuppressive cell populations was a bit complicated and the papers on this point of view are sometimes contradictory to each other. Some reports indicated that immature DCs, phenotypically and functionally impaired by the immunosuppressive environment induced by cancer, may be able to induce Treg cell expansion (Chung et al., 2009). Others suggested that MDSCs expanded in 4T1 or LLC tumor bearing mice are capable of suppressing TGF- β -induced polarization of naive T cells into Treg cells (Centuori et al., 2012). This question could not be addressed in our recent study; therefore, we will set up experiments in the future to make it clear which kind of roles tumor-derived TGF- β played in the relationship between MDSCs and Treg cells in the tumor microenvironment.

Current therapeutic inhibitors that block TGF- β signaling via antisense molecules, neutralizing antibodies and small molecule inhibitors often lack specificity and consequently target systemic TGF- β signaling. So elucidating the role of tumor-derived TGF- β is important, not only for a better understanding of the mechanisms of TGF- β -mediated tumor progression and immune suppression, but also for the design of more effective and specific TGF- β -targeting cancer immunotherapy.

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