CD4+CD25+Foxp3+ Treg and TGF-β play important roles in pathogenesis of Uygur cervical carcinoma

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Summary

Objective: The aim of the study was to evaluate the function of CD4+CD25+ and Foxp3+ T (Treg) cell and related cytokine in the Uygur patients with cervical carcinoma and CIN (cervical intraepithelial neoplasia). *Materials and Methods:* 170 Uygur women were recruited in the study from January 2007 to January 2011. The study group was comprised of normal controls, cases of primary cervical carcinoma/CIN, and cervical carcinoma/CIN treated with surgery. The following parameters were examined: clinicopathologic features of patients, percentage of CD4+CD25+Foxp3+ Treg cell in blood and Foxp3 mRNA expression in CD4+CD25 high T cell concentration of serum cytokine. Women with primary cervical carcinoma/CIN after being treated with surgery were compared to the normal controls. Where appropriate, univariate and multivariate analyses were used to identify the function of the Treg and related cytokine. *Results:* The percentages of CD4+CD25+ Treg were detected as well as in the blood of carcinoma patients and CIN II/III, but the number of cells was much higher compared to both control and CIN I groups ($p < 0.01$). Moreover, a significant correlation between the expression of Foxp3 mRNA and pathological changes was found. The secretion levels of IL-10, and TGF-β correlated positively with the process of carcinoma. Furthermore, after surgical operation, the number of Treg cells and related cytokines were decreased. *Conclusions:* Finally, the authors would like to highlight that CD4+CD25+ Treg, especially the CD4+CD25+Foxp3+ Treg and TGF-β play important roles in Uygur cervical carcinoma, and may have a correlation with survival. Therefore, the inhibitory function of TGF-β depletion of Treg cells in combination with other anti-tumor therapies could optimize eradication of malignancies.

Key words: Cervical carcinoma; CD4+CD25+Foxp3+ Treg; TGF-β; Uygur.

Introduction

Cervical carcinoma is a leading cause of morbidity and mortality among women worldwide, especially in the developing countries [1]. Cervical carcinoma has a positive correlation with human papilloma virus (HPV) [2, 3] and cervical carcinoma cells may have many mechanisms to escape from host immunosurveillance. At present, it was thought that the imbalance of T cell responses were associated with the progress of cervical carcinoma [4]. It has been reported that T cell responses are regulated by CD4+CD25+Foxp3+ 'regulatory' T (Treg) cells. Treg accounts for 10% of the total peripheral T cell and play an important role in maintenance of immunological tolerance to self-antigens and in preventing immune pathologies. Evidences from other carcinoma suggest that increased Treg activity may be associated with poor immune responses to tumor antigens and contribute to immune dysfunction [5]. A recent study on Treg cells in cervical carcinoma patients and CIN showed increased Treg frequencies in peripheral blood of these patients. It suggests that suppression of immunity by Treg will be an impediment in designing therapeutic strategies [5].

In Xinjiang, Uygur women have higher risk for the occurrence of cervical carcinoma Han compared to the population [6]. In this study, to expand the authors' understanding of Treg interference with immune response, the effect of Treg cells and cytokines in the blood from donors was calculated. The authors first reported that the proportion of CD4+CD25+Foxp3+ T cell in Uygur cervical carcinoma was high. They also found that after surgical operation, Treg cells and related cytokines were decreased. Using this data set may help the authors to better understand the mechanism of cervical carcinoma and provide insight for the treatment.

Materials and Methods

Patients

This study recruited 170 Uygur women, including the normal control, and cervical carcinoma, CIN I, and CIN II/III. The cases of cervical carcinoma, CIN I and CIN II/III were diagnosed in the Gynecology and Obstetrics Department of the First Affiliated Hospital of Xinjiang Medical University from January 2007 to January 2011. The characteristics of the patients are shown in Table 1. Peripheral anticoagulation blood was used to measure the phenotype of the cells of each patient by flow cytometry (FCM). Informed consent was obtained from all donors and the Institutional Review Board at Xinjiang Medical University Hospital approved this study.

Preparation of cells and FCM

Cells were isolated according to an established method with slight modifications. Briefly, peripheral blood mononuclear cells (PBMC) from donors were isolated by density centrifugation on Histopaque 1077 (Sigma, St. Louis, MO, USA). The PBMC (1×10^6) were subjected to FCM analysis using the ASR system (Becton Dickinson, Franklin Lakes, NJ). Cells

Revised manuscript accepted for publication March 13, 2012

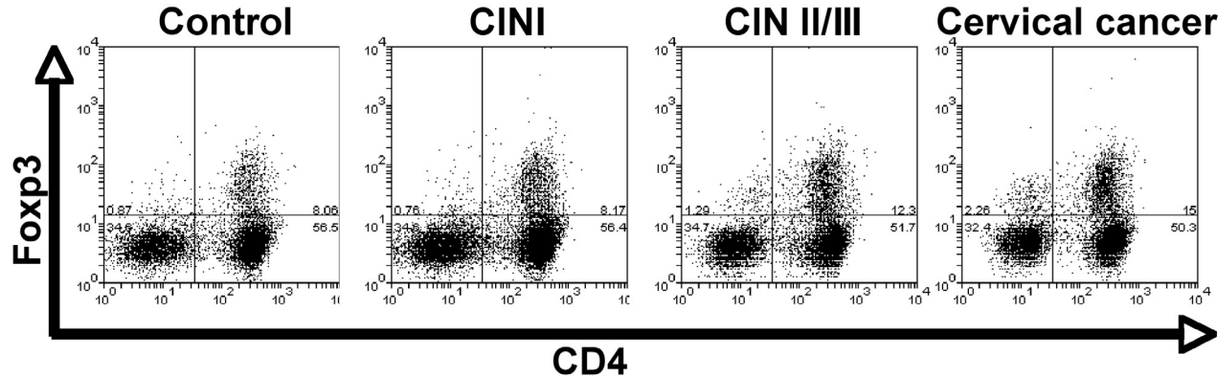

Figure 1. — Percentage of Treg cells in blood T cells.
T cells were analyzed by FCM. CD3+ T cells were first gated and then the CD4+CD25+ and CD4+Foxp3+ Treg cells were analyzed.

were washed with phosphate buffered saline (PBS) containing 0.5% BSA, pre-incubated for 15 minutes with unlabeled isotype control Abs (IgG1 or IgG2b) (eBioscience, San Diego, CA, USA), and then labeled with either fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, PerCP- or PE-cy7-conjugated Abs (eBioscience, San Diego, CA, USA) by incubation on ice for 30 minutes, and then followed by washing with PBS. Cellular debris was then eliminated from the analysis using a gate on forward and side scatter. The cells were subsequently analyzed using ASR flow cytometer. As a control for non-specific staining, isotype-matched irrelevant Abs were used. Analysis of data was performed using Weasel software (The Walter and Eliza Hall Institute, Parkville Victoria, Australia).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

PBMC from donors were isolated by density centrifugation on Histopaque 1077. Then CD4+CD25+ T cell were purified from PBMC by positive selection using a MACS column containing microbeads-conjugated with Abs to CD4 and CD25 (Miltenyi Biotec, Bergisch Gladbach, Germany). RNA was isolated from CD4+CD25+ T cell using TRIzol (Invitrogen, Breda, Netherlands) and first strand cDNA synthesis with oligoDT primers and reverse transcriptase AMV (Roche Diagnostics, Mannheim, Germany) were both performed according to the manufacturer's instructions. Oligonucleotide primers for Foxp3 and β-actin were chosen on the basis of known sequences (5'-ACACCACCCACCACCGCCACT-3 , 5'- TCGGATGATGCCACAGATGAGC-3', 400 bp) and cDNA encoding for the Foxp3 and actin was amplified. The concentration of PCR product were determined by 1% agarose gel stained with ethidium bromide (Sigma, St. Louis, MO, USA). Scan and analysis of the A value of electrophoretic bands by GelDoc 2000 analysis system (Bio-Rad, USA). cDNA were stored at -80°C before further use.

Enzyme-linked immunosorbent assay (ELISA)

The serum was collected then IL-10 and TGF-β concentrations in the serum were measured in triplicate using standard ELISA kits (R&D Systems, Minneapolis, MN, USA), according to manufacturer's instructions with standard cytokine preparations used as the internal controls. The amount of cytokines was quantified by using an xMark spectrophotometer (Bio-Rad, Hercules, CA, USA).

Table 1. — *Summary of clinicopathologic features of patients*.

Patient	Age	Lymph node metastasis*		Tumor size*	
		yes	no	< 40 mm	≥ 40 mm
Control	45.33 ± 6.1	0	30	0	0
CIN I	45.56 ± 6.7	0	40	0	0
CIN II-III	44.12 ± 6.5	0	40	0	0
Cervical cancer	45.45 ± 6.3	0	60	25	35

*N, number of patients/cervical carcinomas.

Statistical analysis

Statistical analysis was performed using SPSS 14.0. Results are presented as the means ± SD. Data were processed using the chi-square test, the Kruskal-Wallis H test, and analysis of variance, depending on the number and distribution of the compared groups. A p value of < 0.05 was considered statistically significant.

Results

Demographic profiles

One hundred and seventy women with or without primary cervical carcinoma/CIN were enrolled in the study. All cervical carcinoma/CIN patients were operated on mainly due to carcinoma/CIN. Tumor size, lymph node metastasis, and depth of invasion were confirmed by biopsy during surgery (Table 1). There were no significant differences between the two groups in terms of age, parity, and body mass index (BMI). For the cervical carcinoma group, all patients underwent radical hysterectomy and pelvic lymph node dissection. Among them, pathological results showed no lymph node metastasis.

Treg cell frequencies in peripheral blood

To investigate the association between CD4+CD25+ Treg cells, and cervical carcinoma or CIN, the number of Treg cells in the blood was determined. There were

Fig. 2A

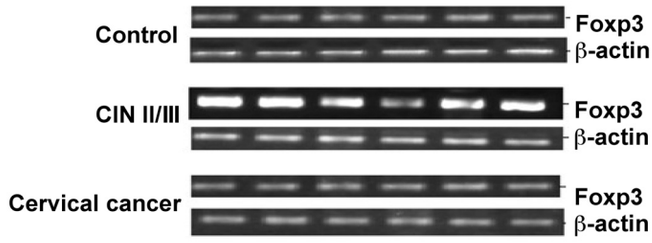

Fig. 2B

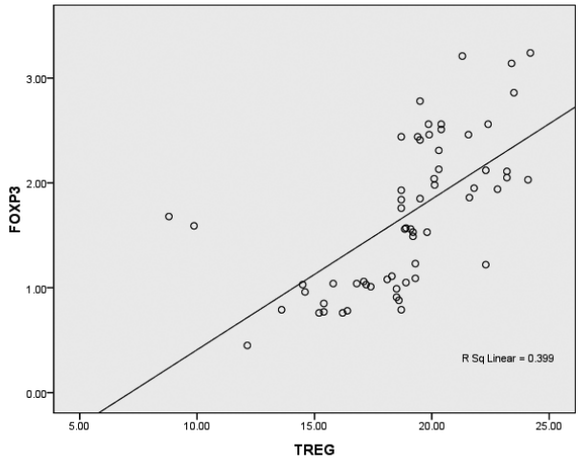

Figure 2. — Expression of Foxp3 mRNA in CD4+CD25+ T cells. A: CD4+CD25+ T cells were purified from PBMC by positive selection using a MACS column containing microbeads conjugated with Abs to CD4 and CD25. RT-PCR was performed to detect the Foxp3 mRNA expression. B: The relevance between the Foxp3 expression and Treg cells in cervical carcinoma.

Table 2. — *Expression of Foxp3 mRNA in CD4+CD25+ T cells*.

Control	CIN groups		Cervical cancer
	CIN I	CIN II-III	
N = 30	N = 40	N = 40	N = 60
1.41 ± 0.79	1.49 ± 0.78	1.61 ± 0.86	1.85 ± 0.89*

The results represent the mean SD of three independent experiments.
*$p < 0.01$ compared to controls.

Table 3. — *Percentage of Treg cells in blood T cells*.

Group	Case	CD3+CD4+T(%)	CD4+/CD8+	CD3+CD8+T(%)	CD4+CD25+ Foxp37(%)
Control	30	56.69 ± 3.02	1.56 ± 0.32	38.49 ± 6.73	7.87 ± 3.52
CIN I	40	55.15 ± 8.18	1.53 ± 0.64	38.70 ± 6.27	8.76 ± 3.62
CIN II-III	40	57.53 ± 4.53	1.58 ± 0.41	37.46 ± 4.67	14.67 ± 4.26*
Cervical cancer	60	56.15 ± 4.22	1.55 ± 0.43	37.83 ± 8.57	17.48 ± 4.58*

The results represent the mean SD of three independent experiments.
*$p < 0.01$ compared to controls.

no differences in total CD3+CD4+ T cell and balance of CD4/CD8 among all groups, but in terms of Treg cells, cervical carcinoma patients had the most abundant Treg cells in the blood (Table 2, Figure 1). In contrast to CIN II/III patients, the Treg cells in cervical carcinoma patients were higher, but not statistically significant ($p > 0.05$). The CIN I group had a higher number of Treg cells in the blood than controls but not significant; however, both groups had a statistically significant comparison to the cervical carcinoma and CIN II/III groups ($p < 0.05$). These results showed that there was a significant association between Treg and cervical carcinoma. Among the enrolled 170 Uygur women, 140 patients with CIN I/II/III or cervical carcinoma underwent surgical therapy. Then the phenotype of Treg cells in the blood was evaluated five days postoperativey (Table 3). Treg cells in all groups had decreased, especially in CIN II/III and cervical carcinoma groups (from 19.7 ± 5.8 to 14.5 ± 4.6 and 20.5 ± 5.8 to 15.7 ± 6.5, respectively).

The expression of Foxp3 mRNA in cervical carcinoma

First, the authors measured Foxp3 expression on T cell (Figure 1). CD4+CD25+Foxp3+ T cell were increased in patients with cervical carcinoma or CIN II/III (Table 1). Subsequently, the expression of Foxp3 mRNA in cervical carcinomas was determined. A positive correlation between Foxp3 expression and stage of CIN and cervical carcinoma was observed ($p < 0.01$) (Figure 2A, Table 2). The expression of Foxp3 mRNA showed a positive correlation with CD4+CD25+ Treg cells (Figure 2B). These results indicate that Foxp3 + Treg cells contribute more to the progress of cervical carcinoma.

Concentration of IL-10, and TGF-β in the serum

The authors assayed the IL-10 and TGF-β secretion in serum. After collecting the serum, the IL-10, and TGF-β concentrations in the serum were measured by ELISA. The levels of IL-10, and TGF-β production in the control group were very low, but in the cervical carcinoma group, there were large amounts of IL-10 and TGF-β (Figure 3A). Considering that both IL-10 and TGF-β are able to act as regulators mediated by Treg cells, IL-10 and TGF-β are associated with Uygur cervical carcinoma (Figure 3B). These results indicate that the suppressive activity in the immune response may be due to the induction of soluble factor production. In fact, a significant increase of IL-10 or TGF-β production was already apparent in early CIN II patients. Moreover, five days after surgery, the concentration of IL-10 and TGF-β decreased dramatically from 728.4 pg ± 47.0 pg and 4890.0 pg ± 200.2 pg to 460.1 pg ± 38.7 pg and 3547 pg ± 138.4 pg, respectively (Table 4).

Conclusion

In China, the incidence of cervical cancer is 14.6/100,000. Among all ethnic groups, the Uygur women have the highest incidence. A census in 2004 showed that the prevalence of cervical cancer in Uygur

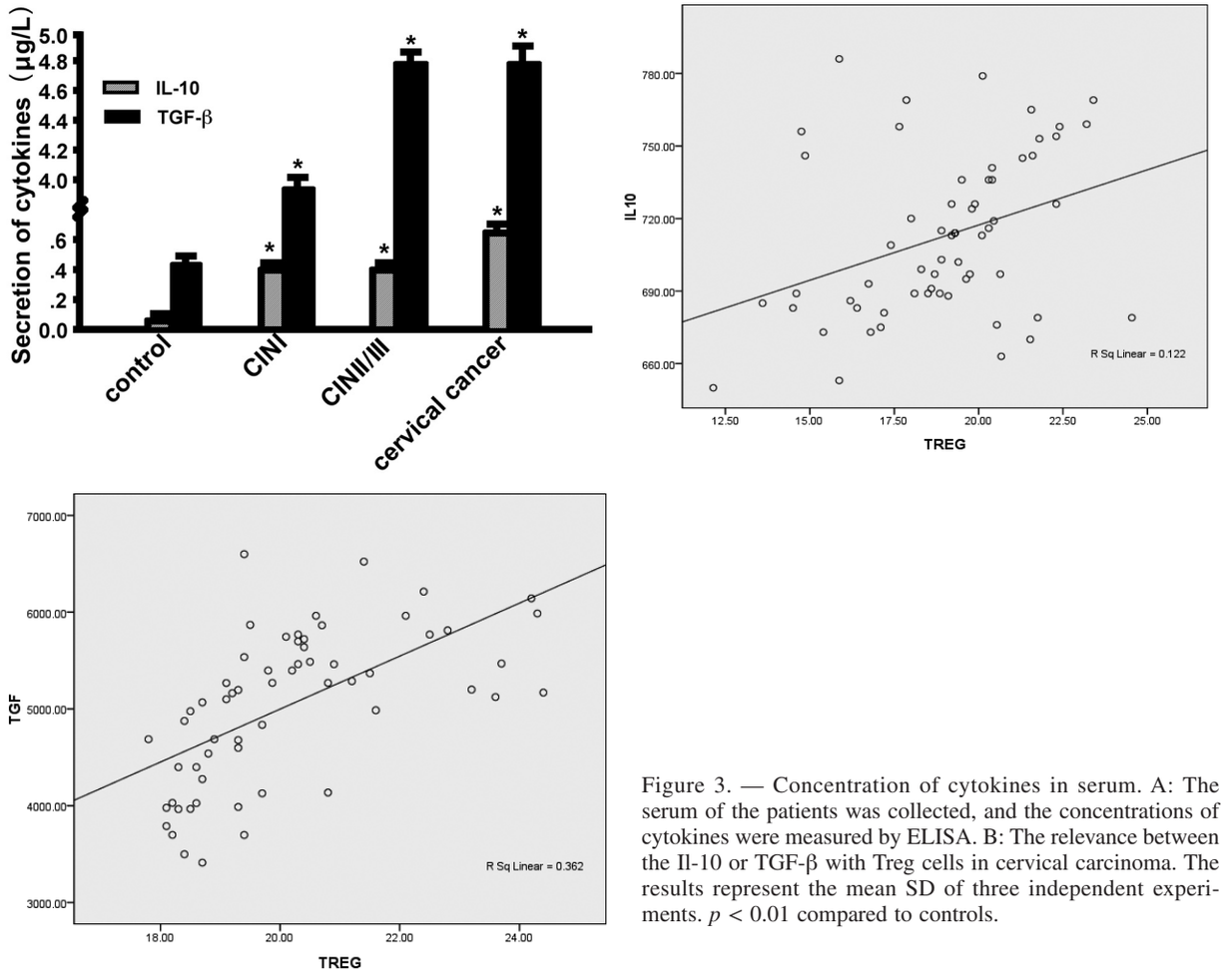

Figure 3. — Concentration of cytokines in serum. A: The serum of the patients was collected, and the concentrations of cytokines were measured by ELISA. B: The relevance between the Il-10 or TGF-β with Treg cells in cervical carcinoma. The results represent the mean SD of three independent experiments. $p < 0.01$ compared to controls.

Table 4. — *Treg and cytokines in surgical patients*.

Surgical	Treg		IL-10 (ng/ml)		TGF-β (ng/ml)	
operation	CIN II-III	Cervical cancer	CIN II-IIII	Cervical cancer	CIN II-III	Cervical cancer
Before	14.7 ± 4.3*	17.48 ± 4.6*	517.4 ± 30.6	728.4 ± 47.0	4908.2 ± 150.3	4890.0 ± 200.2
After	11.5 ± 2.8*	13.70 ± 3.5*	265.2 ± 28.8*	460.1 ± 38.7*	3456.2 ± 170.6*	3547.9 ± 138.4*

The results represent the mean SD of three independent experiments. *$p < 0.01$ compared to controls.

was 527/100,000, which was much higher than the national average. Recently, with the development of or molecular immunology, cervical cancer immunotherapy has become the hotspot for researchers all over the world. More and more clinical studies and animal experimental study confirmed that immunotherapy, cytokine therapy, and adoptive cellular immunotherapy for cervical cancer treatment has an obvious curative effect. However, due to few samples and complicated immune mechanism, it is difficult to assess the exact clinical effect of a certain kind of immunotherapy. There is still a considerable distance for immunotherapy to become a routine treatment. Regulatory T cells (Treg) were found in the peripheral blood circulation of immunocompetent mice by Sakaguchi *et al*. in 1995 [7]. Treg is one of the subsets of the T cell, which has a special function. Treg (CD4+CD25+) cells are essential for immune incompetence of and immune suppression. Treg cells can inhibit T cell immune responses to foreign and self-antigens, thus maintaining their self-tolerance and prevent the immune response to tumor cells, leading to tumor cell immune evasion. Foxp3 is a key control molecule for the development and function of natural CD4+CD25+ Treg cells [8]. The role of Treg cells in tolerance is that natural CD4+CD25+ Treg cells specifically express the transcription factor Foxp3, which controls their development and function in a highly Treg-specific manner [9]. Therefore, Foxp3 can be used as a gold standard for confirming Treg.

The current study found that Treg and tumor occurrence are highly relevant. The key biological characteristics of malignant tumors are the disorder of proliferation and differentiation. From the immunological point of view, the tumor is a host of cells expressing a group of "normal antigens" (over-expression) and/or "abnormal antigens" (genetic modification, mutation or deletion). In vivo, autologous T cells recognize tumor antigens as a normal self-component, thereby resulting in immune neglect and immune escape of the tumor. A mouse tumor model revealed that Treg can inhibit tumor immune response [10]. Recent studies have found that in the area around the infiltrating tumor, exists not only the effective CD8+T or CD4+T cells, but also contains a large number of regulatory T cells, mainly CD4 + CD25 + Treg cells [11].

Further studies have shown that in colon cancer, ovarian cancer, lung cancer, breast cancer, pancreatic cancer, and other tumors, the CD4+CD25+ Treg cells increased in peripheral blood and local tumor [11]. This signifies that CD4+CD25+ Treg cells may have a role in the tumor immune process. Treg cells normally inhibit the generation of effective T cell-dependent anti-tumor immune responses which has been was confirmed by depletion of Treg cells using CD25-specific mAbs [10, 11]. Substantial evidence confirms that in a clinical setting, the prevalence of Treg cells was found to be increased in the peripheral blood and tumour microenvironment of carcinoma patients [12]. In humans, Treg cells have been demonstrated to impair cytotoxic T lymphocyte (CTL) function in the setting of cancer [13]. The results in this study showed that cervical lesions and the CD4+ / CD8+T cell ratio showed no significant difference ($p > 0.05$). However, for the cervical cancer and normal control groups, CD4 + CD25 + Foxp3+ Treg accounted in the proportion of CD4+ T lymphocytes is $20.48 \pm 5.78\%$ and $11.87 \pm 6.52\%$, respectively, and there was significant difference between them ($p < 0.05$). It is speculated that during the development of cervical cancer, CD4+CD25+Foxp3+ Treg cells play an important role. In patients with cervical cancer, Treg cell mediated immune tolerance and tumor growth is highly correlated and this may directly affect the process of tumor development. The number of Treg cells in blood is gradually increased with the order of normal control, CIN I, CIN II-III, and cervical cancer. Moreover, there was a significant difference between CIN II-III, cervical cancer groups and normal control and CIN I groups. This signifies that with the progression of cervical cancer, the number of CD4+CD25+Foxp3+ Treg increased gradually, which have may inhibited immune responses, promoting tumor growth and metastasis. In this study, the authors demonstrated that there are imbalances between Treg cells and effect T cells [14]. There was no significant decrease in CD8+T cells, but both CD4+CD25+Foxp3+Treg cells and Il-10 or TGF-β increased in CIN and cervical carcinoma: they suppressed the anti-tumor immunity. There was a significant relation between Treg cells and Uygur cervical carcinoma. In the current study, the authors evaluated the function of Treg, dendritic cells (DCs), and CTLs around the diseased regions. As it is known the function of DCs and CTLs was impaired. The authors hypothesized that the increased Treg suppressed the DCs and CTL, and caused decreased immunosurveillance. Moreover, the results show that after surgery, Treg cells and related cytokines were decreased. Therefore, depletion of Treg cells in combination with other anti-tumor therapies could optimize eradication of malignancies.

A number of studies have found that Treg and Foxp3 expression increased in many cancers [15, 16]. Foxp3 is a key control molecule for the development and function of natural CD4+CD25+ Treg cells [17]. The role of Treg cells in tolerance is that natural CD4+CD25+ Treg cells specifically express the transcription factor Foxp3, which controls their development and function in a highly Treg-specific manner [18]. Disruption of the Foxp3 gene, which results in production of a structurally abnormal protein, blocks the development of natural Treg cells or produces dysfunctional Treg cells [19], and causes autoimmune disease. Foxp3 is currently the most specific and reliable molecular marker for natural Treg cells in humans. Yagi *et al*. [20] found that in patients with metastatic melanoma, the number of Treg in metastatic lymph nodes was two times higher than normal lymph nodes, and highly expressed in Foxp3. In this study, RT-PCR was used to detect expression levels of Foxp3; the results showed that the expression levels of Foxp3 in Uygur patients gradually increased from normal control, cervical CIN to cervical cancer. There was a statistically significant difference between normal controls, CIN I and CIN II-III, cervical cancer. The results showed that Foxp3 has a positive correlation with the stage of CIN and cervical carcinoma. Combining the Treg findings, this is possible because of Uygur CD4 + CD25 + Treg cell number increased, so that the Foxp3mRNA expression level increased. The inhibition function of Treg not only maintains their constituents in tolerance, but also prevents the body from immune responses, resulting in cervical cancer cell immune evasion. The authors also studied the correlation between Treg and Foxp3, and the data confirmed that there is a positive correlation between them. The results showed that Foxp3 may be the main regulatory genes of CD4 + CD25 + Treg, and Foxp3 is an important switch for Treg cell development.

Chen *et al*. [21] first proved that TGF beta can stimulate activated CD4+CD25+T cells express Foxp3 in vitro. Ghiringhelli *et al*. [22] also found that tumor cells not only secrete TGF - beta and or IL-10, but can stimulate immature myeloid DC changes producing TGF-beta, thereby facilitating a tumor microenvironment, promoting CD4+CD25- T cells transformation into CD4+CD25+Treg cells. IL-10 is essential for induction of antigen-specific Treg in vivo. Lundqvist *et al*. [23] found that mature DC can produce high levels of IL-10 and low levels of IL-2 to induce tumor-specific Treg cell generation. As a soluble mediator, IL-10 is often crucial for the maintenance of homeostasis and plays a pivotal role in Treg cell function. Treg cells from IL-10-/- mice have been shown to be significantly less potent than wild-type counterparts. In cervical carcinoma, patients

produce higher concentrations of IL-10, which have also been shown to exert suppressive effects on DCs [24]. They can decrease anti-tumor immunity indirectly. Moreover, the role of IL-10 also implicates TGF-β, the production and action of these two cytokines being interrelated and involving positive feedback loops. IL-10 may act locally at the site of inflammation, while TGF-β seems to have a more systemic effect on immune response. Although, the requirement for TGF-β expression by Treg cells in vitro and in vivo are controversial [25], the results in this study indicated that TGF beta and IL-10 in patients with cervical cancer and cervical CIN was significantly higher than that in the normal control and CIN I group and had a high correlation with Treg cells and stage of CIN. Moreover, after surgery, TGF-β and IL-10 levels have decreased, suggesting that increased TGF-β and IL-10 promoted the increase of CD4 + CD25 + Treg; this is conducive to tumor growth in vivo. It appears that regulation is dictated primarily by both Treg cells and soluble cytokine, such as IL-10 and TGF-β [26].

In conclusion, the authors provided evidence that Treg cells, especially CD4+CD25+Foxp3+ Treg cells, and soluble cytokine, IL-10 and TGF-β mediated the anti-tumor immune response. Therefore, these factors may promote researchers to investigate their therapeutic potential. Treg cells could be used as an immunotherapeutic tool by blocking their suppressive activity in anti-tumor immunity or vaccine development. Therefore, depletion of Treg cells in combination with other anti-tumor therapies could optimize eradication of malignancies, which may construct a theoretical framework for therapeutic use.

Acknowledgments

This work was supported by Grant, 81101555 from the National Natural Science Foundation of China (NCSF), China. *Contribution* Co-first authors: Qi Xu.

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