

## RESEARCH ARTICLE

# Effects of Gastric Cancer Cells on the Differentiation of Treg Cells

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### Abstract

The aim of this study was evaluated the prevalence of Treg cells in peripheral blood in patients with gastric cancer, and investigate the effect of gastric cancer cells on their differentiation. ELISA was employed to assess the concentrations of TGF- $\beta$  and IL-10 in gastric cancer patients' serum. Then, mouse gastric cancer cells were co-cultured with T lymphocytes or T lymphocytes + anti-TGF- $\beta$ . Flow cytometric analysis and RT-PCR were then performed to detect Treg cells and TGF- $\beta$  and IL-10 expression in gastric cancer cells. Our data showed that the expression of TGF- $\beta$  and IL-10 in the patients with gastric cancer was increased compared to the case with healthy donors. The population of Treg cells and the expression levels of TGF- $\beta$  and IL-10 in the co-culture group were much higher than in the control group (18.6% vs 9.5%) ( $P < 0.05$ ). Moreover, the population of Treg cells and the expression levels of TGF- $\beta$  and IL-10 in the co-culture system were clearly decreased after addition of anti-TGF- $\beta$  (7.7% vs 19.6%) ( $P < 0.01$ ). In conclusion, gastric cancer cells may induce Treg cell differentiation through TGF- $\beta$ , and further promote immunosuppression.

**Keywords:** Gastric cancer cells - immunosuppression - Treg cells - TGF- $\beta$  - IL-10

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### Introduction

Gastric cancer is the fourth most common causes of cancer death in China (Thun et al., 2010). It is well known that patients with gastric cancer have a poor immune response. Recently, a significantly increase of TGF- $\beta$  expression has been described in gastric cancer cells (Achyut et al., 2011; Shen et al., 2012). The present studies have established that TGF- $\beta$  plays a central role in mediating T cell differentiation by enhancing Treg cell development and inhibiting effector TH cell differentiation (Regateiro et al., 2011; Kue et al., 2013).

Treg cells, characterized by co-expression of CD4 and CD25 markers, are thought to be a functionally unique population of T cells and function to maintain immune homeostasis (Nakamura et al., 2007; Lindau et al., 2013; Stelmaszczyk-Emmel et al., 2013). Patients and experimental models with cancer showed that Treg cells down-regulated the activity of effector function against tumors, resulting in T-cell dysfunction in cancer-bearing hosts (Weiss et al., 2012; Sakuishi et al., 2013). An increased population of Treg cells was reported in patients with ovarian cancer (Wicherek et al., 2011), lung cancer (Erfani et al., 2012), and breast cancer (Decker et al., 2012). However, there are no previous reports describing Treg cells in gastric cancer. And little is known about whether gastric cancer cells induced the Treg cells through TGF- $\beta$ .

Thus, the aim of this study was to evaluate the prevalence of Treg cells in peripheral blood in patients with gastric cancer, and further investigate the role of gastric cancer cells on the differentiation of Treg cells.

### Materials and Methods

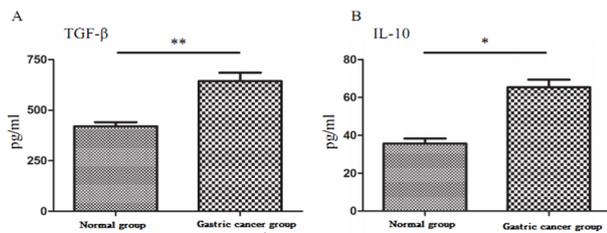
#### *Experimental animals*

C57/BL6 was purchased from SLRC company, Shanghai, China. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Zhumadian Central Hospital.

#### *Determination of TGF- $\beta$ and IL-10 concentrations in serum*

The concentrations of TGF- $\beta$  and IL-10 in serum were measured by ELISA (R&D Systems Inc, Minneapolis, MN, USA) according to the manufacturer's instructions. Serum or supernatants were incubated with biotinylation antibody and then enzyme conjugate at room temperature. Then, stop buffer were added to each well and absorption at wavelength of 450nm measured by a spectrophotometer. A standard curve was generated to calculate the concentration of TGF- $\beta$  or IL-10 for each set of samples

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**Figure 1. Expression of TGF-β and IL-10 in Sterums of Healthy Human and Gastric Cancer Patients** (Number of samples in each group n=6, the assay was repeated for 3 times)

assayed. To control for the TGF-β or IL-10 concentration in the culture medium, we set a well of culture medium without cells and used it as a baseline to be subtracted for all samples assayed.

*Cell preparation*

PBMCs were purified from lymphaden and spleen of mice (C57/BL6) by nylon membrane followed by centrifuged at 1500\*g for 5 min and then resuspended with RPMI 1640 (Gibco, Carlsbad, CA, USA).

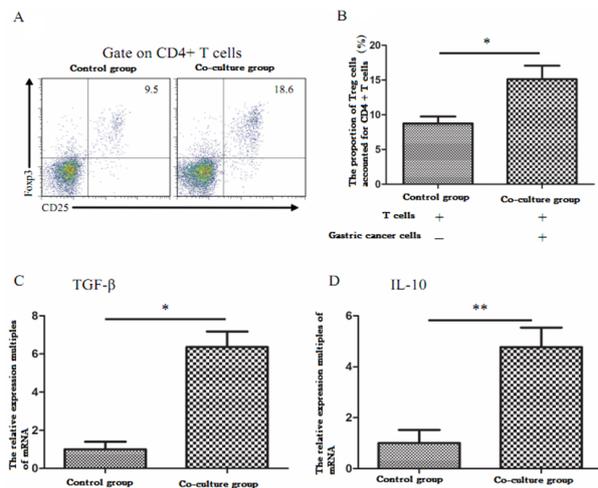
For separation of T cells, PBMCs were further separated with immunomagnetic beads according to the manufacturer’s guidelines. After that, monocytes were cultured in RPMI 1640 supplemented with 10% FCS (Gibco, Carlsbad, CA, USA) and 50 mg/ml penicillin/streptomycin(Gibco, Carlsbad, CA, USA) in a humidified incubator containing 5% CO<sub>2</sub> at 37 °C. Cells were fed three times a week and passaged every 7 days.

*Co-culture of gastric cancer cells with T lymphocytes*

The MFC is a cell line that is established from mice gastric cancer. MFC cell line was maintained in RPMI 1640 as described previously. For comparison, we divided these cells into three groups, co-culture of MFC cells with T lymphocytes were defined as co-culture group, co-culture of MFC cells with T lymphocytes + anti-TGF-β were defined as anti-TGF-β group, without any treatment was defined as control group. MFC cells were mixed with T lymphocytes or T lymphocytes + anti-TGF-β at a certain ratio and then cultured in a humidified incubator containing 5% CO<sub>2</sub> at 37 °C. 42 h after co-culture, cultivation system were added with 1 μg/ml PMA, 50 μg/ml Ionomycin and 0.7 μl/ml Golgistop and then incubated under 37 °C for another 6 h. MFC was purchased from Yanhui Bio Company, Shanghai, China.

*Flow cytometric analysis*

Cells were stained for cell surface molecules to determine their immunophenotype with 0.5 mg/ml FITC-CD4, 0.2mg/ml APC-CD25 and 0.2 mg/ml PE-FoxP3 antibodies (eBioscience, SanDiego, CA, USA). Isolated T cells were stained with titrated amounts of Ab and washed once. Anti-TGF-β mAb (R&D Systems Inc, Minneapolis, MN, USA) was detected by a Pelabeled rabbit-antimouse mAb (DAKO, Glostrup, Denmark), according to the manufacturer’s instructions. Triple- or four-color flow cytometry was performed using FACSCalibur (Becton Dickinson, San Jose, CA, USA). Cells were analyzed using CellQuest software (Becton Dickinson, San Jose, CA, USA).



**Figure 2. The Population of Treg Cells and the Expression of TGF-β and IL-10 in Control and Co-Culture Groups** (Number of samples in each group n=6, the assay was repeated for 3 times)

*RT-PCR*

Total RNA was extracted with miRNeasy Mini Kit (Qiagen, Hilden, Germany), and cDNA was prepared with ReverAid™ First Strand Cdna Synthesis Kit (Thermo Scientific, Rockford, IL, USA), and then RT-PCR was performed using the FastvStart Universal SYBR Green Master Kit (Roche, Basel, Swiss) following the manufacturer’s protocol.

*Statistical analysis*

Statistical analysis was performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). All values were expressed as mean± SD. Differences between the values were determined using Student’s t test. Comparisons were made by using LSD method. A P<0.05 was considered as statistically significant.

**Results**

*Increased concentrations of TGF-β and IL-10 in serum of patients with gastric cancer*

After collecting the blood samples, we detected the expression levels of TGF-β and IL-10 in the patients by ELISA. As shown in Figure 1A, 1B, the concentrations of TGF-β and IL-10 in Gastric cancer group were significantly higher than that in Normal group (P<0.05). These results indicated that there were high expression of TGF-β and IL-10 in gastric cancer patients.

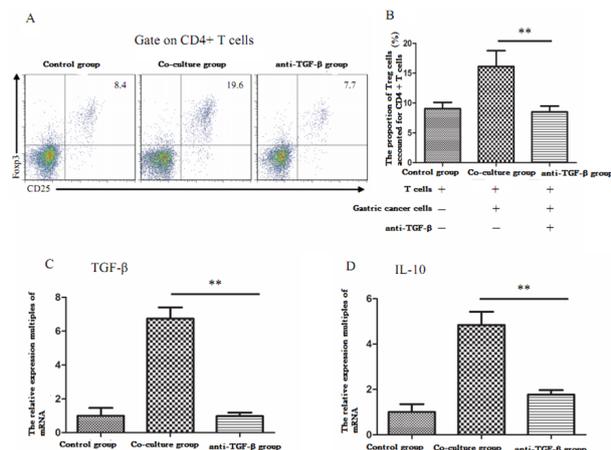
*Induction of gastric cancer cells on Treg cells differentiation*

With the purpose of understanding the induction of gastric cancer cells on Treg cells differentiation, we carried out an assay that of co-culture of gastric cancer cells with T lymphocytes to analysis the change of Treg cells population. Figure 2A, 2B showed that the population of Treg cells in Co-culture group was much higher than Control group (18.6% vs 9.5%). Moreover, the expression levels of TGF-β and IL-10 in Co-culture group were obviously improved than in Control group (P<0.05) (Figure 2C, 2D).

**Table 1. Induction of Gastric Cancer Cells on Treg Cells Differentiation**

	n	Treg cells population	TGF- $\beta$	IL-10
A1: Control group	18	0.084 $\pm$ 0.024	1.09 $\pm$ 0.42	1.21 $\pm$ 0.35
A2: Co-culture group	18	0.196 $\pm$ 0.055	6.72 $\pm$ 0.73	4.82 $\pm$ 0.52
A3: anti-TGF- $\beta$ group	18	0.077 $\pm$ 0.019	1.12 $\pm$ 0.18	1.95 $\pm$ 0.20
Entirety Comparison F, p	60.66, 0.000	766.18, 0.000	453.71, 0.000	
Multiple comparison LSD-t, p	A2vsA1	9.24, 0.000	33.99, 0.000	28.51, 0.000
	A3vsA1	0.58, 0.564	0.18, 0.856	5.84, 0.000
	A3vsA2	9.82, 0.000	33.81, 0.000	22.67, 0.000

n, number of samples  $\times$  number of repeated trial; one-factor analysis of variance was performed for the comparison of each index between the three groups; all data were analyzed by test of normality



**Figure 3. The Population of Treg Cells and the Expression of TGF- $\beta$  and IL-10 in Co-culture and anti-TGF- $\beta$  Groups** (Number of samples in each group n=6, the assay was repeated for 3 times)

#### Induction of TGF- $\beta$ on Treg cells differentiation

We further compared the population of Treg cells and the expression levels of TGF- $\beta$  and IL-10 between Co-culture group and anti-TGF- $\beta$  group, for determining whether gastric cancer cells induced the Treg cells through TGF- $\beta$ . As presented in Figure 3A, 3B, the population of Treg cells in and anti-TGF- $\beta$  group was significantly lower than Co-culture group (7.7% vs 19.6%). Furthermore, we found that the expression levels of TGF- $\beta$  and IL-10 in the co-culture system were clearly decreased after added with anti-TGF- $\beta$  ( $P < 0.01$ ) (Figure 3C, 3D). In addition, there were statistically differences between the Treg cells population and expression of TGF- $\beta$  and IL-10 between the three groups after one-factor analysis of variance, and there were also obviously significant in Co-culture group compared with the other two groups after LSD method ( $P < 0.01$ ) (Table 1).

#### Discussion

It is well known that cell-mediated immunity in cancer hosts is suppressed by many factors (Narita et al., 2013). As an additional explanation for impaired cell-mediated immunity in cancer hosts, the increased prevalence of Treg cells could be included. Considering the present study and previous reports, the fact that an increased population of CD4 / CD25 Tregs is observed in peripheral blood and tumor microenvironments in patients with cancer is established. But there is no clear evidence for the mechanisms of induction of Treg cells in cancer hosts. A

better understanding of the underlying mechanism of Treg cells regulation or of the strategy for controlling Treg cells may lead to more effective immunotherapy for cancer.

In the present study, we showed increased TGF- $\beta$  and IL-10 expression in the patients with gastric cancer in comparison with healthy donors. Santinet et al. (Santinet et al., 2001) found a higher expression levels of TGF- $\beta$  among PBMCs in oophoroma patients. This was similar with the result made in our paper. High expression of TGF- $\beta$  and IL-10 in gastric cancer patients indicated that there were immunosuppression in the patients.

Transforming growth factor-beta (TGF- $\beta$ ) is a pleiotropic cytokine that plays a pivotal role in regulating cell growth and differentiation in a variety of cell types (Li et al., 2006). Although TGF- $\beta$  has been intensively investigated in a variety of tumor types, studies have focused on the effects of TGF- $\beta$  on the malignant cells and very few studies have explored the effects of TGF- $\beta$  on T cell differentiation.

Co-culture assay was performed and results showed that the population of Treg cells and the expression levels of TGF- $\beta$  and IL-10 increased after Co-culture (Mishra et al., 2005). Moreover, we found that Treg cells population and TGF- $\beta$  and IL-10 expression in the co-culture system were significantly decreased after added with anti-TGF- $\beta$ . This result was accordance with the report that Huber (Huber et al., 2006) made in 2006. Data generated from this study strongly suggest that gastric cancer cells may induce the Treg cells through TGF- $\beta$ . Thus, depletion of Tregs may become a successful anticancer strategy, and manipulation of Tregs in terms of their frequency and functional activity should be added to the therapeutic armamentarium for enhancing tumor immunity in humans (Zou, 2006).

In conclusion, our data indicated that gastric cancer cells may play a important role in inducing Treg cells differentiation through TGF- $\beta$ , and further promote the generation of immunosuppression. Nonetheless, further functional studies will be necessary to elucidate the detailed mechanisms.

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The author(s) declare that they have no competing interests.

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