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Original Article

Limited Efficacy of Decreased Tumor-infiltrating Regulatory T Cells after Transforming Growth Factor-beta Blockade in Murine Pancreatic Ductal Adenocarcinoma

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Abstract

Background and objectives: Regulatory T cells (Tregs) are a vital cell subset that induces immune tolerance in the tumor microenvironment by secreting suppressive cytokines and inhibiting innate immune cells. Transforming growth factor-beta (TGF-β) plays an important role in this process. However, the effect of TGF-β blockade on intratumoral Tregs and its specific biological role remains unclear.

Methods: Quantitative and functional changes in Tregs were evaluated after TGF-β blockade with gradient doses of monoclonal antibody 1D11 in a murine pancreatic ductal adenocarcinoma model.

Results: The number of tumor infiltrating Tregs decreased significantly (high dose, low dose and control, 38.6 ± 8.1, 38.6 ± 1.8, 74.6 ± 4.9 /40× field, p = 0.024) after 1D11 administration, while $CD8^+$ T cells in the tumor microenvironment significantly increased in the low dose group but reversed in the high dose group (3.1 ± 1.4, 12.3 ± 2.1, 5.4 ± 0.5 /40× field, p = 0.016). The frequency of $CD4^+$, $CD8^+$ or Treg cells in the peripheral blood and spleen showed no significant change. The typical cytokines TGF-β and inerleukin-10 secreted by Tregs as well as interferon-γ produced by cytotoxic T cells in tumor tissues did not change compared with the controls.

Conclusions: TGF-β blockade with a monoclonal antibody can reduce Tregs in the tumor niche, however, its therapeutic efficacy in PDAC patients remains limited. Further investigation of combination therapies is required.

Keywords: Pancreatic neoplasm; Antitumor immunity; T cell subset; Cytokines; monoclonal antibody.

Abbreviations: DC, dendrite cell; IFN, interferon; IL, interleukin; MDSCs, myeloid derived suppressor cells; PDAC, pancreatic ductal adenocarcinoma; Tc, cytotoxic T cell; TGF-β, transforming growth factor-β; Treg, regulatory T cell.

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) has the worst prognosis among digestive malignancies. Thus, there is an urgent need to discover new effective treatments. With the increasing incidence of PDAC, the 5-year survival is less than 6% in Shanghai and 10% in the US.[1,2] Although surgery is the only treatment to potentially cure PDAC,[3] survival can be improved by neoadjuvant/adjuvant therapy since only less than one-fifth of PDAC patients is eligible to receive surgery.[4–6]

In the last 20 years, immunotherapy has readily gone from the bench to bedside. Various strategies, including monoclonal antibodies, kinase inhibitors, and immune checkpoint therapies,

have been widely applied and shown to have relatively satisfying outcomes. Treatments focusing on the tumor niche and antitumor immunity were considered the most hopeful therapies to improve PDAC prognosis.[7] However, regardless which immune treatment is administrated, the effect is limited by the immunosuppressive microenvironment of PDAC. Regulatory T cells (Tregs) can induce immune tolerance in the tumor niche through contact-dependent (such as inhibiting the maturation of effector T cells and suppressing cytotoxic T cells (Tc) and contact independent (such as secreting immunosuppressive cytokines and combining interleukin-2) approaches.[8] Therefore, Tregs play a vital role in the escape, metastasis, and invasion of PDAC. In contrast, Tc is the representative of antitumor immunity which can be induced by tumor cells. We have previously reported on Treg and Tc distribution in PDAC and their relationships with survival in postoperative patients. Our work indicated that higher intratumoral Tregs and lower peritumoral Tc were both related to worse prognosis of PDAC,[9] consistent with other studies.[10,11]

Transforming growth factor-β (TGF-β) is a multifunctional polypeptide with three isotypes in mammals that also suppress antitumor immunity in the tumor microenvironment.[12] TGF-β can induce naïve T cells to differentiate into Tregs, which is secreted from various cells such as tumor cells, myeloid derived suppressor cells (MDSCs), dendrite cells (DCs), and macrophages.[13–15] On the other hand, TGF-β is an important suppressive functional molecule in Treg-mediated immune regulation.[16–18] In addition, deletion of Smad4/DPC4 is a very common mutation in high grade pancreatic intraepithelial neoplasm and carcinoma cells, which can break the negative feedback of TGF-β on epithelial cell proliferation.[19] In preclinical studies, silencing TGF-β gene expression or blocking its function with antibodies has been shown to effectively reduce intratumoral Tregs.[20] Thus, neutralizing TGF-β may be an effective treatment for PDAC.

1D11 is a murine originated monoclonal antibody that can recognize and neutralize all three isotypes of TGF-β.[21] 1D11 has already been widely used in preclinical studies for breast cancer,[22] glioma,[23,24] and kidney cancer.[25] Some studies indicated that 1D11 could decrease tumor-infiltrating Tregs[25] and inhibit $CD4^+CD25^-$ T cells from differentiating into $CD4^+CD25^+$ Tregs.[26] Furthermore, 11D1 increased the amount and cytotoxic activity of tumor infiltrating Tc by promoting secretion of granzyme B and perforin.[22,27]

We previously collected five PDAC tissue samples and analyzed them using an Agilent whole genome expression array. The results showed that both CD25 and TGF-β expression were upregulated and CD8 expression was downregulated (data not published). Therefore, we hypothesized that Tregs and TGF-β are increased in the tumor microenvironment, following inhibition of $CD8^+$ Tc cells. In this study, we blocked TGF-β signaling with 1D11 and evaluate the effect in a murine PDAC model. Specifically, we analyzed changes in tumor volume, frequencies of T cell subsets in the peripheral blood, spleen and tumor tissues, and intratumoral cytokines.

Materials and methods

Animals and cell line

Six- to eight-week old female C57BL/6 mice were purchased from the SLAC laboratory animal (Shanghai, China) and kept under barrier condition in the Department of Laboratory Animal Science, Fudan University. All protocols were approved by the animal experiment ethics board of Fudan University (Supplementary File 1).

The murine PDAC cell line Panc02 was a kind gift from Johns Hopkins Hospital, USA. The cells were cultured in RPMI-1640 (Gibco, Carlsbad, CA, USA) supplemented with 10% FBS (Gibco, Australia) and 1% penicillin/streptomycin (Gibco, Carlsbad, CA, USA), and incubated at 37°C and 5% CO_2.

Tumor implantation, TGF-β blockade, and sample harvest

Before the experiment, the mice had one week to acclimate to the laboratory environment. To establish the murine PDAC model, Panc02 cells were suspended in serum-free RPMI at the concentration of 1×10^6 /0.1 mL and then subcutaneously injected in the right flank of the mice. Palpable tumors typically formed two to three weeks after inoculation. Eighteen mice were randomly divided into three groups as follows: high dose group (100 μg/dose), low dose group (50 μg/dose), and control group (PBS, phosphate buffer solution). Mice with ulcer on the tumor were precluded because of the possible influence on immunity.

The anti-TGF-β antibody (Clone: 1D11, R&D System, Minneapolis, MN, USA) was diluted with sterilized PBS into 50 μg /0.1 mL and 25 μg /0.1 mL, and PBS alone was administrated as control. Mice in each group were injected with 0.2 mL 1D11 or PBS intraperitoneally every three days, for a total of three times. Before injection, the long (a) and short (b) diameters of the tumors were measured with electronic caliper and the volume was calculated as $a \times b^2/2$.

Within 24−48 hours after the final administration, mice were anaesthetized with 1% pentobarbital and tumor volumes were measured. Blood samples were collected via orbital extraction and anticoagulated with EDTA. Tumor tissues were resected under sterile conditions and laparotomy was performed to harvest the spleen.

Flow cytometry

Erythrocytes in the blood samples were lysed with 1× ammonium chloride lysis buffer. The spleen was processed using the GentleMACS Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany) in protocol m_spleen_02 to obtain single cell suspensions. All cell suspensions were washed and resuspended with stain buffer at about $1-5 \times 10^7$ cells/mL. In this panel, fluorescence combined antibodies included FITC CD3 Molecular Complex (561798), PE-Cy7 CD8a (552877), PerCP-Cy5.5 CD4 (550954), APC CD25 (557192), PE CD127 (552543). All antibodies were from BD (San Jose, CA, USA). After incubation for 20 m at room temperature, the samples were washed and analyzed using a BD FACS Aria II flow cytometer. OneComp eBeads™ (eBioscience, Carlsbad, CA, USA) were applied to calculate compensation. Lymphocytes were gated with FSC×SSC characters and T cells were further gated as CD3+ cells. Under the T cell gate, the proportions of CD4+ T helper (Th) cells, CD4+CD25+CD127low/− Tregs, and CD8+ Tc were also recorded. All analyses were performed using FlowJo v10.0.7 (Tree Star, Inc., USA).

Immunohistochemistry

Tumor tissues were dissected into pieces for different analyses. Samples were fixed with neutral formaldehyde for at least 24 h and embedded in paraffin. Tissues were sectioned at 5 μm thickness and deparaffinated in gradient ethanol. Antigen retrieval was performed with EDTA retrieving solution and endogenous peroxidase was inactivated with 3% H_2O_2. Two primary antibody pairs were

Table 1. Tumor volume during 1D11 administration (n = 4–6)

Intervention	n	Day 1	Day 4	Day 7
PBS	4	17.5±8.5	28.7±6.5	55.1±33.1
1D11 50μg	4	12.1±5.7	20.3±8.2	42.7±35.9
1D11 100μg	6	12.8±8.5	21.5±18.6	75.9±61.6
p-value		0.601	0.402	0.079

PBS, phosphate buffer solution.

applied separately: rabbit anti-mouse FoxP3 (MAB8214, R&D Systems, 1:125, Minneapolis, MN, USA) was mixed with mouse anti-mouse TGF-β (MAB1835, R&D Systems, 1:50, Minneapolis, MN, USA) or rat anti-mouse interleukin (IL) -10 (ab189392, Abcam, 1:100, Cambridge, MA, USA), while rat anti-mouse CD8a (14-0808, eBioscience, 1:100, Carlsbad, CA, USA) was stained alone. The double staining was processed using the MultiVision™ Polymer Detection System (TL-012-MHRA, Thermo Scientific) following the manufacturer's instructions. The rabbit originated primary antibody stained blue and the mouse/rat originated primary antibody stained red. Finally, slides were counterstained with hematoxylin and mounted with Clearmount™ as previously publication.[28] Since the density of different lymphocyte subsets varies across slides, we chose the 4−5 fields with the highest cell density under low power (4×) and then counted CD8+ cells, FoxP3+ cells, FoxP+IL-10+ cells, and FoxP3+TGF-β+ cells in these high-power fields (40×). The means of these fields were finally calculated for statistical analyses.

Enzyme linked immunosorbent assay

Parts of the tumor tissue samples were frozen at -80°C for later procedures. Every 10 mg tissue was incubated with 1.000 mL Radio Immunoprecipitation Assay (RIPA) lysis buffer (Beyotime, Shanghai, China) supplemented with 1 mmol/L phenylmethanesulfonyl fluoride (Beyotime, Shanghai, China) and processed using the GentleMACS Dissociator in protocol Protein_01 for receiving tissue homogenate. Total protein in each sample was measured using a BCA protein assay kit (Beyotime, Shanghai, China), and cytokines including TGF-β, IL-10, and interferon (IFN)-γ were measured using an ELISA kit (Quantikine, R&D Systems, Minneapolis, MN 55413 USA) separately. All cytokines were normalized to the total protein concentration.

Statistical analysis

Data are shown as mean ± standard deviation. Statistics were performed using IBM SPSS Statistics Version19. Data were analyzed using the one-way ANOVA test or rank sum test as appropriate. Once the significance among the groups was indicated, the LSD method was used for further comparison. A p-value <0.05 was considered as statistical significance.

Results

Tumor volume variation after TGF-β blockade

Sixteen to nineteen days after implantation, a palpable tumor was found on the flank of mice. On the first day of antibody administration, the average tumor sizes were 17.5 ± 8.5, 12.1 ± 5.7, and 12.8 ± 8.5 mm^3 in the control, low-dose, and high-dose groups, respectively. During TGF-β blockade, the average volume of tumors increased to 28.7 ± 6.5, 20.3 ± 8.2, and 21.5 ± 18.6 mm^3 on day 4 and 55.1 ± 33.1, 42.7 ± 35.9, and 75.9 ± 61.6 mm^3 on day 7. No significant difference was found between the TGF-β blockade and control groups (p = 0.601, 0.402, and 0.079 on days 1, 4 and 7; Table 1).

Distribution of T cell subsets in the peripheral blood and spleen

No significant change of T cell subsets was found in the peripheral blood or spleen following systemic administration of the TGF-β blocking antibody (Fig. 1). In the high-dose, low-dose, and control groups, the average proportions of Th were 53.1 ± 4.2%, 54.7 ± 3.6%, and 54.4 ± 1.8%, respectively; Tregs were 5.64 ± 0.31%, 5.55 ± 0.22%, and 5.81 ± 0.40%, respectively; and Tc were 31.7 ± 1.8%, 30.3 ± 0.8%, and 31.3 ± 3.1%, respectively in the peripheral blood (p = 0.728, 0.514, and 0.603, respectively between doses). The average proportions of Th cells were 55.5 ± 4.0%, 55.6 ± 2.1%, and 55.4 ± 1.4%, respectively; Tregs were 8.44 ± 1.30%, 8.99 ± 0.52%, and 9.00 ± 1.17%, respectively; and Tc were 25.2 ± 1.7%, 27.1 ± 3.1%, and 27.8 ± 2.2%, respectively the in spleen (p = 0.990, 0.700, and 0.270, respectively between doses).

Variations in tumor-infiltrating T lymphocytes

We observed that the numbers of tumor-infiltrating lymphocytes in the tumor microenvironment were significantly different after TGF-β blockade. In the high-dose, low-dose, and control groups, the average numbers of tumor-infiltrating $FoxP3^+$ cells were 38.6 ± 8.1, 38.6 ± 1.8, and 74.6 ± 4.9. These numbers were significantly lower (p = 0.03 for the high-dose group; p = 0.04 for the low-dose group) than the control, but there was no significant between two doses (p = 0.90). In all groups, the tumor-infiltrating $FoxP3^+$ cells were significantly decreased after TGF-β blockade (p = 0.024, one-way ANOVA; Fig. 2a). Additionally, the average number of tumor-infiltrating $CD8^+$ cells was significantly different in the high-dose (3.1 ± 1.4), low-dose (12.3 ± 2.1), and control (5.4 ± 0.5) groups (p = 0.016; Fig. 2a). Moreover, the number of Tc cells increased in the low-dose group (p = 0.020) and decreased in the high-dose group (p = 0.924) compared with control. This difference requires further investigation.

Cytokine variations in tumor tissues

In the high-dose, low-dose, and control groups, the average concentrations of TGF-β1 were 965.2 ± 225.7, 1,774.1 ± 707.7, and 1,002.3 ± 133.4 pg/mg, respectively; IL-10 was 13.2 ± 3.2, 18.1 ± 3.0, and 14.4 ± 3.0 pg/mg; and IFN-γ was 21.8 ± 6.2, 28.4 ± 10.5, and 15.1 ± 5.6 pg/mg, respectively (Fig. 2b). None of these results was statistically significant (p = 0.056, 0.094, and 0.082, respectively between doses). In addition, the cell number per high-power field in the immunohistochemistry double staining analysis was calculated and is presented in Table 2, as well as in Figure 2c and d.

Discussion

In this study, we tested the hypothesis that administration of 1D11 could reduce the number and function of Tregs in PDAC by block-

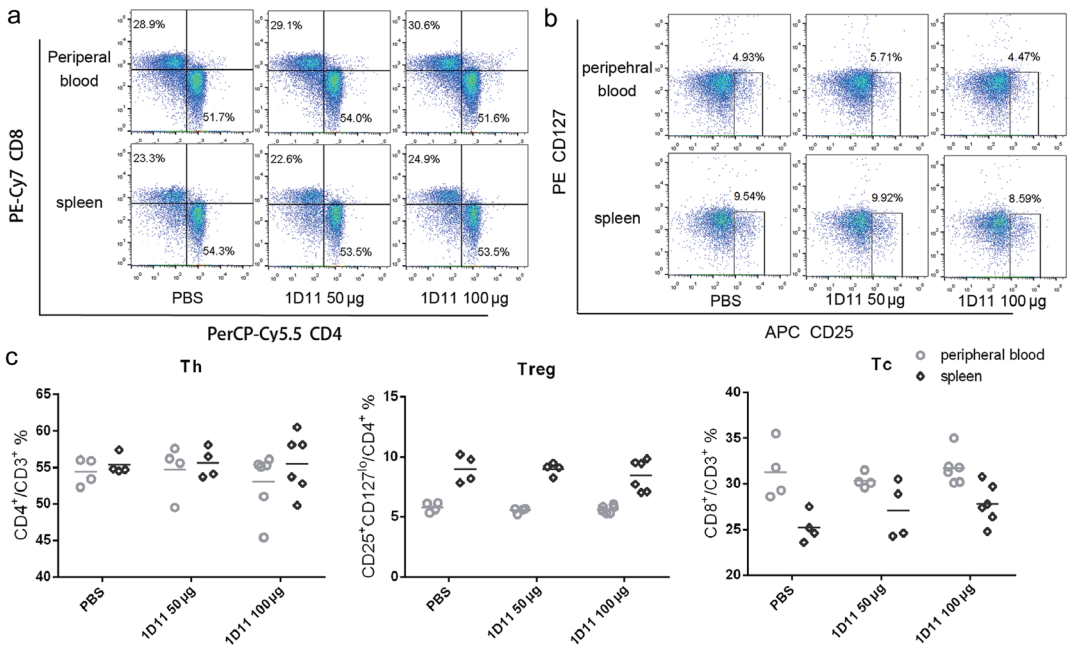

Fig. 1. T cell subset frequencies in peripheral blood and spleen after TGF-β blockade. The frequencies of $CD4^+$ Th, $CD8^+$ Tc (a), and $CD25^+CD127^{low/-}$ Tregs (b) gated on the $CD3^+$ gate in peripheral blood and spleen were assessed by flow cytometry. There was no significant change after 1D11 administration (c).

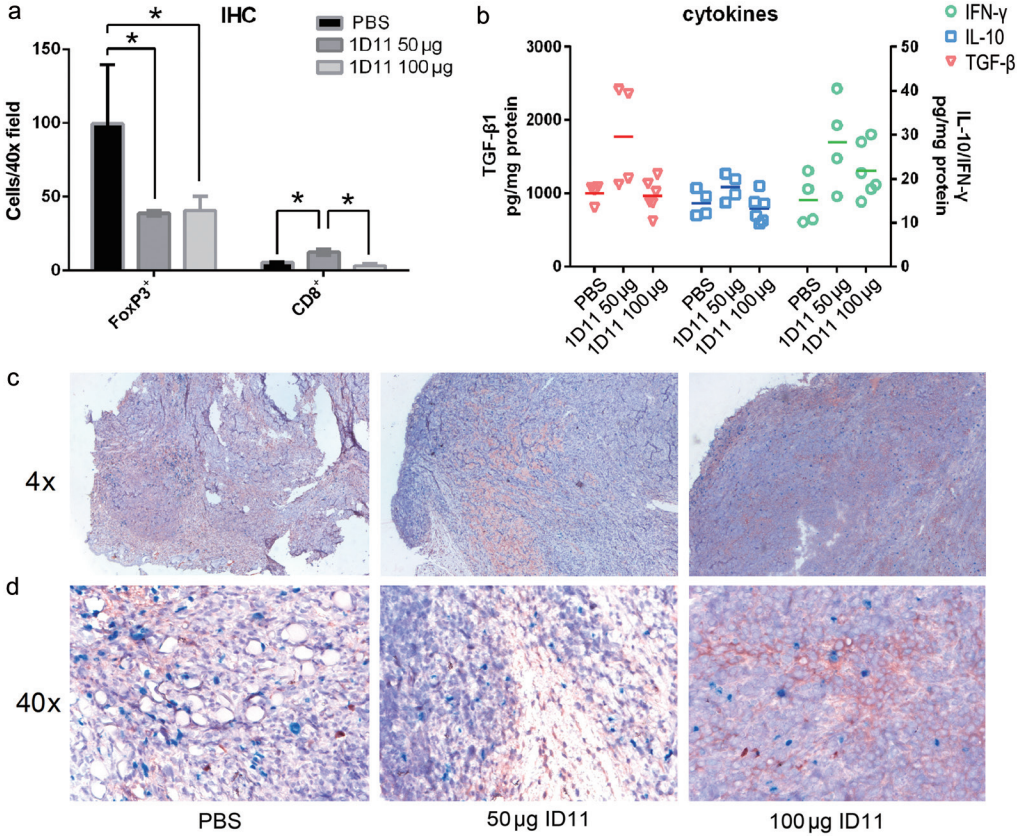

Fig. 2. Variations in tumor infiltrating Tregs, Tc and typical cytokines after TGF-β blockade. The tumor infiltrating Tregs decreased significantly after 1D11 administration while the Tc cells showed the opposite trend (a). The typical functional cytokines of Tregs, such as TGF-β and IL-10, and IFN-γ, were mainly produced by $CD8^+$ Tc and did not vary significantly (b). Representative images of double stained slides (c, d). *$p < 0.05$ where statistics could be calculated.

Table 2. FoxP3 and TGF-β/IL-10 double stained by IHC (n = 4)

Intervention	IL-10$^+$	FoxP3$^+$IL-10$^+$	TGF-β$^+$	FoxP3$^+$TGF-β$^+$
PBS	2.9	0.9	7.0	0.7
50 μg 1D11	2.3	0.8	3.5	0.0
100 μg 1D11	4.6	0.4	3.2	0.2

IHC, immunohistochemistry; PBS, phosphate buffer solution; TGF-β. transforming growth factor-β; IL-10, interleukin-10.

ing TGF-β blockade. However, the results did not support our hypothesis as we observed that only the tumor-infiltrating Tregs were changed significantly. Nor did we observe any significant changes in the T cell subset distribution in peripheral blood or spleen or the typical functional Treg and Tc cell cytokines in the tumor niche. Even in the high-dose antibody group there were no significant changes in the number of tumor-infiltrating Tc.

Tumor growth was not inhibited by 1D11 compared with the control group in this study; however, this finding was not surprising. The effects of monoclonal antibodies have been controversial in previous cancer studies. Two studies focused on glioma showed contrasting results: one identified that 1D11 could only delay the progression of tumor after radiotherapy,[29] while the other showed that a single 1D11 treatment could result in complete remission of tumor in immune sufficient mice.[30] Some studies indicated that TGF-β blockade may induce apoptosis or inhibit resistance to apoptosis.[25,31] In the murine models established with different breast cancer cell lines, the anti-proliferation and induction of apoptosis effect of 1D11 varied considerably;[32] thus, we propose that the tumor inhibitive ability of 1D11 is dependent on tumor type, host immune status, and experimental design.

Numerous studies have already demonstrated that TGF-β blockade could effectively decrease tumor-infiltrating Tregs and inhibit cytokine secretion in animal models. In a renal carcinoma model, 1D11 inhibited the differentiation of Tregs induced by TGF-β.[26] Another murine sarcoma model also revealed that 1D11 administration after resection of the primary site could significantly decrease Tregs in the metastases, reduce serum IL-10 levels, and increase metastases-infiltrating Tc cells and serum IFN-γ,[23] which indicated that TGF-β blockade may confer antitumor immunity through inhibiting Tregs. In a murine pancreatic cancer model established in the Rag-/- mouse and Panc02 which lack mature T cells, 1D11 inhibited FoxP3 expression induced by TGF-β.[33] Our results only indicated that intratumoral Tregs could be reduced by neutralizing TGF-β and that the effect on intratumoral Tc remains uncertain. Previous studies reported that 1D11 could elevate the amount and activity of CD8$^+$ Tc in tumor niches,[24] while there was no change in Tc distribution in the spleen.[22]

Given that the approaches (intravenous or intraperitoneal) and doses (from 5 to 5,000 μg/g body weight) of 1D11 has varied across studies,[27,30,34] it is not surprising that our 1D11 intervention had no effect on lymphocyte distribution in the peripheral blood and spleen or intratumoral cytokines. However, changes in functional cytokines secreted by T cells after TGF-β blockade remains controversial. Several clinical studies showed that cytokines secreted by Tregs may only partially change. A study from Sweden indicated that Tregs proliferated in gastric cancer mucosa with high-levels of IL-10 and only little TGF-β.[35] In contrast, a meta-analysis of 1,407 type 1 diabetes patients and 1,373 healthy controls from 40 case-control studies confirmed that peripheral Tregs and serum TGF-β were reduced in the diabetes patients while the serum IL-10 levels remained in normal ranges.[36] These results suggest that the classical cytokines secreted by a specific T cell subset may not always be related to immune regulatory function. Thus, our results suggest that TGF-β blockade may alter Treg function in a cytokine independent manner in the PDAC model.

Previous studies have also showed that a discrete DC subset both expanded Tregs and suppressed Tc to establish an immunosuppressive microenvironment conducive to metastasis.[37] Given similar results in murine glioma model[29] as well as other models,[22,38] we propose that neutralization of TGF-β may perform better at preventing metastasis compared to treating a primary tumor. A recent study reported that TGF-β failed to alter the frequencies of MDSCs or DCs in the primary tumor but had an effect in liver metastases in a murine model. Expression of programmed death-ligand 1 in macrophages was also increased by TGF-β in another experiment, which indicates that TGF-β plays a suppressive role in the microenvironment of PDAC.[39] Furthermore, combination of adoptive and innate immunotherapy showed a promise in tumor treatments.[40] We also attempted to neutralize TGF-β and block CD25, and found that this combination therapy was beneficial. The Treg frequency in the blood, spleen, and tumor decreased with this approach and tumor growth was also significantly suppressed. This effect has been shown to be further enhanced with an anti-PD-1 antibody.[41] In addition, since the stromal plays a relatively important role in PDAC, and TGF-β plays an important role in tumor angiogenesis, cell apoptosis, collagen synthesis, and stromal cell differentiation, more attention should be given to these non-immune related aspects to comprehensively evaluate the effect of TGF-β blockade in PDAC.[42] Moreover, a recent report revealed that the tumor-promoting function of TGF-β was induced by MUC1 overexpression.[43] As MUC1 is not expressed in the Panc02 cell line, the function of TGF-β requires further investigation in this model.

Several limitations in our work should be mentioned. Since this was a preclinical study and the sample volume was quite small, some conclusions need to be further confirmed. We also failed to be able to conduct statistical analysis for our double staining to confirm the functional cytokines secreted by Tregs and Tc because only few double-stained cells could be found, however, with advancements in flow cytometry, we can more effectively assay these cytokines in the future.

In conclusion, our study elucidated that tumor-infiltrating Tregs could be suppressed by TGF-β blockade using a monoclonal antibody in a murine PDAC model. However, TGF-β blockade monotherapy may create limited therapeutic efficacy in PDAC patients, so further combination therapies should be investigated.

Supporting information

Supplementary material for this article is available at https://doi.org/10.14218/CSP.2022.00001.

Supplementary File 1. ARRIVE Checklist.

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Conflict of interest

One of the authors, Dr. Ning Pu, has been an editorial board member of *Cancer Screening and Prevention* since November 2021. The authors declare no other competing interests.

Author contributions

Contributed to study concept and design (WCW and GCZ), acquisition of the data (NP and GCZ), assay performance and data analysis (NP, AN, HLY, ZL and GCZ), drafting of the manuscript (GCZ, NP and ZL), critical revision of the manuscript (WHL and WCW), supervision (WCW).

Ethical statement

The protocol of this study was approved by the Animal Experiment Ethics Board of Fudan University.

Data sharing statement

The data that support the findings of this study are available upon request from the corresponding author.

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