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ORIGINAL ARTICLE

Integrin alphavbeta6 promotes tumor tolerance in colorectal cancer

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Abstract Tumor immune tolerance plays a critical role in tumor cell survival; the establishment of tumor immune tolerance is incompletely understood yet. Integrin alphavbeta6 (avb6) is involved in tumor growth and metastasis. This study aimed to observe the effect of avb6 on the development of tumor tolerance in colorectal cancer (CRC). In this study, 28 CRC patients were recruited. The frequencies of tolerogenic dendritic cells (TolDC), regulatory T cells (Treg), and CD8+ T cells in surgically removed CRC tissue were assessed by flow cytometry. The levels of avb6 in CRC tissue were measured by enzyme-linked immunoassay (ELISA). The effect of avb6 on inducing TolDCs and Tregs was evaluated with the cell culture model. The results showed that in surgically removed CRC tissue, we detected higher frequencies of TolDC and Tregs, lower frequency CD8+ T cells and high levels of avb6 as compared with non-CRC tissue. CRC protein extracts could induce TolDC development that could be blocked by anti-avb6 antibody. CRC-derived DCs could convert naïve CD4+ T cells to Tregs. Peripheral CD8+ T cells from CRC patients still retained the ability to produce granzyme B and to proliferate in response to CRC tumor antigen in culture that was abolished by the presence of CRC-derived Tregs. We conclude that CRC-derived avb6 is involved in the establishment of tumor immune tolerance in local tissues.

Keywords Colon · Cancer · Immune tolerance · Integrin · T lymphocyte

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Introduction

The immune system has dual roles in tumor. It can inhibit tumor growth by destroying tumor cells such as via CD8+ cytotoxic T-cells' activities [1]; it also can facilitate tumor outgrowth such as the over-proliferation of regulatory T cells (Treg) in the tumor that can suppress the antitumor immune cells such as tumor-specific CD8+ T cells [2]. Tumor-infiltrating CD8+ T cells in colorectal cancer (CRC) play an important role in the elimination of CRC cells; the frequency of CD8+ T cell has prognostic significance for CRC patients [1]. Tregs have the ability to inhibit the generation of antitumor immunity via suppression of tumor-specific effector T-cell responses and facilitate the establishment of the immune tolerance to tumor cells in the body [3]; the tumor cells thus gain the opportunity to progress. It is reported that the number of Tregs is increased in CRC [4]; they may play a fundamental role in CRC cell survival. However, the causative factors in the increase in Tregs in CRC remain unclear.

Dendritic cells (DC) initiate immune responses by dictating other immune cells' activities. A subset of DC expressing transforming growth factor (TGF)beta; the latter has the ability to convert naïve CD4+ T cells to forhead

fox P3 (Foxp3)+ CD4+ CD25+ Tregs [5]; and this subset of DC is designated tolerogenic DC (TolDC). It is reported that TolDCs also express aldehyde dehydrogenases (ALDH), the retinoic acid (RA)-synthesizing enzyme [5]. Both DC-derived TGFbeta and RA are critical in the induction of Tregs [5]. However, the driving force on the development of TolDCs is incompletely understood.

Integrin alphavbeta6 (avb6) belongs to a molecular family that is heterodimeric epithelial cell-derived proteins that regulate cell growth, migration, and survival [6]. Avb6 binds to latent TGFbeta, cleaves its latency peptide, and converts it to the active form [6]. Published data indicate that avb6 is involved in the pathogenesis of tumor and tumor metastasis [7]. Whether avb6 is also involved in the induction of tumor tolerance has not been investigated.

A link between Tregs and cancers has been noted for decades. Depletion of Tregs is proposed as a potential therapeutic remedy for cancers [8]. Recent reports indicate that interleukin (IL)-10-producing DCs are involved in tumor tolerance by promoting the Treg development [9]. In preliminary studies, we found high level of avb6 in the CRC. Based on the information above, we hypothesized that CRC-derived avb6 may promote the generation of TolDCs; the latter induces Treg development that contributes to the tumor immune tolerance. In this study, high levels of avb6 were detected in CRC tissue that could induce TolDC development and promote the Treg generation.

Materials and methods

Reagents

Antibodies of CD8 (YTS169.4; isotype: Rat IgG2b), Fxop3 (F-9; isotype: Mouse IgG1), and integrin beta6 (N-20; isotype: Goat IgG) were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). CD4+, CD25+, and CD3+ T-cell isolating reagents were purchased from Myltenyi Biotech (Shanghai, China). Fluorescence-labeled antibodies including Foxp3, CD8, CD11c, TGFbeta, and isotype-matched IgG were purchased from BD Bioscience (Shanghai, China). The ALDEFLUOR® Kit was purchased from StemCell Tech (Vancouver, BC, Canada). ELISA kit of granzyme B was purchased from R&D Systems (Shanghai, China). Endotoxin levels in all reagents were detected using the Limulus assay (Limulus amebocyte lysate QCL 1000, Bio Whittaker, Walkersville, MD, USA). The reagents used in this study contained <0.2 U endotoxin/10 μg reagents.

Patients

Twenty-eight patients with CRC were recruited to this study at the China PLA General Hospital. The diagnosis of CRC was made by physicians' clinical observation and CRC biopsy histology examination by pathologists. The demographic information of the patients was listed in Table 1. All the patients were treated by surgical operation to remove the tumor as well as to eliminate the draining lymph nodes if necessary. Informed consent to be included in this study was obtained from each patient. The study procedures were approved by the Human Research Ethic Committee at the China PLA General Hospital.

Protein extraction

Surgically removed CRC tissue and non-CRC tissue were collected, cut into small pieces, and homogenated in lysis buffer at 4°C. After centrifuged (17,600 ×g) at 4°C for 20 min, the supernatant was collected. The protein contents were measured by Bradford assay.

Western blotting

Proteins were separated (40 μg/well) using a precast NuPAGE gel system and blotted onto nitrocellulose membrane. The membrane was incubated with primary antibody (0.05–0.1 μg/ml) in 5% nonfat milk in TBS at 4°C overnight. This was followed by incubation with HRP-conjugated second antibody for 1 h at room temperature with shaking. Reactions were developed using the Pierce ECL chemiluminescence substrate kit. Results were recorded with X-ray film.

Immune cell isolation

Surgically removed CRC tissue was cut into small pieces (2 × 2 × 2 mm) and treated with a predigestion solution

Table 1 Demographic data of cancer patients

Number	28
Sex (m/f)	13/15
Age (y)	53 (32–80)
Location	
Ascending colon	6
Transverse colon	8
Descending colon	7
Sigmoid colon	6
Rectum	1
Size (mm)	
Small (10–29)	11
Medium (29–40)	12
Large (≥50)	5
Stage	
I	4
II	11
III	13

(1× HBSS containing 5 mM EDTA and 1 mM DTT) at 37°C for 30 min under slow rotation. The tissue was collected by centrifugation (800×g, 10 min) and incubated in digestion solution (dissolve 0.05 g of collagenase D, 0.05 g of DNase I, and 0.3 g of dispase II in 100 ml of 1 × PBS) at 37°C for 60 min under slow rotation. Cells were filtered with a cell strainer (40 μm in diameter). Isolation of indicated immune cells was performed with commercial magnetic cell sorting (MACS) kits following the manufacturer's instruction. CD4+ CD25high T cells were sorted by FACSAdvantage (the Foxp3+ Tregs were over 95% as checked by flow cytometry). On the other hand, the peripheral blood samples (40 ml/patient) were collected; mononuclear cells (PBMC) were isolated by gradient density centrifugation. CD3+ CD4+, CD3+ CD8+ T cells, and CD11c+ DCs were isolated with commercial reagent kits following the manufacturers' instruction.

Flow cytometry

Cells were collected and incubated with primary antibodies on ice for 30 min (for the intracellular staining, cells were fixed with 1% paraformaldehyde on ice for 30 min and incubated with permealization reagents for 30 min). The stained cells were analyzed using a BD FACS Canto flow cytometer (BD Bioscience, San Jose, CA, USA).

Cytokine measurement

The levels of granzyme B in culture supernatant were measured by ELISA with commercial reagent kits following manufacturers' instruction.

CD8+ T-cell proliferation assay

CD8+ T cells and DCs were isolated from the peripheral blood by MACS. After stained with CFSE, CD8+ T cells were cultured with DCs (CD8+ T cell:DC = 10:1), with or without culture with Tregs (CD8+ T cell:Treg = 1:1) in the presence of CRC protein extracts (1 μg/ml) for 96 h. Cells were collected at the end of culture and analyzed by flow cytometry.

Statistical analysis

Data are summarized as means ± SD. The values were analyzed using the two-tailed unpaired Student's t test when the data consisted of two groups or by ANOVA when three or more groups were compared. The Pearson correlation assay was performed with the data of LC and normal control tissue. $P < 0.05$ was accepted as statistically significant.

Results

The levels of avb6 in colorectal cancer (CRC) tissue are increased and correlated with the frequency of Tregs

Tumor-infiltrating Tregs play an important role in tumor survival by facilitating tumor cells to escape from the immune surveillance [10]. Integrin avb6 is involved in the pathogenesis of cancer [11]. Avb6 plays a role in the activation of TGFbeta; the latter is a critical molecule in Tregs' development and function [12]. Thus, avb6 might be involved in the pathogenesis of CRC. In the first attempt, we observed the correlation between the frequency of Tregs and the expression of avb6 in the CRC. The results showed that about 5% CD4+ CD25+ Foxp3+ Tregs was detected in CRC tissue, while only about 1% Tregs was detected in non-CRC tissue ($P < 0.01$) (Fig. 1a) as well as in the marginal non-disease tissue (proved by pathologists) from surgical removed colon tissue of three patients with advanced Crohn's disease complicated with colon obstruction (the frequencies of Treg were 1.2, 0.9, and 1.0%, respectively). We also extracted proteins from the same batch of CRC specimens and examined the expression of avb6 in the CRC. As shown by Western blotting, the levels of avb6 were significantly higher in CRC tissue than that in non-CRC tissue (Fig. 1b). The correlation assay was performed with the data on Treg number and avb6 levels; the results showed a positive correlationship between the number of Treg and the levels of avb6 ($r = 0.663$, $P < 0.01$).

CRC-derived avb6 has the ability to induce tolerogenic DC development

The finding that Tregs were increased in CRC tissue prompted us to assess the frequency of Tregs in isolated peripheral blood cells from the same subjects. As shown by flow cytometry, however, the frequency of peripheral Tregs from CRC patients was not significantly different from that in healthy control subjects (Fig. 1c). The fact implies that the causal factors in the increase in Tregs exist inside CRC tissue. Published data indicate that TolDCs have the ability to induce Treg development [13]; we postulated that TolDCs might be increased in the CRC. We next assessed the frequency of TolDCs in CRC tissue. The data from flow cytometry showed that the frequency of CD11c+ MHCII+ ALDH+ TGFbeta+ DCs (TolDCs) [5] was markedly more in CRC tissue than that in non-CRC tissue (Fig. 2a). Since both RA and TGFbeta are important molecules in the induction of Tregs [5], avb6 has the ability to convert the latent TGFbeta to the active form of TGFbeta [14], we inferred that CRC-derived avb6 might be important in the generation of TolDCs in CRC tissue. To test the hypothesis, we isolated PBMCs from healthy subjects; the PBMCs were

cultured in the presence of GM-CSF and IL-4 for 6 days. CD11c+ MHCII+ DCs were then isolated by MACS and further cultured in the presence of CRC-extracted proteins (0.02 mg/ml; the proteins contained avb6, 23.5 pg/mg protein, as measured by ELISA) for 6 days. The cells then were analyzed by flow cytometry. The results showed that CRC protein extracts could induce development of TolDCs that did not occur in BSA-treated DCs (Fig. 2b). Considering that avb6 might be responsible for the induction of TolDCs, a batch of PBMC was treated with CRC protein extracts as well as anti-avb6 antibody (0.01 mg/ml). Indeed, few TolDCs were induced (Fig. 2b). Collectively, the data indicate that CRC-derived avb6 has the ability to generate TolDCs.

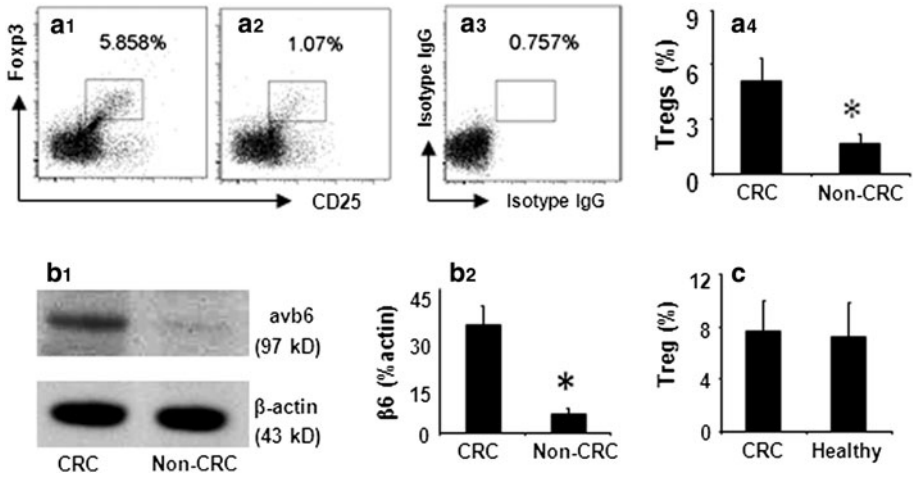

Fig. 1 High frequency of CD4+ CD25+ Foxp3+ Tregs and high levels of avb6 CRC in CRC tissue. **a** CD4+ T cells were isolated from surgically removed CRC tissue (**a1**) and the marginal non-CRC tissue (**a2**) from 28 CRC patients. Cells were stained with antibodies of CD25 and Foxp3 and analyzed by flow cytometry. *Dot plots* indicate the frequency of CD4+ CD25+ Foxp3+ Tregs in CRC tissue. **a3** Is isotype control. **a4** *Bars* indicate the summarized data of **a1** and **a2**. **b** Proteins were extracted from CRC and non-CRC tissue and analyzed by Western blotting. **b1** Immune blots show beta6 levels in extracted proteins. **b2** *Bars* indicate the summarized densitometrical data in **b1**. **c** CD3+ CD4+ T cells were isolated from the peripheral blood mononuclear cells (PBMC) of CRC patients or healthy subjects and analyzed by flow cytometry. *Bars* indicate the frequency of Foxp3+ Tregs. In **a4**, **b2**, and **c**, data were presented as mean ± SD. $*P < 0.01$, compared with CRC tissue. Data in **c** represent 3 experiments

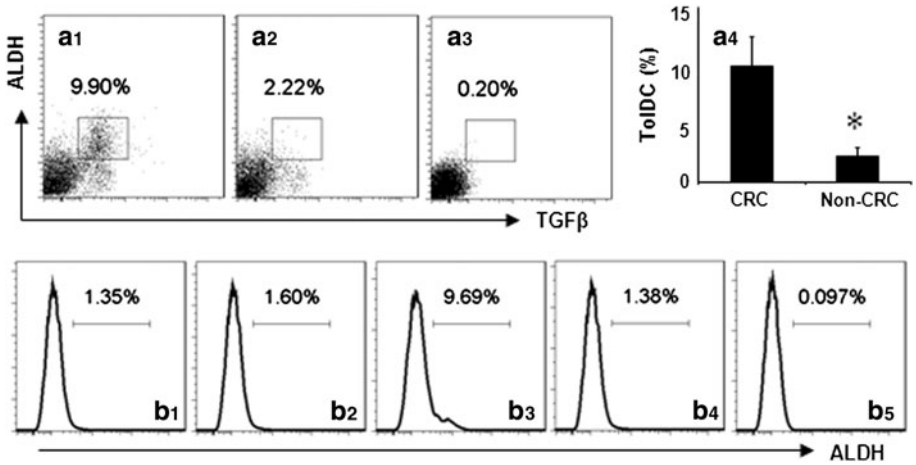

Fig. 2 Increases in TolDCs in CRC. CRC specimens were obtained as described in Fig. 1. Single cells were prepared from the specimens; CD11c+ cells were isolated from the single cells with MACS, stained with antibodies of TGFbeta and ALDH kit and analyzed by flow cytometry. **a** *Dot plots* show the frequency of TolDCs in CRC tissue (**a1**) and non-CRC tissue (**a2**). **a3** Shows results from cells stained with isotype IgG. **a4** *Bars* show the summarized data of **a1** and **a2** (mean ± SD; $*P < 0.01$, compared with CRC group). **b** PBMCs were isolated from healthy subjects; the PBMCs were cultured in the presence of GM-CSF (20 ng/ml) and IL-4 (10 ng/ml) for 6 days. CD11c+ DCs were then isolated by MACS and further cultured for 6 days in the presence of saline (**b1**) or bovine serum albumin (**b2**) or CRC protein extracts (0.02 mg/ml) (**b3**) or CRC extracts neutralized with anti-avb6 antibody (**b4**). Cells were collected at the end of culture and stained with ALDH kit and analyzed by flow cytometry. The histograms show the frequency of CD11c+ ALDH+ DCs (TolDCs). **b5** Shows isotype IgG staining control

CRC environment induces Foxp3+ Treg development

As mentioned above, high frequency of Tregs was detected in CRC tissue (Fig. 1a), but not in the peripheral blood cells (Fig. 1c), it was possible that the Tregs could be generated within CRC. To test the hypothesis, we isolated PBMCs from healthy subjects; the PBMCs were cultured in the presence of GM-CSF, IL-4, and CRC-extracted proteins for 9 days; and the cells were then stained with antibodies of CD4, CD25, and Foxp3 and analyzed by flow cytometry. The results showed that the number of CD4+ CD25+ Foxp3+ Treg was markedly increased as compared with those treated with BSA, which could be partially blocked in the presence of anti-TGFbeta antibody or retinoic acid receptor antagonist LE540 and completely blocked by both (Fig. 3). Using as a control, some CD4+ CD25- T cells were stimulated with protein extracted from surgically removed colon tissue (the marginal normal tissue, proved by pathologists) from Crohn's disease with colon obstruction. No detectable increase in Tregs was observed (Fig. 3c). The data indicate that CRC-derived avb6 induces TolDC development; the TolDCs then induces Treg development.

CRC-derived Tregs suppress CRC-specific CD8+ T cells

Cytotoxic CD8+ T cells play a critical role in the elimination of tumor cells in the body. The survival of tumor cells in tumor-bearing subjects implies the reduction or dysfunction of CD8+ T cells in local tissue. We next assessed the number of CD8+ T cells in CRC tissue by flow cytometry. The results showed significantly less CD8+ T cells in CRC-derived CD3+ T cells than those in non-CRC-derived CD3+ T cells (Fig. 4a). To clarify whether the CD8+ T cells were also reduced in the peripheral system, we isolated CD3+ T cells from the blood and examined by flow cytometry. However, the frequency of CD8+ T cell in CRC

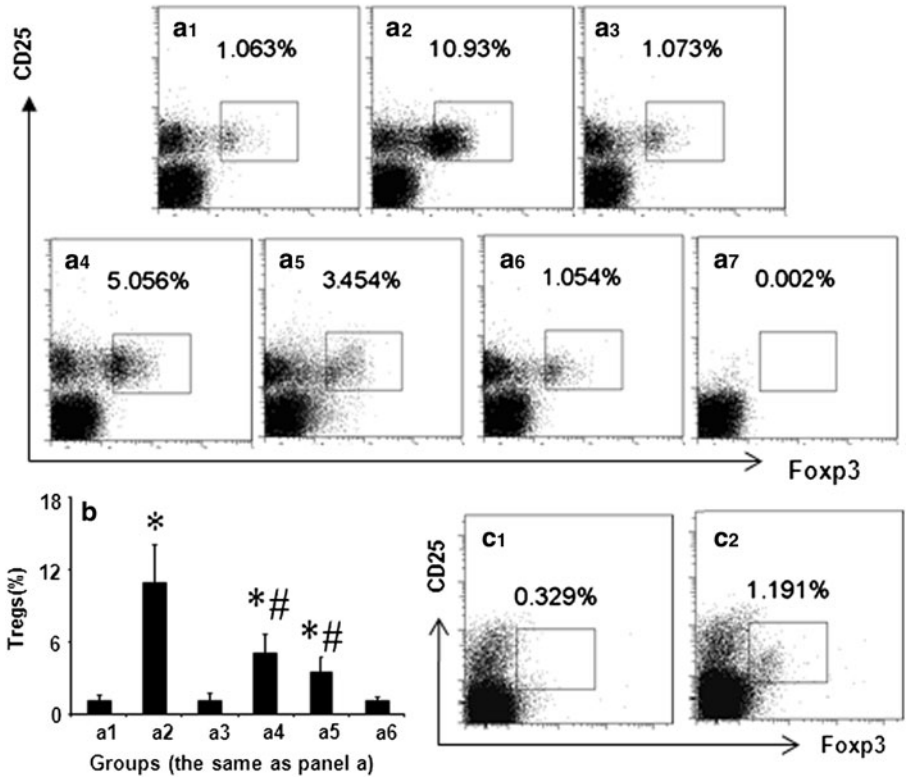

Fig. 3 CRC-derived retinoic acid and TGFbeta induce Treg development. PBMCs were prepared from healthy subjects and treated with CRC extracts (containing tumor antigens) for 6 days. CD4+ T cells were isolated from the PBMCs and stained with antibodies of CD25 and Foxp3 and analyzed by flow cytometry. **a** *dot plots* show the frequency of CD4+ CD25+ Foxp3+ Tregs (the gated cells) in cells cultured with (**a1**) medium alone, or (**a2**) CRC extracts (0.02 mg/ml), or (**a3**) CRC extracts neutralized by anti-avb6 antibody (100 ng/ml), or (**a4**) CRC extracts together with anti-TGFbeta antibody (100 ng/ml), or (**a5**) LE540 (100 ng/ml), or (**a6**) both anti-TGFbeta and LE540. **a7** is isotype control. **b** Summarized data from **a**. Data represent 3 experiments. $^{*}P < 0.01$, compared with group **a1**. $^{\#}P < 0.05$, compared with group **a2**. **c** The procedures were the same as **a** except the cells were stimulated with proteins from colon specimens that were obtained from surgically removed colon tissue from three Crohn's diseases with colon obstruction. The marginal normal tissue was used. **c1** Cells were stained with antibodies of CD25 and Foxp3. **c2** Cells were stained with antibodies of CD25 and isotype IgG using as a control

patients was not significantly less than that in healthy control subjects (Fig. 4b). The results indicate that the frequency of CD8+ T cell was mainly suppressed within the tumor tissue of CRC patients, but not in the peripheral blood.

We next tested the immune suppressor effect of CRC-derived Tregs on peripheral CD8+ T cells from the same CRC patients. CFSE-labeled peripheral CD8+ T cells were cultured in the presence of DCs and CRC protein extracts (0.02 mg/ml, containing the tumor-specific antigens) or non-CRC protein extracts (0.02 mg/ml, using as a control) for 4 days (samples were obtained from the same patients). The results showed that in the presence of CRC protein extracts (containing tumor-specific antigen), but not the non-CRC protein extracts, more than 30% CD8+ T cells proliferated 2–3 times (Fig. 4c); the levels of granzyme B, the immune suppressive molecule, were significantly increased in culture medium (Fig. 4d); however, in the presence of CRC-isolated Tregs, the proliferation of CD8+ T cells and the levels of granzyme B in culture medium were markedly suppressed (Fig. 4c, d). In addition, non-CRC protein could not induce Tregs to suppress CD8+ T cells (Fig. 4c, d). The results indicate that CRC-specific CD8+ T cells still exist in CRC patients, however, that can be suppressed by CRC-specific Tregs.

Discussion

The present data indicate that high levels of avb6 are detected in CRC tissue that is correlated with the frequency of TolDCs and Tregs in CRC tissue. CRC protein extracts can induce TolDC development. DCs isolated from the CRC tissue facilitate the generation of Tregs. CRC-derived Tregs suppress PBMC-derived CD8+ T cells from CRC patients in the presence of CRC protein extracts (containing the tumor-specific antigens).

Tumor progression depends upon multiple changes in tumor, such as loss or attenuating tumor immunogenicity, induces changes in the microenvironment surrounding tumors, or changes the systemic immune surveillance, e.g.,

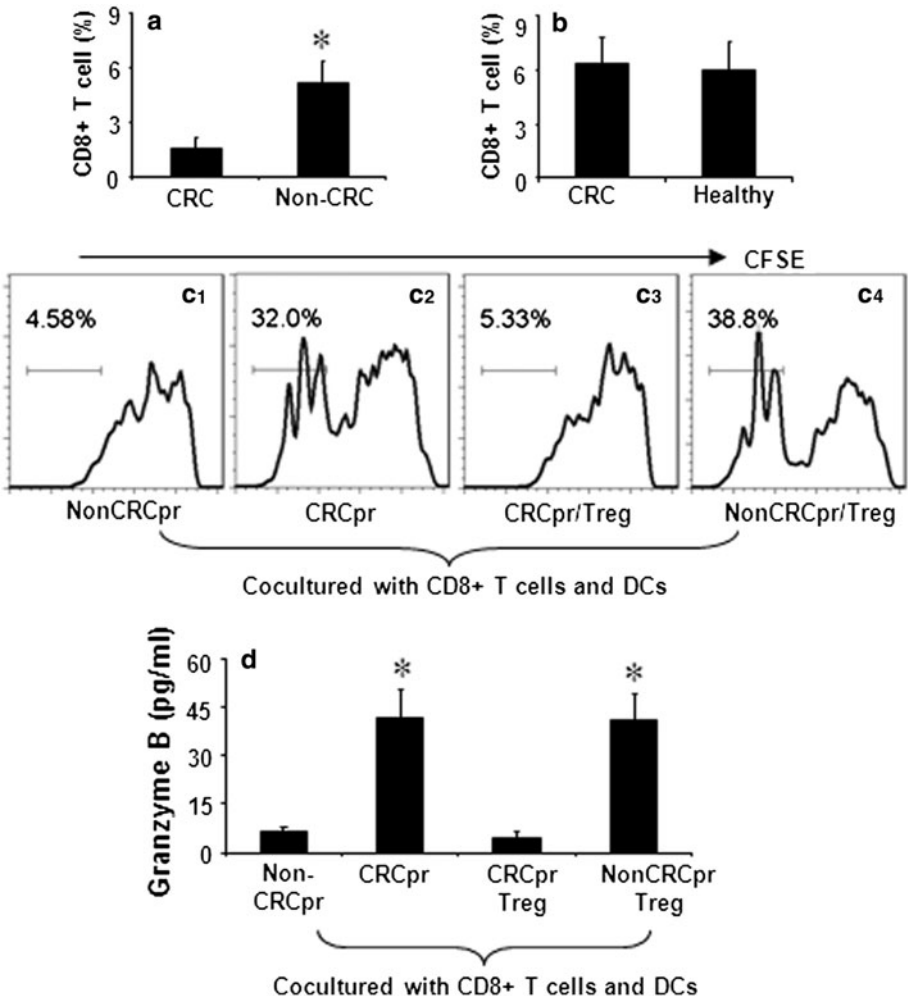

Fig. 4 CRC-specific Tregs suppress CRC-specific CD8+ T cells. **a**, **b** CD3+ T cells were isolated from CRC (**a**) and PBMCs (**b**) and analyzed by flow cytometry. *Bars* indicate the frequency of CD8+ T cells in isolated CD3+ T cells. **c** CD8+ T cells were isolated from PBMCs of CRC patients, labeled with CFSE, and cultured with non-CRC protein extracts (non-CRCpr) (**c1**) or CRC protein extracts (CRCpr) (**c2**) or CRC protein extracts and Treg (**c3**) or non-CRC protein extracts and Treg (**c4**) for 4 days. The CFSE-dilution was assessed with flow cytometry. The histograms indicate the CFSE-fluorescence intensity. **d** Culture supernatants in **c** were analyzed by ELISA. *Bars* indicate the levels of granzyme B in culture supernatants. Data in *bar graphs* were presented as mean ± SD. $^*P < 0.05$, compared with CRC group (**a**) or non-CRCpr group (**d**). Data represent 3 experiments

induces tumor tolerance in the body [15]. Tumor tolerance is crucial in tumor cells' survival by escaping from immune surveillance. To prevent or inhibit tumor tolerance has shed light on the improvement of tumor treatment [16]. It has been recognized that Tregs are an important subset of T cells in the initiation and maintenance of tumor tolerance [17]. Our data are in line with previous studies [18] by demonstrating that a relative large number of Tregs were detected in CRC tissue. Since Tregs are one of the major components of immune tolerance, the fact anatomically suggests that tumor tolerance may be established in the CRC. It is suggested that although therapeutic strategies designed to eliminate Treg from the body could not be enough to manage established tumors [19], it can be useful in a setting of minimal residual disease [20].

Since Tregs play a role in facilitating tumor growth in the body [2], to remove the causal factors from the body that induces Treg development is also of significance. Several molecules, including TGFbeta, IL-10, RA, etc. [5], have been recognized that play roles in the induction of Tregs. Most of the molecules can be produced by DCs. DCs having high levels of these molecules are designated TolDCs [5]. The present data show that abundant TolDCs are detected in CRC tissue, which implies that the TolDCs may be responsible for the increase in Tregs in CRC tissue by promoting Treg development. The inference is supported by subsequent data of this study; using CRC-derived proteins (containing tumor-specific antigens) induced a large number of TolDCs and Tregs in in vitro experiments.

The present data also show that CRC-derived TolDCs have high levels of ALDH and TGFbeta; both molecules are indispensable in the induction of Tregs in in vivo experiments [5]. Published data indicate that after synthesis, TGFbeta exists as a precursor, the latent TGFbeta; it requires to be converted to TGFbeta before gaining the immune regulatory ability [6]. Several molecules have been recognized having the ability to convert the latent TGFbeta to TGFbeta including avb6, avb8, plasmin, or some other proteases [21]. Our data show high levels of avb6 in CRC tissue; the fact implies that the avb6 may be the molecule-inducing naïve DCs to develop to TolDCs. Indeed, the inference is supported by the present data, adding CRC protein extracts to culture media successfully induced TolDCs that was abolished by the presence of anti-avb6 antibody.

CD8+ T cells are the major antitumor immune cells in the body. Activated CD8+ T cells release a number of molecules such as granzyme B and perforin to induce tumor cell death that prevents tumor growth in healthy individuals [22]. However, the number of CD8+ T cell is significantly less in CRC tissue than that in healthy tissue as observed in the present study. Others also published supportive data [18]. The finding demonstrates that the CD8+ T cells are suppressed in CRC tissue. However, the frequency of the CD8+ T cells in the peripheral blood in CRC patients is not much different from healthy subjects; those CD8+ T cells from CRC patients still retain the ability to be activated upon exposure to tumor-specific antigens. The production of granzyme B indicates that the peripheral-specific CD8+ T cells have the antitumor's ability, which may be inhibited upon contacting CRC-specific Tregs; the deduction is supported by subsequent data that culture with CRC-derived Tregs, the peripheral-specific CD8+ T cells stopped proliferating, and the production of granzyme B was also suppressed.

Taken together, the present data show that high frequencies of Tregs and TolDCs are observed in CRC tissue; the number of CD8+ T cells is markedly less in CRC tissue than non-CRC tissue. Importantly, high levels of avb6 are detected in CRC tissue that can induce the development of TolDCs and Tregs, which can be prevented by neutralizing avb6. The data demonstrate that the tumor-specific immune tolerance is established in the CRC; avb6 plays a critical role in the development of immune tolerance that may be disrupted by neutralizing the avb6.

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Conflict of interest None to declare.

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