

## Tumor-Derived Chemokine CCL5 Enhances TGF- $\beta$ -Mediated Killing of CD8<sup>+</sup> T Cells in Colon Cancer by T-Regulatory Cells

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### Abstract

Chemokine CCL5/RANTES is highly expressed in cancer where it contributes to inflammation and malignant progression. In this study, we show that CCL5 plays a critical role in immune escape in colorectal cancer. We found that higher levels of CCL5 expression in human and murine colon tumor cells correlated with higher levels of apoptosis of CD8<sup>+</sup> T cells and infiltration of T-regulatory cells (T<sub>reg</sub>). In mouse cells, RNA interference (RNAi)-mediated knockdown of CCL5 delayed tumor growth in immunocompetent syngeneic hosts but had no effect on tumor growth in immunodeficient hosts. Reduced tumor growth was correlated with a reduction in T<sub>reg</sub> infiltration and CD8<sup>+</sup> T-cell apoptosis in tumors. Notably, we found that CCL5 enhanced the cytotoxicity of T<sub>reg</sub> against CD8<sup>+</sup> T cells. We also found tumor growth to be diminished in mice lacking CCR5, a CCL5 receptor, where a similar decrease in both T<sub>reg</sub> cell infiltration and CD8<sup>+</sup> T-cell apoptosis was noted. TGF- $\beta$  signaling blockade diminished apoptosis of CD8<sup>+</sup> T cells, implicating TGF- $\beta$  as an effector of CCL5 action. In support of this concept, CCL5 failed to enhance the production of TGF- $\beta$  by CCR5-deficient T<sub>reg</sub> or to enhance their cytotoxic effects against CD8<sup>+</sup> T cells. CCR5 signaling blockade also diminished the *in vivo* suppressive capacity of T<sub>reg</sub> in inhibiting the antitumor responses of CD8<sup>+</sup> T cells, in the same way as CCL5 signaling blockade. Together, our findings establish that CCL5/CCR5 signaling recruits T<sub>reg</sub> to tumors and enhances their ability to kill antitumor CD8<sup>+</sup> T cells, thereby defining a novel mechanism of immune escape in colorectal cancer. *Cancer Res*; 72(5); 1092–102. ©2012 AACR.

### Introduction

Colorectal cancer (CRC) is one of the most common cancers worldwide and a major cause of cancer-related death (1). The presence of tumor-infiltrating lymphocytes (TIL) could predict the clinical outcome in patients with CRC (2–5), as evidenced by high density of CD8<sup>+</sup> and CD45RO<sup>+</sup> T cells in TILs associated with decreased invasiveness, lower stage, and improved survival (2).

T-regulatory cells (T<sub>reg</sub>) play an indispensable role in the maintenance of immunologic tolerance and in control of excessive immune responses (6, 7). The percentage of T<sub>reg</sub> cells is inversely related to the outcomes of various malignant

diseases (4, 5, 8–10). Furthermore, depletion of T<sub>reg</sub> cells can enhance antitumor immune responses and improve the efficacy of tumor immunotherapy in several mouse tumor models (11, 12). For patients with CRC, increased numbers of T<sub>reg</sub> cells had been shown in peripheral blood, tumor-draining lymph node (DLN), and tumor and these T<sub>reg</sub> cells could suppress the autologous effector T-cell responses (13–16). In CT26 mouse colon tumor model, we have shown that T<sub>reg</sub> cells increased in number as the progression of tumor and these T<sub>reg</sub> cells could suppress antitumor functions of CD8<sup>+</sup> T cells (17). Furthermore, Casares and colleagues also reported that functional inhibition of T<sub>reg</sub> cells by the Foxp3-inhibitory peptide P60 enhanced antitumor immunotherapy in CT26 mouse colon tumor model (18). All these evidence highlights the important regulatory role of T<sub>reg</sub> cells in CRC either in human or in mouse model.

CCL5/RANTES is one of the C-C chemokines and interacts with the G-protein-coupled receptors CCR1, CCR3, and CCR5. CCL5 is a potent chemoattractant for T lymphocytes, monocytes, natural killer cells, and eosinophils (19). It has been reported that CCL5 is highly expressed in various tumors and promotes tumor growth and metastasis by inducing tumor cell proliferation, angiogenesis, or expression of matrix metalloproteinases (20–22). In addition, CCL5 has been shown to diminish antitumor immune responses by increasing the presence of tumor-associated macrophages and T<sub>reg</sub> cells in

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tumors (22, 23). Recent studies have shown that CCL5 was highly expressed in colon tumor tissues when compared with paired normal tissues in early-stage tumor of patients with CRC (24). However, whether the tumor-helping role of CCL5 is through evading the immune attack or not remains unclear.

Thus, the purpose of this study was to investigate the possible tumor-helping role of CCL5 in evading the immune attack in CRC. We found that CCL5 was highly expressed in human colorectal tumor tissues, as well as in CT26 mouse colon tumor cells. Knockdown of CCL5 secretion from CT 26 cells resulted in decreased apoptosis of tumor-infiltrating CD8<sup>+</sup> T cells and delayed growth of tumor in mice. Interestingly, CCL5 expression could not only promote migration of T<sub>reg</sub> cells to tumors but also enhance their killing ability on CD8<sup>+</sup> T cells. Furthermore, this enhancement of killing ability was associated with increased production of TGF- $\beta$  by T<sub>reg</sub> cells. These results provide a better understanding of interplay between tumor cells and T<sub>reg</sub> cells and identify an unrevealed mechanism by which tumor cells induce immune tolerance in CRC.

## Materials and Methods

### Study population

Healthy control subjects ( $n = 20$ ) were recruited from health check-up center in Chang Gung Memorial Hospital, Taiwan. A total of 25 human colon cancer tissues and peripheral blood samples were collected immediately after a regular colon cancer operation for patients with CRC. About  $1 \times 1 \text{ cm}^2$  size tumor specimens from each patient were used for the present study. All patients in this study had provided written informed consent. This study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethical committees of Chang Gung Memorial Hospital.

### Mice

BALB/c mice and nude mice were purchased from the National Laboratory Animal Center of Taiwan (Taipei, Taiwan). RAG-1-deficient mice (BALB/c background), CCR3-deficient mice (BALB/c background), and CCR5-deficient mice (C57BL/6 background) were purchased from Jackson Laboratory. CCR5-deficient mice were fully backcrossed to BALB/c background for 10 generations. All mice were maintained in the animal house of Chang Gung Memorial Hospital and used in experiments at ages 8 to 10 weeks. All animal breeding and experiments were in accordance with guidelines of the institutional animal ethics committee.

### Cell lines and reagents

Both CT26 mouse colon carcinoma cell line and NIH 3T3 mouse fibroblast cell line were purchased from the American Type Culture Collection and maintained in our laboratory. Peptide P17, a known inhibitor of TGF- $\beta$  (25) was synthesized by Kelowna and GzmB inhibitor I (z-AAD-CMK) was purchased from Merck.

### CCL5 small hairpin RNA transfection

Knockdown of CCL5 was done by RNAi using GIPZ lentiviral shRNAmir starter Kits following manufacturer's instruction

(Open Biosystem). Two CCL5 (NM\_013653) RNA interferences were used with 3 RNA interference targets glyceraldehyde-3-phosphate dehydrogenase (GAPDH) lentiviral shRNAmir, EG5 lentiviral shRNAmir, and Nonsilencing control shRNAmir. After 48 hours of transfection, CT26 cells were selected in 60  $\mu\text{g}/\text{mL}$  puromycin to enrich pure population. CCL5 knock-down CT26 cells were determined by ELISA (R&D Systems) of culture supernatants.

### Quantitative real-time PCR

Cell lines or tumor tissues were homogenized with 1 mL TRI reagent to extract total RNA. cDNA was synthesized by reverse transcription of total RNA (Epicentre). Quantitative real-time PCR (qRT-PCR) was carried out as previously described (26). Previously described primers of the targeted genes were used in the study (26, 27).

### Flow cytometry

All antibodies were purchased from BD Biosciences or eBiosciences, except for anti-LAP monoclonal antibody (mAb; R&D Systems) and anti-TGF- $\beta$  mAb (IQ Products). Single-cell suspensions were stained for surface or intracellular proteins and cytokines as previously described (17). For intracellular cytokine staining,  $1 \times 10^6$  cells were stimulated with 50 ng/mL phorbol 12-myristate 13-acetate and 500 ng/mL ionomycin in the presence of GolgiStop for 5 hours, followed by surface and intracellular staining.

### Cell isolation

Human peripheral blood mononuclear cells were separated from blood samples by Ficoll density (Pharmacia). Tumors were chopped into small pieces and incubated with collagenase IV (0.1%; Sigma-Aldrich) in Hank's Balanced Salt Solution (HBSS) for 30 minutes at 37°C. After passing through nylon mesh, single-cell suspensions were separated with Ficoll, and leukocytes were recovered from the interphase.

### Cell purification

Single-cell suspensions were prepared from spleen of BALB/c mice bearing CT26 tumors, and CD8<sup>+</sup> T cells were enriched using magnetic microbeads conjugated with anti-mouse CD8 (Miltenyi Biotech) by AutoMACS (Miltenyi Biotech). For CD4<sup>+</sup>CD25<sup>+</sup> T-cell purification, single-cell suspensions were first enriched for CD4<sup>+</sup> T cells via negative selection by the CD4 isolation kit (Miltenyi Biotech). Enriched CD4<sup>+</sup> T cells were then labeled with CD25-PE and sorted using FACSARIA (BD Biosciences). Cell purity (>90%) for all populations was confirmed by flow cytometry.

### Tumor experiments

In peptide P17 treatment experiments, BALB/c mice were inoculated subcutaneously with  $1 \times 10^5$  CT26 cells and 3 days later, mice were treated with/without peptide P17 (intraperitoneally; 100  $\mu\text{g}/\text{mouse}$ ) 5 times per week in a 14-day interval. In adoptive transfer experiments, CD4<sup>+</sup>CD25<sup>+</sup> T cells were isolated spleen from BALB/c or CCR5-deficient mice bearing CT26 tumors, and these cells ( $2 \times 10^5$  cells) were cotransferred with CD8<sup>+</sup> T cells ( $1 \times 10^6$  cells) into CCR5-deficient mice

bearing CT26 tumors or CCL5 knockdown CT26 tumors generated 1 day earlier by subcutaneous inoculation ( $1 \times 10^5$  cells). In all case, the tumor size was measured twice a week and volume was calculated [(length  $\times$  width<sup>2</sup>)/2].

#### T<sub>reg</sub> cell killing ability assay *in vitro*

CD4<sup>+</sup>CD25<sup>+</sup> T cells and CD8<sup>+</sup> T cells were enriched from spleen or DLN of BALB/c mice bearing CT26 tumors. CD4<sup>+</sup>CD25<sup>+</sup> T cells were cultured with interleukin (IL)-2 (2,000 U/mL) in presence or absence of CCL5 (500 ng/mL) for 18 hours and were then washed twice in RPMI-1640 medium. For the analysis of T<sub>reg</sub> cell killing ability, these CD4<sup>+</sup>CD25<sup>+</sup> T cells were cultured with CD8<sup>+</sup> T cells ( $1 \times 10^5$  each) in presence of plate-bound anti-CD3 antibody (10  $\mu$ g/mL) and soluble anti-CD28 antibody (2  $\mu$ g/mL) for 48 hours. The apoptosis of CD8<sup>+</sup> T cells was measured by flow cytometry using Annexin V staining. To determine the mechanisms underlying the killing ability of T<sub>reg</sub> cells against CD8<sup>+</sup> T cells, anti-mouse FasL mAb (10  $\mu$ g/mL, MFL-3; eBiosciences), GzmB inhibitor I (10  $\mu$ g/mL), peptide P17 (10  $\mu$ g/mL), or anti-mouse TGF- $\beta$  mAb (10  $\mu$ g/mL,

ID11; R&D Systems) was added in the coculture of CD8<sup>+</sup> T cells and CCL5-pretreated T<sub>reg</sub> cells.

#### Statistical analysis

Two-tailed unpaired Student *t* test was used for statistical analyses of differences between groups, and a paired *t* test was used to determine pairwise differences. The calculations were made with GraphPad Prism 5 Software. Differences were recognized as significant at *P* less than 0.05.

#### Results

#### Increased CCL5 expression, increased infiltration of T<sub>reg</sub> cells, and increased apoptosis of CD8<sup>+</sup> T cells in human colorectal cancer tissues

First, we examined CCL5 expression in both tumor part and nontumor part of colon from patients with CRC using qRT-PCR. The CCL5 mRNA was significantly increased in tumor tissues compared with their nontumor counterpart (Fig. 1A). We next investigated phenotypes of T<sub>reg</sub> cells and CD8<sup>+</sup> T cells in peripheral blood lymphocytes (PBL) and TILs in patients

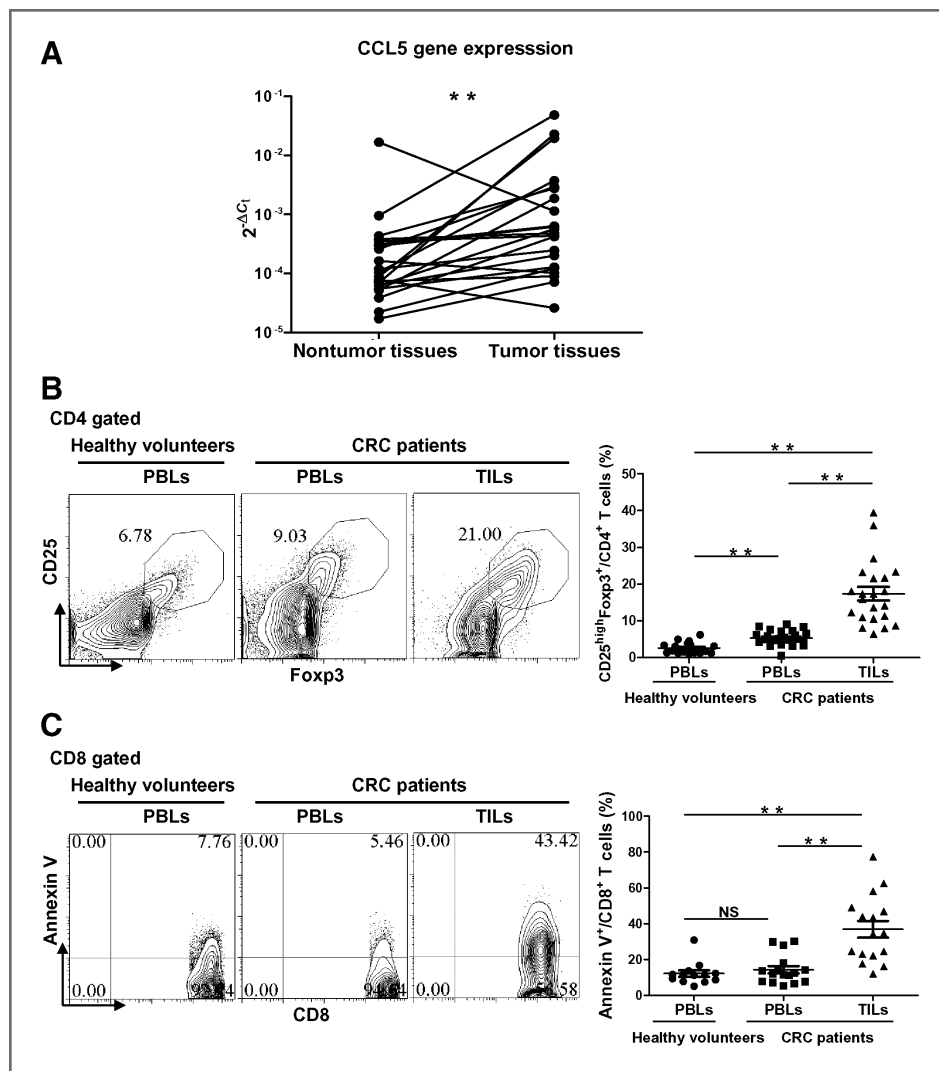


Figure 1. Increased CCL5 expression, infiltration of T<sub>reg</sub> cells, and apoptosis of CD8<sup>+</sup> T cells in human colorectal tissues. A, the CCL5 gene expression in tumor part and nontumor part of colon in 22 patients with CRC was measured by qRT-PCR. Relative expression levels were adjusted by the level of  $\beta$ -actin mRNA for each sample. All data represent mean  $\pm$  SEM. \*\*, *P* < 0.01 and NS, not statistically significant.

with CRC and found that the frequency of  $T_{reg}$  cells increased in PBLs and TILs of patients with CRC compared with PBLs of healthy volunteers. In addition,  $T_{reg}$  cells accumulated in tumor due to higher percentage of  $T_{reg}$  cells in the TILs than in PBLs in patients with CRC (Fig. 1B). On the other hand, the percentage of Annexin V<sup>+</sup>CD8<sup>+</sup> T cells in TILs was significantly increased compared with PBLs of patients with CRC or healthy volunteers (Fig. 1C). These results indicate increased CCL5 expression, increased infiltration of  $T_{reg}$  cells, and increased apoptosis of CD8<sup>+</sup> T cells in the tumor microenvironment in patients with CRC.

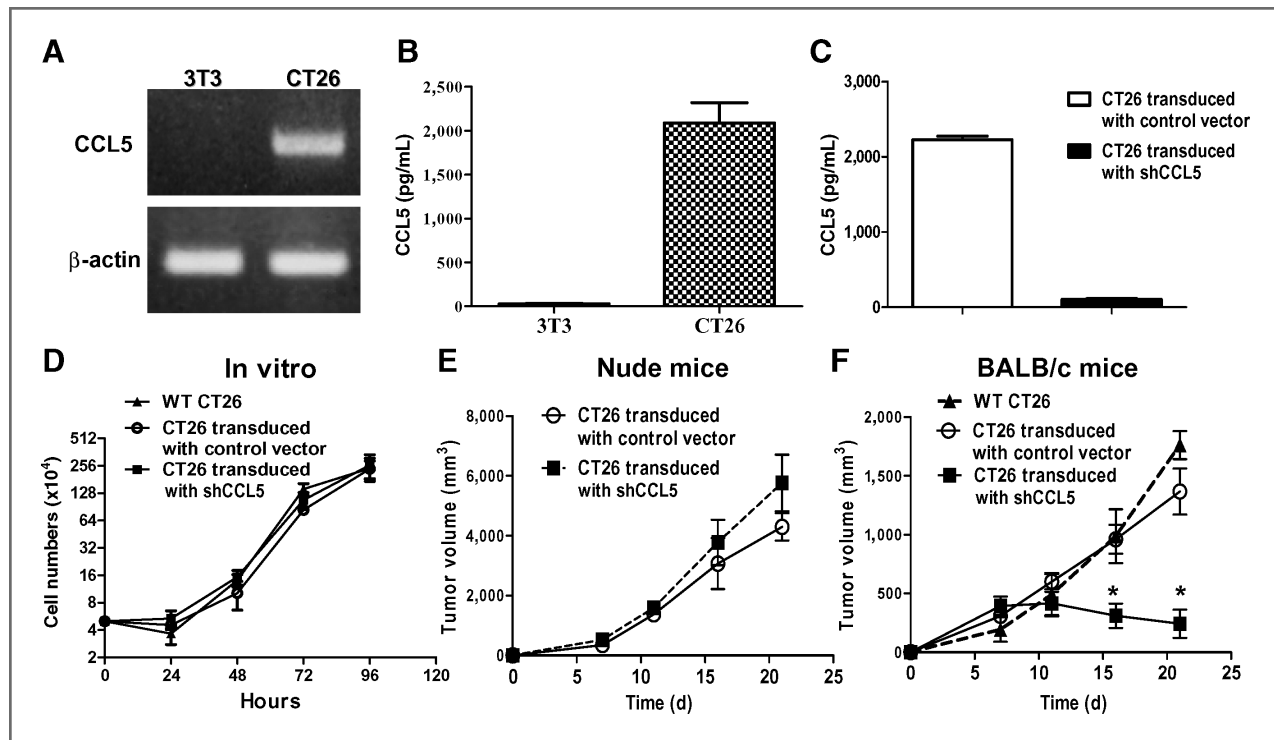
### CCL5 contributes to the tumor development in the mouse CT26 colon tumor model

For further exploring the possible role of CCL5 in the colorectal tumor development, we chose the CT26 mouse colon cancer model because of high CCL5 mRNA expression and CCL5 protein secretion by CT26 cells (Fig. 2A and B). We then knocked down CCL5 secretion from CT26 cells by lentiviral small hairpin RNA (shRNA; CCL5<sup>low</sup> CT26 cells, Fig. 2C). The *in vitro* growth pattern of CCL5<sup>low</sup> CT26 cells was similar to CT26 cells transfected with control lentiviral vector (control CT26 cells, Fig. 2D). Regarding their *in vivo* behavior, the tumor growth of CCL5<sup>low</sup> CT26 cells was also similar to control CT26 cells when these cells were subcutaneously inoculated into

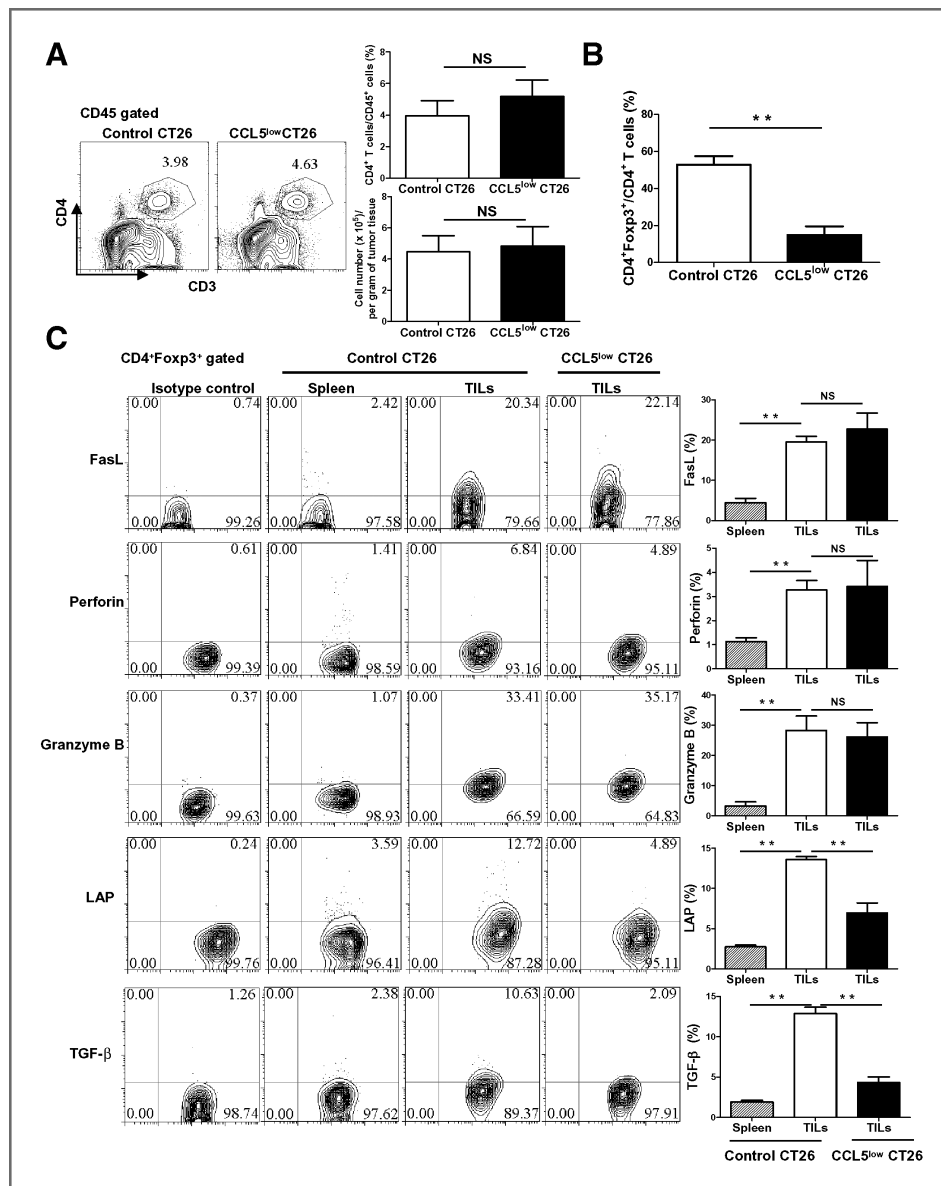
nude mice and RAG-1-deficient mice (Fig. 2E and Supplementary Fig. S1). However, in immunocompetent BALB/c mice, the tumor growth was significantly impaired in CCL5<sup>low</sup> CT26 cells (Fig. 2F). These results suggest that CCL5 contributes to tumor development in immunocompetent mice possibly via affecting the adaptive immune system.

### Knockdown of CCL5 secretion from tumors reduces expression of TGF- $\beta$ by tumor-infiltrating $T_{reg}$ cells

Because of chemoattracting nature of CCL5 toward T lymphocytes (19), it was worthy of further investigation of T cells in the tumor microenvironment between mice inoculated with CCL5<sup>low</sup> CT26 cells and with control CT26 cells. As shown in Fig. 3A, there was no difference in the percentage and absolute number of CD4<sup>+</sup> T cells between CCL5<sup>low</sup> CT26 tumors and control CT26 tumors. However, the percentage of  $T_{reg}$  cells in CCL5<sup>low</sup> CT26 tumors was significant lower than that in control CT26 tumors (Fig. 3B). We also examined the phenotypes of  $T_{reg}$  cells in these mice.  $T_{reg}$  cells in both CCL5<sup>low</sup> CT26 tumors and control CT26 tumors expressed similar levels of effector molecules, like FasL, perforin, and Granzyme B (Fig. 3C). However,  $T_{reg}$  cells in CCL5<sup>low</sup> CT26 tumors showed a marked reduction in the expression of latency-associated peptide (LAP) and TGF- $\beta$  (Fig. 3C). These results suggest that CCL5 expression could not only promote the recruitment of



**Figure 2.** Knockdown of CCL5 secretion from tumors results in delayed tumor growth in mice. A, RT-PCR analysis of CCL5 expression in CT26 cells and NIH 3T3 cells.  $\beta$ -Actin was used as a control. B, CCL5 levels in culture supernatants from CT26 cells and NIH 3T3 cells were measured by ELISA. C, CT26 cells were transfected with a lentiviral vector producing shRNA to CCL5. CCL5 knockdown CT26 cells (CCL5<sup>low</sup> CT26) were confirmed by ELISA of culture supernatants. D, *in vitro* proliferation of wild-type (WT) CT26 cells (- $\blacktriangle$ -), CCL5<sup>low</sup> CT26 cells (- $\blacksquare$ -), and CT26 cells transfected with control vector (control CT26; - $\circ$ -). E, nude mice were inoculated with either  $1 \times 10^5$  control CT26 cells (- $\circ$ -) or  $1 \times 10^5$  CCL5<sup>low</sup> CT26 cells (- $\blacksquare$ -). Growth pattern of tumors are shown. F, BALB/c mice were inoculated with  $1 \times 10^5$  WT CT26 cells (- $\blacktriangle$ -),  $1 \times 10^5$  control CT26 cells (- $\circ$ -), or  $1 \times 10^5$  CCL5<sup>low</sup> CT26 cells (- $\blacksquare$ -). Growth pattern of tumors are shown. Data show mean  $\pm$  SEM of  $n = 6$  mice. Results are representative of 3 independent experiments. \*,  $P < 0.05$ .



**Figure 3.** Knockdown of CCL5 secretion from tumors results in reduced production of TGF-β by tumor-infiltrating T<sub>reg</sub> cells. Three weeks after tumor inoculation, (A) the percentage and absolute number of CD4<sup>+</sup> T cells and (B) the percentage of CD4<sup>+</sup> Foxp3<sup>+</sup> T<sub>reg</sub> cells in TILs from control CT26 tumors or CCL5<sup>low</sup> CT26 tumors were determined by flow cytometry. C, different expression of effector molecules on T<sub>reg</sub> cells from spleen or TILs from control CT26 tumors or CCL5<sup>low</sup> CT26 tumors. Data show mean ± SEM of *n* = 6 mice. Results are representative of 3 independent experiments. \*\*, *P* < 0.01 and NS, not statistically significant.

T<sub>reg</sub> cells but also enhance the production of TGF-β by T<sub>reg</sub> cells in tumor microenvironment.

**Knockdown of CCL5 secretion from tumors reduces the apoptosis of tumor-infiltrating CD8<sup>+</sup> T cells**

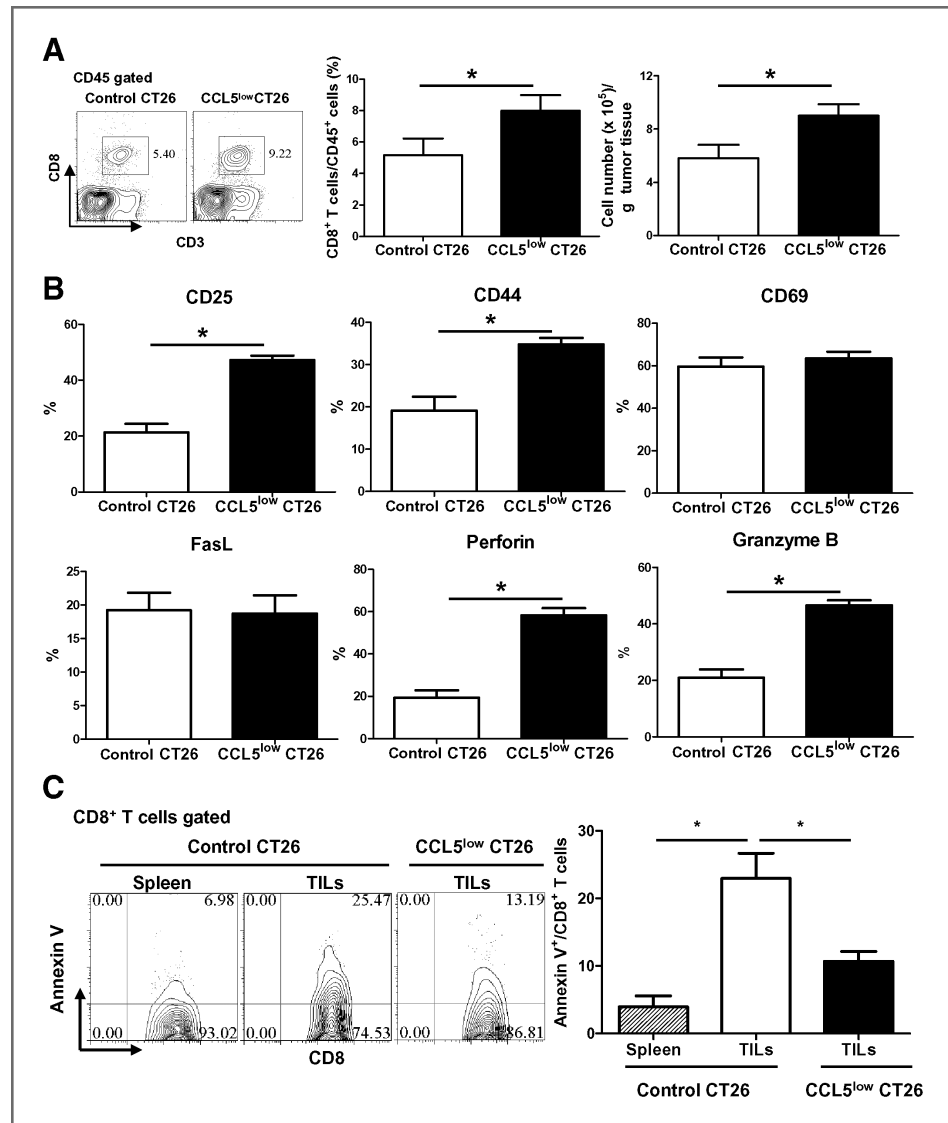
We also compared the phenotypes of CD8<sup>+</sup> T cells in both CCL5<sup>low</sup> CT26 tumors and control CT26 tumors. The percentage and absolute number of CD8<sup>+</sup> T cells were increased in CCL5<sup>low</sup> CT26 tumors when compared with control CT26 tumors (Fig. 4A). In addition, CD8<sup>+</sup> T cells in CCL5<sup>low</sup> CT26 tumors were highly activated, which expressed increased levels of CD25, CD44, perforin, and Granzyme B when compared with control CT26 tumors (Fig. 4B). Numerical reduction in CD8<sup>+</sup> T cells in control CT26 tumors might be from their enhanced mortality. The apoptosis of CD8<sup>+</sup> T cells in tumor was significantly higher than that in spleen (Fig. 4C). As well as, the

knockdown of CCL5 secretion by tumors led to reduced apoptosis of tumor-infiltrating CD8<sup>+</sup> T cell (Fig. 4C). Taken together, these results suggest that CCL5 expression in tumor affects the phenotypes of T<sub>reg</sub> cells and CD8<sup>+</sup> T cells in tumor microenvironment.

**CCL5 enhances T<sub>reg</sub> cell-mediated CD8<sup>+</sup> T-cell apoptosis in a TGF-β-dependent manner**

Considering the correlation between CCL5 and apoptosis of CD8<sup>+</sup> T cells, we first showed CCL5 could only lead to a marginal increase in the apoptosis of CD8<sup>+</sup> T cells *in vitro* (Fig. 5A). We then examined whether T<sub>reg</sub> cells could induce the apoptosis of CD8<sup>+</sup> T cells or not. T<sub>reg</sub> cells were isolated from spleen or DLN of mice bearing control CT26 tumors and incubated with CCL5 *in vitro*. CCL5-pretreated T<sub>reg</sub> cells could enhance the apoptosis of CD8<sup>+</sup> T cells *in vitro* when compared

**Figure 4.** Knockdown of CCL5 secretion from tumors results in a reduced apoptosis of tumor-infiltrating CD8<sup>+</sup> T cells. **A**, three weeks after tumor inoculation, the percentage and absolute number of CD8<sup>+</sup> T cell in TILs from control CT26 tumors or CCL5<sup>low</sup> CT26 tumors were determined by flow cytometry. **B**, different expression of activated/effector molecules on CD8<sup>+</sup> T cells in TILs from control CT26 tumors or CCL5<sup>low</sup> CT26 tumors. **C**, the percentage of Annexin V<sup>+</sup>CD8<sup>+</sup> T cells in spleen or TILs from control CT26 tumors or CCL5<sup>low</sup> CT26 tumors. Data show mean ± SEM of *n* = 6 mice. Results are representative of 3 independent experiments. \*, *P* < 0.05.



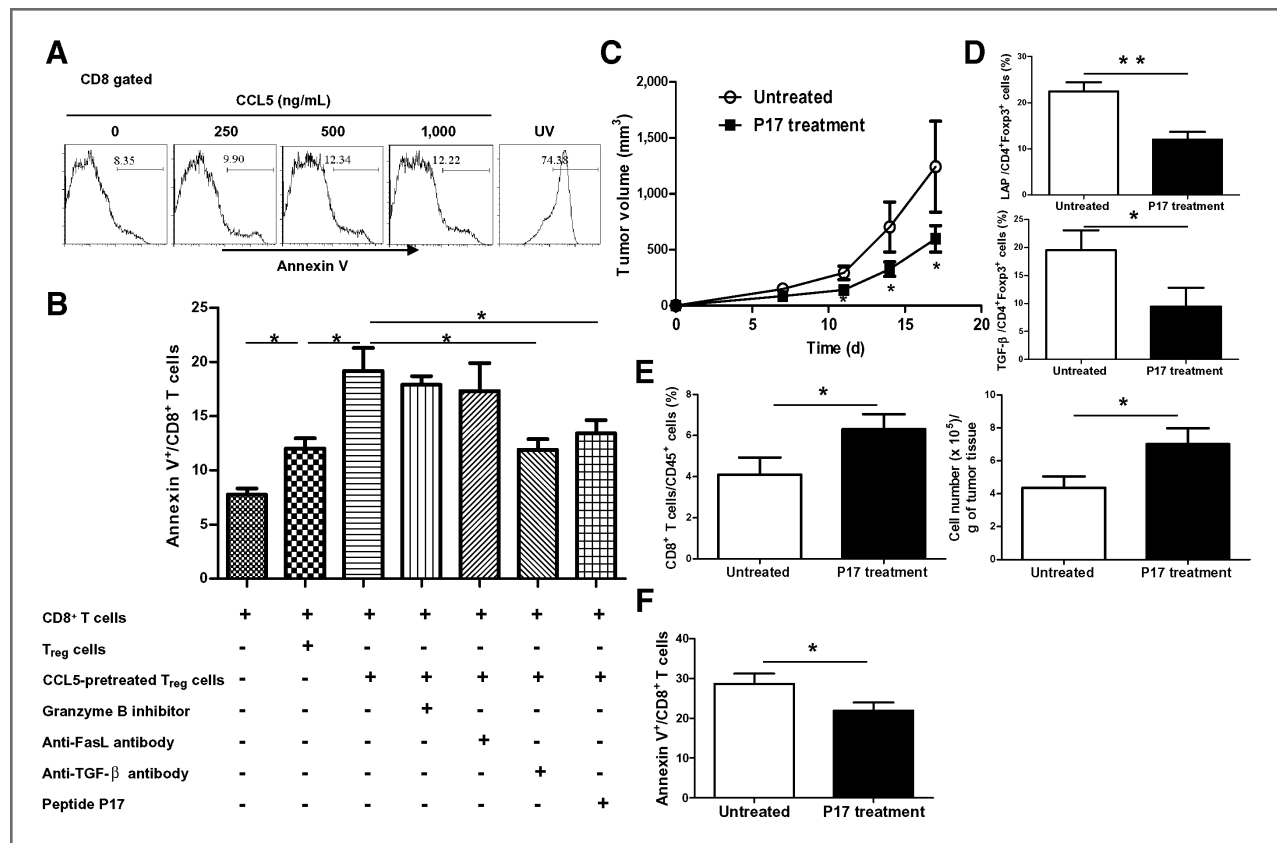
with T<sub>reg</sub> cells without CCL5 pretreatment or with CD8<sup>+</sup> T cells alone (Fig. 5B and Supplementary Fig. S2). In addition, we found that this enhanced apoptosis of CD8<sup>+</sup> T cells was blocked by anti-TGF-β-neutralizing antibody but not Granzyme B inhibitor and anti-FasL-neutralizing antibody (Fig. 5B and Supplementary Fig. S2). Peptide P17 has been shown to inhibit TGF-β production of T<sub>reg</sub> cells *in vitro* and *in vivo* (25). We further confirmed the role of TGF-β on the apoptosis of CD8<sup>+</sup> T cells by incubating these cells with peptide P17. Peptide P17 also blocked CCL5-pretreated T<sub>reg</sub> cell-mediated CD8<sup>+</sup> T-cell apoptosis *in vitro* (Fig. 5B and Supplementary Fig. S2). Furthermore, mice treated with peptide P17 had a delay in tumor growth and a reduction in the expression of TGF-β and LAP on tumor-infiltrating T<sub>reg</sub> cells (Fig. 5C and D). Administration of peptide P17 also increased the absolute number of CD8<sup>+</sup> T cells and reduced the apoptosis of CD8<sup>+</sup> T cells in tumors (Fig. 5E and F). In addition, the expression of IFN-γ in tumor-infiltrating CD8<sup>+</sup> T cells was increased in mice treated

with peptide P17 (Supplementary Fig. S3). Taken together, these results indicate that CCL5 enhances T<sub>reg</sub> cell-mediated CD8<sup>+</sup> T cell apoptosis through TGF-β signaling.

**CCR5-deficient mice exhibit slower tumor growth by restricting the killing ability of T<sub>reg</sub> cells on CD8<sup>+</sup> T cells**

We next examined the expression of CCL5-associated receptors CCR3 and CCR5 on both T<sub>reg</sub> cells and CD8<sup>+</sup> T cells in tumor. Only a minor proportion of CD8<sup>+</sup> T cells (<5%) expressed CCR3 or CCR5 (Supplementary Fig. S4). However, CCR5 but not CCR3 was highly expressed on CD4<sup>+</sup>Foxp3<sup>+</sup> T cells compared with CD4<sup>+</sup>Foxp3<sup>-</sup> T cells (Supplementary Fig. S4). Furthermore, the tumor growth was significantly delayed in CCR5-deficient mice than in BALB/c mice or CCR3-deficient mice (Fig. 6A). Furthermore, the frequencies of both T<sub>reg</sub> cells and apoptotic CD8<sup>+</sup> T cells in TILs were significantly reduced in CCR5-deficient mice than in BALB/c mice or CCR3-deficient mice (Fig. 6B and C). In addition, tumor-infiltrating T<sub>reg</sub> cells

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**Figure 5.** CCL5 enhances killing ability of T<sub>reg</sub> cells on CD8<sup>+</sup> T cell through TGF-β signaling. **A**, CD8<sup>+</sup> T cells were isolated from spleen of BALB/c mice bearing control CT26 tumors and stimulated with anti-CD3 mAb and anti-CD28 mAb in the absence or presence of CCL5 for 48 hours. The expression of Annexin V<sup>+</sup> on CD8<sup>+</sup> T cells was determined by flow cytometry. **B**, T<sub>reg</sub> cells were isolated from spleen of BALB/c mice bearing control CT26 tumors and cultured with IL-2 in absence or presence of CCL5 for 18 hours. These T<sub>reg</sub> cells were cocultured with CD8<sup>+</sup> T cells and this coculture was stimulated with anti-CD3 mAb and anti-CD28 mAb in the presence or absence of the indicated mAb or inhibitor for 48 hours. The expression of Annexin V<sup>+</sup> on CD8<sup>+</sup> T cells was determined by flow cytometry. **C**, BALB/c mice were inoculated with  $1 \times 10^5$  CT26 tumor cells and 3 days later, mice were left untreated or received 10 administrations of peptide P17 in a 14-day interval. Growth pattern of tumors are shown. **D**, the expression of TGF-β and LAP by T<sub>reg</sub> cells, **(E)** the percentage and absolute number of CD8<sup>+</sup> T cells, and **(F)** the percentage of Annexin V<sup>+</sup>CD8<sup>+</sup> T cells in TILs were determined by flow cytometry after peptide P17 treatment. Data show mean ± SEM of  $n = 6$  mice. Results are representative of 3 independent experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

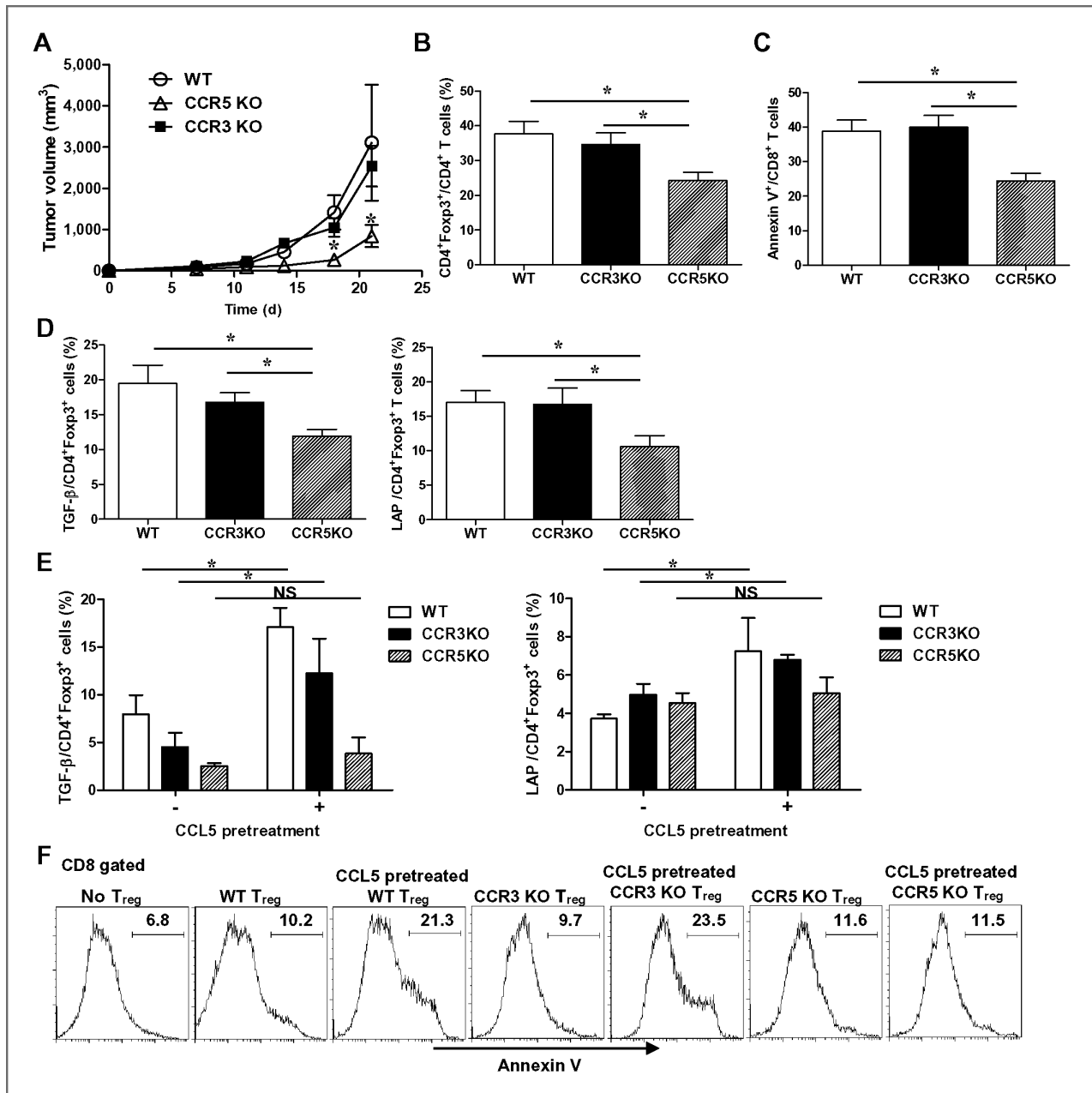
expressed lower levels of TGF-β and LAP in CCR5-deficient mice than in control mice or CCR3-deficient mice (Fig. 6D). We further examined the *in vitro* effect of CCL5 on the expression of TGF-β and LAP by T<sub>reg</sub> cells. CCL5 pretreatment significantly enhanced the expression of TGF-β and LAP on T<sub>reg</sub> cells from BALB/c mice and CCR3-deficient mice but not from CCR5-deficient mice (Fig. 6E).

We then examined whether the CCL5/CCR5 signaling was important for T<sub>reg</sub> cell-mediated CD8<sup>+</sup> T-cell apoptosis. CCL5 pretreatment significantly enhanced T<sub>reg</sub> cell-mediated CD8<sup>+</sup> T-cell apoptosis *in vitro* when T<sub>reg</sub> cells were from BALB/c mice and CCR3-deficient mice but not when T<sub>reg</sub> cells were from CCR5-deficient mice (Fig. 6F). We also examined whether the CCL5/CCR5 signaling was required for suppression ability of T<sub>reg</sub> cells *in vivo*. We sorted wild-type T<sub>reg</sub> cells and CD8<sup>+</sup> T cells from spleen of BALB/c mice bearing control CT26 tumors and cotransferred them into CCR5-deficient mice bearing control CT26 tumors or CCL5<sup>low</sup> CT26 tumors. The cotransfer of T<sub>reg</sub> cells could suppress antitumor responses of CD8<sup>+</sup> T cells in mice bearing control

CT26 tumors but not in mice bearing CCL5<sup>low</sup> CT26 tumors (Fig. 7A). On the other hand, we also cotransferred CCR5-deficient T<sub>reg</sub> cells with CD8<sup>+</sup> T cells into CCR5-deficient mice bearing control CT26 tumors. However, CCR5-deficient T<sub>reg</sub> cells failed to suppress antitumor response of CD8<sup>+</sup> T cells when compared with wild-type T<sub>reg</sub> cells (Fig. 7B). These results indicate the CCL5/CCR5 signaling contributes to the colorectal tumor progression through enhancing suppressive capacity of T<sub>reg</sub> cells.

## Discussion

CCL5 is known to be highly expressed in tumor microenvironment and contributes to tumor development in diverse human and murine tumors (20–23). Herein, we showed that in human CRC, apoptosis of CD8<sup>+</sup> T cells and infiltration of T<sub>reg</sub> cells was increased along with higher expression of CCL5. In addition, we explored the role of CCL5 in tumor growth using CT26 mouse colon tumor model. We found that the secretion of CCL5 from tumors was helpful for tumor growth through

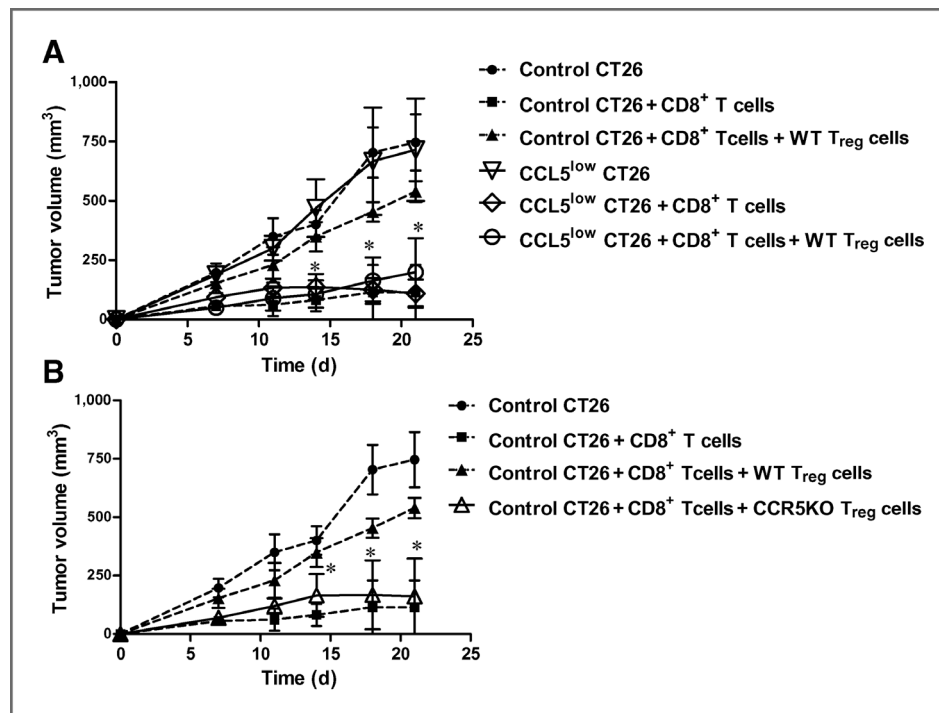


**Figure 6.** Reduced tumor growth in CCR5-deficient mice is associated with perturbed killing ability of T<sub>reg</sub> cells. A, a total of  $1 \times 10^5$  CT26 cells were inoculated in BALB/c mice (WT, -○-), CCR5-deficient mice (CCR5KO, -△-), and CCR3-deficient mice (CCR3KO, -■-). The growth of the tumors in different groups is depicted. Three weeks after tumor inoculation, the percentage of (B) CD4<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells and (C) Annexin V<sup>+</sup>CD8<sup>+</sup> T cells and (D) the expression of TGF-β and LAP in T<sub>reg</sub> cells in TILs were determined by flow cytometry. E, T<sub>reg</sub> cells were isolated from spleen of BALB/c mice, CCR3-deficient mice, or CCR5-deficient mice bearing control CT26 tumors and cultured with IL-2 in the absence or presence of CCL5 for 18 hours. These T<sub>reg</sub> cells were stimulated with anti-CD3 mAb and anti-CD28 mAb for 24 hours, and the expression of TGF-β and LAP by T<sub>reg</sub> cells were determined by flow cytometry. F, T<sub>reg</sub> cells were cocultured with CD8<sup>+</sup> T cells and this coculture was stimulated with anti-CD3 mAb and anti-CD28 mAb for 48 hours. The expression of Annexin V<sup>+</sup> on CD8<sup>+</sup> T cells was determined by flow cytometry. Data show mean  $\pm$  SEM of  $n = 6$  mice. Results are representative of 3 independent experiments. \*,  $P < 0.05$  and NS, not statistically significant.

modulating adaptive immune system because this advantage was lost in nude mice and RAG-1-deficient mice. In addition, knockdown of CCL5 secretion from tumors reduced T<sub>reg</sub> cell infiltration into tumor microenvironment and, interestingly, also reduced the TGF-β expression in T<sub>reg</sub> cells. Furthermore, CCL5 could enhance the killing ability of T<sub>reg</sub> cells on CD8<sup>+</sup> T

cells through TGF-β signaling. Similar observations were redisplayed by CCR5-deficient mice. These results indicate the tumor could secrete CCL5 to enhance the T<sub>reg</sub> cell suppressive function in tumor microenvironment through CCL5/CCR5 signaling and contribute to the immune-evasion behavior of tumor.





**Figure 7.** The CCL5/CCR5 signaling is required for T<sub>reg</sub> cells in controlling antitumor responses of CD8<sup>+</sup> T cells. CCR5KO T<sub>reg</sub> cells were isolated from spleen of CCR5-deficient mice bearing control CT26 tumors. WT T<sub>reg</sub> cells and CD8<sup>+</sup> T cells were isolated from spleen of BALB/c mice bearing control CT26 tumors. A, CCR5-deficient mice were inoculated with  $1 \times 10^5$  control CT 26 cells or CCL5<sup>low</sup> CT26 cells, and 1 day later, CD8<sup>+</sup> T cells ( $1 \times 10^6$ ) alone (-■-; -◇-), or together with WT T<sub>reg</sub> cells ( $2 \times 10^5$ ; -▲-; -○-) were cotransferred into these mice. Control group of mice were inoculated with tumors but without cell transferred (-●-; -▽-). B, CD8<sup>+</sup> T cells ( $1 \times 10^6$ ) alone (-■-), or together with WT T<sub>reg</sub> cells ( $2 \times 10^5$ ; -▲-), or together with CCR5KO T<sub>reg</sub> cells ( $2 \times 10^5$ ; -△-) were cotransferred into CCR5-deficient mice bearing control CT26 tumors generated 1 day earlier. Control group of mice were inoculated with tumors but without cell transferred (-●-). Growth pattern of tumors are shown. Data represent show mean  $\pm$  SEM of  $n = 4$  mice. Results are representative of 2 independent experiments. \*,  $P < 0.05$ .

The induction of apoptosis on effector CD8<sup>+</sup> T cells is one of the mechanisms used by tumors to engineer an immune evasion (28). It has been reported that melanoma tumor cells could stimulate TILs to secrete CCL5, which participates in activating apoptosis pathways in TILs through CCR5 (29). On the other hand, gastric tumors could also stimulate CD4<sup>+</sup> T cells to secrete CCL5 and induce FasL-Fas-mediated apoptosis of CD8<sup>+</sup> T cells (21). However, all these evidence were only showed *in vitro* and no *in vivo* corresponding phenomenon had been shown. In addition, the influence of tumor-derived CCL5 on the tumor microenvironment had not been investigated before. In our study, exogenous CCL5 alone could only marginally increased the apoptosis of CD8<sup>+</sup> T cells from tumor-bearing mice *in vitro*. On the contrary, knockdown of CCL5 secretion from tumor could significantly reduce the apoptosis of CD8<sup>+</sup> T cells in TILs. Therefore, the direct effect of tumor-derived CCL5 on the apoptosis of CD8<sup>+</sup> T cells could not explain the high apoptosis of CD8<sup>+</sup> T cells in the tumor microenvironment in our tumor model.

It has been reported that breast tumor cell-derived CCL5 induced tumor-supporting function in T cells by increasing their ability to highly express matrix metalloproteinases (22). In our study, we showed that an indirect effect of CCL5 on the apoptosis of CD8<sup>+</sup> T cells was through enhancing the killing ability of T<sub>reg</sub> cells on CD8<sup>+</sup> T cells. T<sub>reg</sub> cells have been shown

to induce apoptosis of effector T cells as a mechanism of suppression through killing molecules like perforin, FasL, and Granzyme B (30, 31). We previously reported that tumor-infiltrating T<sub>reg</sub> cells expressed higher levels of killing-associated molecules, such as perforin, FasL, and Granzyme B (17). However, the expression of these killing molecules in tumor-infiltrating T<sub>reg</sub> cells from CCL5<sup>low</sup> CT26 tumor was unaltered when compared with T<sub>reg</sub> cells from control CT26 tumor. The inhibition of Granzyme B and FasL did not diminish the killing ability of T<sub>reg</sub> cells *in vitro*. Recent studies have shown that TGF- $\beta$  is a key factor in controlling the apoptosis of effector CD8<sup>+</sup> T cells in distinct viral and bacterial infection models (32–34). Consistent with this, we observed that the inhibition of TGF- $\beta$  production by T<sub>reg</sub> cells, using a peptide P17, diminished the apoptosis of CD8<sup>+</sup> T cells *in vitro* and *in vivo*. This observation emphasizes the critical role of TGF- $\beta$  in the induction of CD8<sup>+</sup> T cell apoptosis in forging the immunosuppressive tumor microenvironment.

The importance of CCR5 for the chemotaxis of T<sub>reg</sub> cell had been shown in several disease models (23, 35–37). In this study, we found CCR5-deficient mice had delayed tumor growth and a decrease in T<sub>reg</sub> cell infiltration in tumor microenvironment. In addition, CCL5 failed to enhance not only the expression of TGF- $\beta$  by CCR5-deficient T<sub>reg</sub> cells but also their killing ability on CD8<sup>+</sup> T cells. Therefore, these findings indicate that

CCL5/CCR5 signaling could not only induce the migration of  $T_{reg}$  cells into the tumor microenvironment but also increase the synthesis of TGF- $\beta$  in these  $T_{reg}$  cells that in turn enhance the killing on CD8<sup>+</sup> T cells. In addition, the tumor-derived CCL5 plays a major role in forging the immunosuppressive tumor microenvironment through this mechanism. On the other hand, several CCR5 antagonists have been used for treatment of HIV infection in clinical trials (38). Thus, this observation suggests that a therapeutic *targeting* on CCR5 signaling by these CCR5 antagonists may be useful for the future clinical application in colorectal cancer immunotherapy.

In conclusion, we show a new mechanism that tumors adapt to manipulate antitumor immune responses by secreting CCL5 that in turn attract the  $T_{reg}$  cells into tumor

microenvironment and enhance their killing ability on CD8<sup>+</sup> T cells. This phenomenon promotes an immunosuppressive tumor microenvironment that helps in tumor progression.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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