Blockade of TNF-\(\alpha\) signaling benefits cancer therapy by suppressing effector regulatory T cell expansion

Li-Yuan Chang\(^1\), Yung-Chang Lin\(^2,3\), Jy-Ming Chiang\(^2,4\), Jayashri Mahalingam\(^1\), Shih-Huan Su\(^2\), Ching-Tai Huang\(^2,5\), Wei-Ting Chen\(^1,2\), Chien-Hao Huang\(^1\), Wen-Juei Jeng\(^1\), Yi-Cheng Chen\(^1,2\), Shi-Ming Lin\(^1,2\), I-Shyan Sheen\(^1,2\), and Chun-Yen Lin\(^1,2,*\)

\(^1\)Division of Hepatology; Department of Gastroenterology and Hepatology; Linkou Medical Center; Chang Gung Memorial Hospital; Kweishan, Taoyuan, Taiwan; \(^2\)College of Medicine; Chang Gung University; Kweishan, Taoyuan, Taiwan; \(^3\)Department of Hematology/Oncology; Linkou Medical Center; Chang Gung Memorial Hospital; Kweishan, Taoyuan, Taiwan; \(^4\)Colorectal Surgery Section; Department of Surgery; Linkou Medical Center; Chang Gung Memorial Hospital; Kweishan, Taoyuan, Taiwan; \(^5\)Department of Infectious Disease; Linkou Medical Center; Chang Gung Memorial Hospital; Kweishan, Taoyuan, Taiwan

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**Introduction**

Treg cells are abundant in the tumor microenvironment and one of the major players for tumor-induced immune suppression.\(^1\) Recent studies have highlighted the fact that Treg cells are phenotypically and functionally heterogeneous and could be divided into effector Treg cells and naive Treg cells in both human and mouse models.\(^2,3\) Previously, we reported CD103\(^+\) effector Treg cells accumulated in the tumor microenvironment and had potent suppression ability in the mouse CT26 colon tumor model.\(^4,5\) In human studies, Sakaguchi’s group demonstrated CD45RA\(^-\)Foxp3\(^{\text{high}}\) Treg cells as effector Treg cells which are increased in patients with cancer.\(^1,6\) We have also shown these CD45RA\(^-\)Foxp3\(^{\text{high}}\) effector Treg cells were highly suppressive, accumulated in tumor tissues and correlated with disease progression in patients with CRC.\(^7\) This highlights the important regulatory role of tumor-associated effector Treg cells in both human and mouse models.

However, the underlying mechanisms for the expansion of these tumor-associated effector Treg cells were still unknown. Herein, we show effector Treg cells can inhibit the antitumor immune responses in a non-tumor specific way. In addition, TNF-\(\alpha\)/TNFR2 signaling can expand the effector Treg cells in both patients and murine models of CRC and HCC. Furthermore, blockade of TNF-\(\alpha\)/TNFR2 signaling selectively prevented the rapid resurgence of Treg cells after cyclophosphamide-induced lymphodepletion and inhibited the growth of established tumors. Thus, we propose a novel mechanism in which TNF-\(\alpha\) could promote tumor-associated effector Treg cell expansion and suggest a new cancer immunotherapy strategy using TNF-\(\alpha\) inhibitors to reduce effector Treg cells expansion after cyclophosphamide-induced lymphodepletion.

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**Results**

Mice bearing 28-d primary tumors facilitating the growth of secondary tumor is tumor-nonspecific

Concomitant tumor immunity is a phenomenon in which a host with a primary tumor rejects an inoculum of the same tumor.
at a distant site. This effect is lost during tumor progression as shown in our previous studies. We investigated whether this loss of concomitant tumor immunity is tumor specific or not. We chose CT26 cells and BNL cells for these studies. As shown in Fig. 1A, the mice bearing a 7-d primary CT26 tumor were capable of inducing potent concomitant tumor immunity to the growth of secondary CT26 tumors, but not to the growth of secondary BNL tumors (Group 1 and 3 in Fig. 1A). However, this concomitant tumor immunity was lost when the secondary same tumors (CT26) were given on 28 d (Group 2 in Fig. 1A). Interestingly, the growth of different tumors (BNL) was significantly enhanced (Group 4 in Fig. 1A). This phenomenon still held true if the primary tumor was BNL and the secondary tumor was CT26 (Fig. 1B). These results indicate the mice with 28-d tumor could facilitate the growth of a second tumor challenge and, contrary to concomitant tumor immunity, this effect is tumorspecific.

Effector Treg cells are required for the facilitation of secondary tumor growth in mice bearing large tumors

We then demonstrated this loss of concomitant immunity is mediated by adaptive immunity because this phenomenon could not be found in RAG1<sup>−/−</sup> mice (Fig. 2A). Recently, we have shown effector Treg cells with higher CD103 expression were increased in CT26 tumor-bearing mice and were responsible for inhibiting CD8<sup>+</sup> T cell-mediated antitumor immune responses. We therefore investigated the phenotypes of these Treg cells in these animal models. The frequencies of splenic

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**Figure 1.** Loss of tumor immunity is not tumor-specific. (A) $1 \times 10^5$ CT26 cells were inoculated into the flanks of BALB/c mice on day 0. On day 7, secondary tumor challenge with $1 \times 10^5$ CT26 cells (Group 1) or $2 \times 10^6$ BNL cells (Group 3) were inoculated into the contralateral flank of mice. On day 28, secondary tumor challenge with $1 \times 10^5$ CT26 cells (Group 2) or $2 \times 10^6$ BNL cells (Group 4) were inoculated into the contralateral flank of mice. The graphs show growth pattern of secondary challenge tumor in mice with (□) or without (control, [] primary CT26 tumor inoculation. (B) $2 \times 10^5$ BNL cells were inoculated into the flanks of BALB/c mice on day 0. On day 7, secondary tumor challenge with $2 \times 10^5$ BNL cells (Group 1) or $1 \times 10^5$ CT26 cells (Group 3) were inoculated into the contralateral flank of mice. On day 28, secondary tumor challenge with $2 \times 10^5$ BNL cells (Group 2) or $1 \times 10^5$ CT26 cells (Group 4) were inoculated into the contralateral flank of mice. The graphs show growth pattern of secondary challenge tumor in mice with (□) or without (control, [] primary BNL tumor inoculation. Data show mean ± SEM of $n = 5$ and are representative of three independent experiments. *$p < 0.05$, **$p < 0.01$. 
CD103^+ Treg cells increased with tumor progression in both BNL and CT26 tumor-bearing mice (Figs. 2B and C). These CD103^+ Treg cells had activated/memory phenotype with higher expression of CD69, LAG-3, CD44, ICOS, CTLA-4, GITR, and CCR5, and lower expression of CD62L (Fig. 2D). Furthermore, treating these mice with CD25-depleting PC61
antibody led to a reduction in Treg cells and efficiently inhibited the facilitation of different tumor growth (Figs. 3A and B).

CD8<sup>+</sup> T cells were then isolated from spleens of day 28 BNL tumor-bearing mice (BNL CD8<sup>+</sup> T cells) or day 28 CT26 tumor-bearing mice (CT26 CD8<sup>+</sup> T cells) and combined with each of three Treg populations: CD4<sup>+</sup>CD25<sup>+</sup> T cells from day 28 CT26 tumor-bearing mice (CT26 Treg cells), CD4<sup>+</sup>CD25<sup>+</sup> T cells from day 28 BNL tumor-bearing mice (BNL Treg cells) or CD4<sup>+</sup>CD25<sup>+</sup> T cells from naive mice (naive Treg cells). These individual populations were co-transferred into BALB/c mice one day after BNL or CT26 tumor inoculation. As shown in these individual populations, CD4<sup>+</sup>CD25<sup>+</sup> T cells from day 28 BNL tumor-bearing mice (BNL Treg cells) or CD4<sup>+</sup>CD25<sup>+</sup> T cells from naive mice (naive Treg cells). These individual populations were co-transferred into BALB/c mice one day after BNL or CT26 tumor inoculation. As shown in these individual populations, CD4<sup>+</sup>CD25<sup>+</sup> T cells from day 28 BNL tumor-bearing mice (BNL Treg cells) and in the spleens in both CT26 tumor-bearing mice and BNL tumor-bearing mice (Fig. 4A). Transcripts of cytokine receptor expression in CD103<sup>+</sup> Treg cells were analyzed for the purpose of searching for possible molecules that could expand these CD103<sup>+</sup> Treg cells. Within these transcripts, the Tnfrsf1b (TNFR2) transcript was most intriguing because TNF-α has recently been shown to promote Treg cell expansion and activation in the inflammatory microenvironment through TNFR2 in an antigen-nonspecific way. Correlating with the gene expression results, by flow cytometric analysis, the CD103<sup>+</sup> Treg cells expressed higher levels of TNFR2 when compared with CD103<sup>+</sup> Treg cells in spleen (Fig. 4B) and tumor microenvironment (Fig. 4C). Parallelized with these results, the levels of TNF-α was increased in the tumor microenvironment and in the spleens in both CT26 tumor-bearing mice and BNL tumor-bearing mice with tumor progression (Fig. 4D). In addition, blocking the TNF-α signaling either by anti-TNF receptor 2 antibodies or by a soluble TNF receptor 2 fusion proteins (sTNF2-Fc) efficiently prevented CD103<sup>+</sup> Treg cell expansion induced by TNF-α <i>in vitro</i> (Figs. 4E and F). For further examining the <i>in vivo</i> suppression ability of TNF-α-pretreated Treg cells, these Treg cells were co-transferred with CT26 CD8<sup>+</sup> T cells into the mice one day after CT26 tumor inoculation. As shown in Fig. 4G, Treg cells with TNF-α pretreatment were more potent than Treg cells without TNF-α pretreatment in suppressing CD8<sup>+</sup> T cell-mediated antitumor responses. Hence, TNF-α/TNFR2 signaling could promote effector T cell expansion in tumor-bearing mice and inhibit antitumor immunity.

High serum TNF-α level is associated with an increased proportion of CD4<sup>+</sup>Foxp3<sup>high</sup>CD45RA<sup>+</sup> effector Treg cells in peripheral blood of patients with colorectal cancer and hepatocellular carcinoma

To further investigate the relationship between TNF-α and Treg cells in human cancers, we examined serum TNF-α levels and the proportion of CD45RA<sup>+</sup>Foxp3<sup>high</sup> effector Treg cells in peripheral blood of patients with CRC or HCC. The proportion of CD45RA<sup>+</sup>Foxp3<sup>high</sup> effector Treg cells, but not CD45RA<sup>+</sup>Foxp3<sup>low</sup> naive Treg cells, was significantly increased in peripheral blood in both CRC and HCC patients (Fig. 5A) and expressed high levels of CTLA-4, CCR5 and TNFR2 in both CRC and HCC patients (Fig. 5B). Serum levels of TNF-α in both CRC and HCC patients were significantly higher than healthy volunteers (Fig. 5C) and positively correlated with the proportion of CD45RA<sup>+</sup>Foxp3<sup>high</sup> effector Treg cells both CRC and HCC patients (Figs. 5D and E). Similar to the mice model, TNF-α could expand CD45RA<sup>+</sup>Foxp3<sup>high</sup> effector Treg cells and could be inhibited by sTNFR2-Fc <i>in vitro</i> (Figs. 5F and G). Taken together, these results indicate that TNF-α is also capable of mediating the expansion of human CD45RA<sup>+</sup>Foxp3<sup>high</sup> effector Treg cells.

**Blockade of TNF-α/TNFR2 signaling inhibits effector Treg cell recovery from cyclophosphamide-induced lymphodepletion and enhances antitumor efficacy**

Recent studies have shown a re-expansion of Treg cells from lymphodepletion suppress the effective antitumor immunity developed after irradiation and/or cyclophosphamide treatment. Therefore, blockade the TNF-α/TNFR2 signaling could possibly prevent the re-expansion of Treg cells after irradiation and/or cyclophosphamide treatment. Mice with CT26 were treated with cyclophosphamide and decreased numbers of CD4<sup>+</sup>Foxp3<sup>+</sup> and CD8<sup>+</sup> T cells were found in the spleen and tumor draining lymph node (DLN) (Figs. 6A, B and C) but not for the CD103<sup>+</sup> Treg cells (Fig. 6D). These results suggested CD103<sup>+</sup> Treg cells made a quick recovery from cyclophosphamide-induced lymphodepletion and then diminished the antitumor efficacy of cyclophosphamide. In addition, a blockade of TNF-α/TNFR2 signaling by sTNFR2-Fc after cyclophosphamide treatment could strongly inhibit the tumor growth (Fig. 6A) with decreased numbers of CD103<sup>+</sup> T cells in the spleen and DLN (Fig. 6D). By contrast, the absolute numbers of CD4<sup>+</sup>Foxp3<sup>+</sup> and CD8<sup>+</sup> T cells as well as IFNγ secretion by CD8<sup>+</sup> T cells were not affected by sTNFR2-Fc treatment alone (Figs. 6B, C and E). Taken together, these results indicate that blockade of TNF-α/TNFR2 signaling inhibits effector Treg cell expansion during recovery from cyclophosphamide-induced lymphodepletion and enhances their antitumor efficacy.
Discussion

It has been reported that human and murine Treg cells preferentially express high levels of TNFR2.\textsuperscript{10,11} TNF-\(\alpha\)/TNFR2 interaction could expand Treg cells and enhance their suppressive function \textit{in vitro}.\textsuperscript{10,11} Similarly, Grinberg-Bleyer et al. demonstrated that pathogenic effector T cells boosted the expansion and suppressive function of Treg cells \textit{in vivo} through TNF-

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Cells (Treg cells) were sorted from spleen of naive mice (naive Treg cells), day 28 BNL tumor-bearing mice and day 28 CT26 tumor-bearing mice were determined by flow cytometry. ELISA measurements show the TNF-α levels in the spleen and tumor from naive mice, day 7 tumor-bearing mice and day 28 tumor-bearing mice in both murine CT26 and BNL tumor models. CD4+CD25+ T cells were isolated from spleen of naive mice and stimulated with plate-bound anti-CD3 (10 μg/ml) and soluble anti-CD28 (2.5 μg/ml) in the absence or presence of TNF-α for 72 h. The expression of CD103 on CD4+Foxp3+ T cells was determined by flow cytometry. CD4+CD25+ T cells were isolated from spleen of naive mice and stimulated as described in Figure 4E, with TNF (25 ng/ml) in the presence of 1 μg/ml anti-TNF-α mAb or sTNFR2-Fc for 72 h. The expression of CD103 on CD4+Foxp3+ T cells was determined by flow cytometry. CD4+CD25+ T cells were isolated from spleen of naive mice and stimulated as described in Figure 4E, with medium alone (control Treg cells) or with TNF-α (25 ng/ml) for 72 h. CD8+ T cells were sorted from spleen of day 28 CT26 tumor-bearing mice. BALB/c mice were inoculated with 1×10^5 CT26 cells, and 1 day later, CD8+ T cells (1×10^5) were transferred alone (ريد) co-transferred with control Treg cells (1×10^5) (أ)، or TNF-α treated Treg cells (1×10^5) (في) into these mice. Control mice were inoculated with CT26 tumor cells but without adoptive transfer of T cells (النود). The graph shows the growth pattern of the tumor in different groups. Data show mean ± SEM of n = 5 and are representative of three independent experiments. *p < 0.05, **p < 0.01.
a/TNFR2 interaction in murine models of diabetes. In addition, TNFR2-deficient Treg cells failed to suppress pathogenic Th1 responses and colon inflammation in a mouse colitis model induced by transfer of naive CD4+ T cells into Rag1−/− mice. Interestingly, a recent report had shown loss of either TNF-α and TNFR2 on immune cells resulted in decreased tumor metastasis and reduced numbers of Treg cells in the tumor microenvironment in B16F10 melanoma tumor model. These data therefore provide direct in vitro and in vivo evidence that TNF-α/TNFR2 pathway plays a critical role in the expansion and function of Treg cells in both CRC and HCC tumor of human and murine models. Furthermore, TNF-α/TNFR2 interaction promoted the expansion of effector Treg cells and enhanced their suppressive activity against CD8+ T cell-mediated antitumor immune responses. Most importantly, blockade of TNF-α/TNFR2 signaling inhibits effector Treg cell expansion during recovery from cyclophosphamide-induced lymphodepletion and markedly inhibit the growth of established tumors. Our data suggest that TNF-α/TNFR2 interaction likely induces the proliferative expansion of tumor-associate effector Treg cells and contributes to tumor progression.

The basis for the expansion of Treg cells in the tumor microenvironment remains elusive. In addition to expansion, the accumulation of Treg cells in the tumor microenvironment is caused by other mechanisms, such as conversion and trafficking. For the conversion into Treg cells, TGF-β is known to be critical to conversion of naive CD4+ T cells into Foxp3+ Treg cells. However, recent study showed TNF-α/TNFR2 interaction could impair TGF-β-induced Treg cell differentiation through
activation of Akt and inhibition of TGF-β-induced Smad3 phosphorylation in mouse model of experimental autoimmune encephalomyelitis (EAE). Blockade of TNF-α signaling could increase TGF-β-induced Treg cell differentiation, leading to the amelioration of EAE. These observations suggest that TNF-α/TNFR2 signaling may not facilitate the conversion into Treg cells but, on the contrary, impair the generation of TGF-β-induced Treg cells. As for the trafficking of Treg cells, it had been described that chemokine/chemokine receptor interaction like CCL22 plays an important role in recruitment of Treg cells into tumor microenvironment for protecting tumors from immune attacks. Similar observations had been described as well. It is not presently clear whether TNF-α/TNFR2 pathway could enhance the migration of Treg cells into the tumor microenvironment through chemokine/chemokine receptor interactions. Our recent study showed that effector Treg cells had more potent in vivo suppressive ability in inhibiting CD8+ T cell-mediated antitumor immune response. This potent in vivo suppression ability of effector Treg cells is not due to the stronger suppression ability per cell but possibly due to the tissue-migration ability through CCR5 expression. Thus, it is possible that TNF-α enhances in vivo suppressive function of effector Treg cells through increasing their tissue-migration ability. Further study is warranted to elucidate this possibility.

A lot of evidence has demonstrated that the induction of Treg cell suppressive function is antigen specific and requires TCR stimulation, but the suppressive function of Treg cells is not antigen specific. However, the issue is not clear yet, because there

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**Figure 6.** Blockade of TNF-α/TNFR2 signaling enhances antitumor efficacy of cyclophosphamide through diminishing the expansion of effector/memory Treg cells. (A) BALB/c mice were divided into four groups that received either: (●) no treatment as control; (▲) sTNFR2-Fc alone; (square) cyclophosphamide (CP) alone; or (▼) sTNFR2-Fc and CP. In all experiments, 1 × 10⁵ CT26 cells were inoculated into the flanks of BALB/c mice on day 0. 150 mg/kg CP was given i.p. on day 7 and day 14 after CT26 tumor inoculation. On day 7, mice with or without CP treatment were left untreated or received 10 administrations of sTNFR2-Fc (25 mg/kg) in a 14-d interval. Growth pattern of tumors are shown. Data show mean ± SEM of n = 5 and are representative of three independent experiments. *p < 0.05, **p < 0.01. On day 28 after CT26 tumor cell inoculation, absolute number of CD8+ T cells (B), CD4+Foxp3+ T cells (C) and CD103+CD4+Foxp3+ T cells in the spleen and tumor-draining lymph node (DLN) of mice with different experimental treatments were determined by flow cytometry. (E) Estimation of IFNγ production by CD8+ T cells purified from spleens of mice with different experimental treatments after stimulation with irradiated CT26 cells by ELISA. Each dot represents one individual sample and data show mean ± SEM, *p < 0.05, **p < 0.01 and NS, not statistically significant.
is also evidence of antigen-specific suppression by Treg cells in vivo.26-28 In this study, we found that Treg cells were activated and increased as the primary tumor progressed in both murine CT26 and BNL tumor models. Interestingly, these Treg cells could inhibit CD8+ T cell-mediated antitumor immune responses in a tumor-independent manner in vivo. Here, our results support the hypothesis that tumor-associated effector Treg cell-mediated suppression is completely nonspecific.

It had been reported that Foxp3-expressed CD4+ T cells in the tumor microenvironment of cancer patients are mainly effector Treg cells.9,29 Here, we further explored this notion and focused on these effector Treg cells. The effector T reg cells, defined as CD103+ Treg cells, as the tumor-associated Treg cells in murine tumor model had also been proposed in our lab43 and others.29 CD45RA- Foxp3high Treg cells was proposed as human counterpart of effector Treg cell by Miyara and colleagues recently.6 Subsequently these CD45RA- Foxp3high Treg cells were found to be increased in peripheral blood and accumulated in tumor microenvironment in human malignancies like CRC7 and other malignancies, similar to the behavior for tumor-associated Treg cells.1,30 In the present study, we described these human CD45RA- Foxp3high effector Treg cells express high levels of CTLA-4, CCR5 and TNFR2 in both CRC patients and HCC patients and accumulated in the tumor microenvironment, similar to phenotype of tumor-associated Treg cells in tumor bearing mice. Furthermore, in human Treg cells, like in murine model, TNF-α could also induce the expansion of CD45RA- Foxp3high effector Treg cells through interacting with TNFR2 in vitro. These evidence suggest that human CD45RA- Foxp3high effector Treg cells are the human counterpart of murine CD103 expressed tumor-associated Treg cells.

TNF-α can be detected in malignant cells and stromal cells in human cancer biopsies.31-34 In addition, the level of serum TNF-α was inversely related to the outcomes of various malignant diseases.32,35-37 However, the role of TNF-α in tumor development is quite complicated with both tumor-promoting and tumor-inhibiting role.33,38 Blockade of the TNF-α effect has been tested in phase I and phase II clinical trials with TNF-α antagonists as single agents, but only a small number of patients showed stable disease after treatment.39-42 Consistent with this, we observed that TNF-α antagonist sTNF2-Fc treatment alone did not inhibit the growth of established tumors in mice.

Interestingly, our data showed sTNF2-Fc in combination with cyclophosphamide treatment strongly inhibited tumor growth compared with cyclophosphamide treatment alone and sTNFR2-Fc treatment alone. This is because TNF-α blockade could significantly inhibit the expansion of tumor-associated Treg cells during recovery from cyclophosphamide-induced lymphodepletion. The rapid recovery of Treg cells after lymphodepletion is reported as main reason of treatment failure due to inhibition of antitumor immune responses by these recovered Treg cells.9 Though these Treg cells could be depleted with anti-CD25 mAb, the increased risk of inducing severe autoimmune disease is high due to depletion of all endogenous Treg cells. Here, we provide another tumor immunotherapeutic strategy by targeting only effector Treg cells but not all Treg cells through TNF-α inhibitors to efficiently diminish the expansion of effector Treg cells after lymphodepletion.

In conclusion, this study demonstrates that tumor-associated effector Treg cells are tumor-nonspecific. Furthermore, TNF-α/TNFR2 signaling not only induced expansion of effector Treg cells but also enhanced their functions in inhibiting antitumor responses of CD8+ T cells. Targeting TNF-α/TNFR2 signaling after cyclophosphamide treatment could significantly inhibit the expansion of effector Treg cells during recovery from lymphopenia and enhance therapeutic efficacy of cyclophosphamide. Thus, this combination therapy may contribute to cancer immunotherapy approaches.

Materials and Methods

Mice and cell lines
BALB/c mice were purchased from the National Laboratory Animal Center of Taiwan (Taipei, Taiwan). RAG-1−/− mice (C.129S7(B6)-Rag1tm1Mom/J) and Foxp3-GFP reporter mice (C. Cg-Foxp3tm2Tch/J) were purchased from Jackson Laboratory. All mice were maintained in the animal house of Chang Gung Memorial Hospital and used in experiments at ages 8–10 weeks. All animal breeding and experiments were in accordance with guidelines of the institutional animal ethics committee. BALB/c-derived murine HCC cell line BNL 1ME A.7R.1 (BNL) and CT26 colon carcinoma cell line were purchased from the American Type Culture Collection and maintained in our laboratory. BNL cells and CT26 cells were regularly cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) and RPMI 1640 medium (Invitrogen) containing 10% fetal bovine serum (FBS) (Invitrogen) and 1% penicillin/streptomycin (Invitrogen).

Human study population
From 2010 to 2012, 45 patients with CRC and 47 patients with HCC who visited Chang Gung Memorial Hospital, Linkou Medical Center, were enrolled in this study. The diagnoses of CRC and HCC were confirmed by pathological examinations and/or typical image studies. 38 healthy controls were also enrolled during the same period. Peripheral blood mononuclear cells (PBMCs) from these patients were isolated by the Ficoll/Paque™ PLUS density gradient centrifugation method (Pharmacia).

Ethics statements
All patients and healthy controls enrolled in this study provided written informed consent. This study protocol conformed to the ethical guidelines of the Declaration of Helsinki (59th World Medical Association General Assembly, Seoul, October 2008) and was approved by the ethical committees and IRB of Chang Gung Memorial Hospital.

Cell isolation, cell purification, and adoptive transfer
To isolate murine splenocytes, donor mice (wild-type mice or day-28 tumor bearing mice) were sacrificed and their spleens were harvested under sterile conditions. Single cell suspensions
were prepared and CD8⁺ T cells were sorted using magnetic microbeads conjugated with anti-mouse CD8 (Miltenyi-Biotect, Germany) by AutoMACS (Miltenyi-Biotech, Germany). For CD4⁺CD25⁺ T cell purification, single-cell suspensions were firstly enriched for CD4⁺ T cells via negative selection using the CD4⁺ isolation kit (Miltenyi-Biotech, Germany). Enriched CD4⁺ T cells were then labeled with CD25-PE (PC61, BD Biosciences), and sorted using FACSARIA (BD Biosciences). Cell purity (>90%) for all populations was confirmed by flow cytometry. Different isolated populations of T cells were adoptively transferred into mice or used in vitro. For adoptive transfer, purified T cells in appropriate numbers were resuspended in 0.2 mL of HBSS (Invitrogen) and then transferred intravenously into different mice through the tail vein.

**In vitro incubation with TNF-α**

Mouse CD4⁺CD25⁺ T cells were enriched from splenocytes of wild-type BALB/c mice. 1 × 10⁶ mouse CD4⁺CD25⁺ T cells were stimulated with 10 μg/mL plate-bound anti-CD3 (17A2, BD Biosciences), soluble 2.5 μg/mL anti-CD28 (37.51, BD Biosciences) and 10 μg/mL IL-2 (R&D System) and with or without recombinant mouse TNF-α (0–50 ng/mL; R&D System). In some experiments, 10 μg/mL hamster IgG (HTK888, BioLegend) or 10 μg/mL anti-TNFFR2 mAb (TR75-32.4, BioLegend) or 1 μg/mL sTNFR2-Fc (Etanercept; Pfizer, Taiwan) was added. In human experiments, 1 × 10⁶ PBMCs were from healthy donors and incubated with recombinant human TNF-α (0–80 ng/mL; R&D System, USA) and with or without 1 μg/mL sTNFR2-Fc (Etanercept; Pfizer, Taiwan). In all experiments, cells were cultured in a 96-well round-bottom plate in RPMI1640 medium with L-glutamine (Invitrogen), supplemented with 10% FBS (Invitrogen), 1% HEPES (Invitrogen), 1% penicillin/streptomycin (Invitrogen) and 0.1% β-mercaptoethanol (Invitrogen) for 72 h before being subjected to flow cytometry or adoptive transfer.

**Flow cytometry**

Single-cell suspensions were stained for surface and intercellular proteins with appropriately diluted Abs. Intracellular staining with Foxp3 and CTLA-4 was performed after treatment with fixation and permeabilization buffers (eBiosciences), according to the manufacturer’s protocol. The following anti-mouse antibodies were purchased from the indicated sources: CD4-PerCP (L3T4, BD Biosciences), CD103-FITC (M290, BD Biosciences), CD69-PE (H1.2F3, BD Biosciences), CD62L-PE (MEL-14, BD Biosciences), LAG3-PE (C9B7W, BD Biosciences), CCR5-PE (C34-3448, BD Biosciences), CD44-PE(IM7, BD Biosciences), CD120b (TR75-89, BD Biosciences), GITR-PE (DTA-1, BD Biosciences), ICOS-PE (7E.17G9, BD Biosciences), Foxp3-APC (FJK-16s, eBiosciences) and CTLA-4-PE (UC10-4F10-11, BD Biosciences). The following anti-human antibodies were purchased from the indicated sources: CD4-PerCP (SK1, BD Biosciences), CD45RA-FITC (HI100, BD Biosciences), Foxp3-APC (PCH101, eBiosciences), CTLA-4-PE (BNI3, BD Biosciences), CCR5-PE (2D7, BD Biosciences) and CD120b-PE (kTNFR-M1, BD Biosciences). Acquisition was performed using FACSCalibur (BD Biosciences), and data analysis was conducted using FlowJo software (Tree Star, USA).

**Tumor experiments**

A total of 1 × 10⁵ CT26 cells or 2 × 10⁶ BNL cells were harvested, washed in HBSS, and inoculated subcutaneously into the left flanks of naive mice (BALB/c mice or RAG1<−/> mice; primary tumor). In cases of secondary challenge (secondary tumor), 1 × 10⁵ CT26 cells or 2 × 10⁶ BNL cells were inoculated into the contralateral flanks of mice that received primary injections. In PC61 treatment experiments, 2 × 10⁵ BNL cells were inoculated into the flanks of BALB/c mice on day 0. On day 28, secondary tumor challenge with 1 × 10⁵ CT26 cells were inoculated into the contralateral flanks of mice with primary BNL tumor inoculation. 0.25 mg PC61 or PBS was administered intraperitoneally on day 27 and 30 after primary BNL tumor inoculation. BALB/c mice without primary BNL tumor inoculation were inoculated with 1 × 10⁵ CT26 cells and were treated with 0.25 mg anti-CD25 (PC61) or PBS according to the same schedule. In sTNFR2-Fc (Etanercept; Pfizer, Taiwan) treatment experiments, 1 × 10⁶ CT26 cells were inoculated into the flanks of BALB/c mice on day 0. 150 mg/kg cyclophosphamide (Sigma, USA) was given intraperitoneally on day 7 and day 14 after CT26 tumor inoculation. On day 7, mice with or without cyclophosphamide treatment were left untreated or received 10 administrations of sTNFR2-Fc (25 mg/kg) in a 14-d interval. In all cases, tumor were measured twice a week, and volume was calculated (length × width²/2).

**TNF-α determination**

Tissue lysates were prepared by homogenization of excised tissue in RIPA buffer containing HALT protease/phosphatase inhibitor (Pierce Biotechnology). Lysates were centrifuged at 18,000 rpm for 10 min and supernatants were removed for assay. Total protein concentration in supernatants was determined with a bicinchoninic acid assay (Pierce Biotechnology), according to the manufacturer’s recommendations. Supernatants were collected and assayed for TNF-α production by ELISA (BD Biosciences), according to manufacturer instructions. Results are presented as TNF-α concentration standardized to sample protein concentration. Plasma TNF-α level in healthy subjects and all patients were evaluated by cytometric bead array assays (BD Biosciences), according to the manufacturer’s recommendations. Samples were analyzed in a FACSCalibur flow cytometer and the data were analyzed using the BD CBA analysis software. Values were extrapolated from a standard concentration curve and are expressed as pg/mL.

**In vitro cytokine production assay**

Purified CD8⁺ T cells (1 × 10⁶ cells/mL) from spleen of experimental mice were restimulated in vitro by coculturing them with irradiated CT26 tumor cells (1 × 10⁵ cells/mL) for 48 h. Supernatants were collected and assayed for IFNγ production by ELISA according to manufacturer instructions (BD Biosciences).
DNA microarray

CD103+CD4+GFP+ T cells, CD103−CD4+GFP+ T cells and CD4++GFP− T cells were isolated by FACSAria separation from spleens of Foxp3-GFP mice bearing a day 28 CT26 tumor. Total RNA from CD103+CD4+GFP+ T cells, CD103−CD4+GFP+ T cells and CD4++GFP− T cells were labeled with Cy5, hybridized to a mouse Oligo Microarray (Mouse Whole Genome OneArray™; Phalanx Biotech Group, Hsinchu, Taiwan). The data were analyzed according to the manufacturer’s protocol.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Author Contributions

Study concept and design: LY Chang, YC Lin and CY Lin; acquisition of data: LY Chang, SH Su and J Mahalingam; analysis and interpretation of data: LY Chang, J Mahalingam and CY Lin; drafting of the manuscript: LY Chang, YC Lin and CY Lin; critical revision of the manuscript for important intellectual content: LY Chang, YC Lin and CY Lin; statistical analysis: LY Chang; administrative, technical, or material support: JM Chang, CT Huang, WT Chen, CH Huang, WJ Jeng, YC Chen, SM Lin, IS Sheen; study supervision: YC Lin and CY Lin.

Statistical analysis

Two-tailed unpaired Student’s t test was used for statistical analyses of differences of the groups and a paired t test was used to determine pairwise differences. Multiple linear regression analysis was used to determine serum TNF-α level explaining variation in the percentage of CD45RA Foxp3highCD4 effector Treg cells and CD45RA Foxp3mediumCD4+ resting Treg cells. The calculations were made using GraphPad Prism 5 Software. Differences were recognized as significant at p < 0.05.

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