Blockade of TNFR2 signaling enhances the immunotherapeutic effect of CpG ODN in a mouse model of colon cancer

Yingjie Nie,1,2 Jiang He,3 Hidekazu Shirota,1 Anna L. Trivett,1 De Yang,1 Dennis M. Klinman,1 Joost J. Oppenheim,1,8 Xin Chen3,1*

Through the tumor necrosis factor (TNF) receptor type II (TNFR2), TNF preferentially activates, expands, and promotes the phenotypic stability of CD4+Foxp3+ regulatory T (T_{reg}) cells. Those T_{reg} cells that have a high abundance of TNFR2 have the maximal immunosuppressive capacity. We investigated whether targeting TNFR2 could effectively suppress the activity of T_{reg} cells and consequently enhance the efficacy of cancer immunotherapy. We found that, relative to a suboptimal dose of the immunostimulatory Toll-like receptor 9 ligand CpG oligodeoxynucleotide (ODN), the combination of the suboptimal dose of CpG ODN with the TNFR2-blocking antibody M861 more markedly inhibited the growth of subcutaneously grafted mouse CT26 colon tumor cells. This resulted in markedly fewer TNFR2+T_{reg} cells and more interferon-γ-positive (IFN-γ+) CD8+ cytotoxic T lymphocytes infiltrating the tumor and improved long-term tumor-free survival in the mouse cohort. Tumor-free mice were resistant to rechallenge by the same but not unrelated (4T1 breast cancer) cells. Treatment with the combination of TNFR2-blocking antibody and a CD25-targeted antibody also resulted in enhanced inhibition of tumor growth in a syngeneic 4T1 mouse model of breast cancer. Thus, the combination of a TNFR2 inhibitor and an immunotherapeutic stimulant may represent a more effective treatment strategy for various cancers.

INTRODUCTION

Overcoming the immunosuppressive tumor microenvironment is key to achieving effective cancer immunotherapy (1, 2). Tumor-infiltrating CD4+Foxp3+ regulatory T (T_{reg}) cells are potent immunosuppressive cells that represent a major cellular mechanism of tumor immune evasion and play a major role in dampening naturally occurring and therapeutically induced antitumor immune responses (3). Accumulation of T_{reg} cells within tumor tissues, and the resultant high ratio of T_{reg} cells to effector T (T_{eff}) cells, is correlated with poor prognosis of cancer patients, including those with lung cancer (4), breast cancer (5), colorectal cancer (6), pancreatic cancer (7), and other malignancies. Elimination of T_{reg} activity, by either reducing their number or down-regulating their immunosuppressive function using checkpoint inhibitors, has become an effective strategy to enhance the efficacy of cancer therapy (8, 9).

Tumor necrosis factor (TNF) receptor type II (TNFR2) is predominantly present on the maximally suppressive subset of mouse and human T_{reg} cells (10, 11). There is now compelling evidence that the interaction of TNF with TNFR2 promotes the proliferative expansion, suppressive function, and phenotypical stability of T_{reg} cells (12–18). In mouse Lewis lung carcinoma and 4T1 breast tumor model, most of the tumor-infiltrating T_{reg} cells are highly suppressive TNFR2+T_{reg} cells (10, 19). In humans, the proportion of TNFR2+T_{reg} cells is also increased in the peripheral blood of lung cancer patients and in the tumor-associated ascites of ovarian cancer patients (20, 21). Recent analysis of single-cell RNA sequencing showed that the expression of TNFR2 is one of the most markedly increased genes on T_{reg} cells, as compared with CD4+T_{eff} cells and CD8+ cytotoxic T lymphocytes (CTLs) in metastatic melanoma patients, and increased TNFR2 gene expression is associated with exhaustion of CD8+ CTLs (22). Furthermore, the amount of TNFR2 present on the surface of T_{reg} cells is associated with greater lymphatic invasion, a higher incidence of tumor metastasis, a higher clinical stage, and poorer response to treatment in patients with lung cancer and acute myeloid leukemia (AML) (20, 23, 24). This clinical and experimental evidence suggests that the highly suppressive TNFR2+T_{reg} cells associated with tumors play a major role in tumor immune evasion. Meanwhile, TNFR2 is also found on several tumor cells, including colon cancer (25), Hodgkin lymphoma (26), myeloma (27), renal carcinoma (28), and ovarian cancer (29), leading many to consider TNFR2 an oncogene. Antagonistic antibody targeting TNFR2 induces the death of both T_{reg} cells and OVAR3 ovarian cancer cells, which have abundant surface TNFR2 (29). On the basis of these observations, we proposed that TNFR2 behaves as an immune checkpoint activator and oncoprotein (30).

TNF can be induced by various immunotherapies, including dendritic cell (DC)–based interventions, tumor vaccines, and Toll-like receptor (TLR) agonists (31–33). Such immunotherapy-induced TNF may, in turn, increase TNFR2 on T_{reg} cells (34), resulting in the expansion and activation of tumor-associated T_{reg} cells through TNFR2. For example, by activating DCs, the TLR9 agonistic CpG oligodeoxynucleotides (ODNs) have the capacity to induce antitumor immune responses in mouse models (35–37). CpG ODNs promote the maturation and improve the function of professional antigen-presenting cells while supporting the generation of antigen-specific B cells and CTLs (38). Intratumoral injection of CpG ODN also induces the differentiation and reduces the immunosuppressive activity of myeloid-derived suppressor cells (MDSCs) (39), therefore enhancing the host’s response to cancer (40). However, treatment with CpG ODN can also induce human or mouse T_{reg} cells that have potent immunosuppressive function, which, in turn, dampens host immune responses against the tumor (41, 42). CpG ODN treatment can increase the production
of TNF in cultured murine DCs (43) or human peripheral blood mononuclear cells (44), which is likely responsible for the activation of Treg cells. Therefore, by reducing Treg activity, blockade of TNFR2 may enhance the antitumor effect of an immunotherapeutic such as CpG ODN.

Here, we tested this hypothesis and found that the combination of a blocking antibody (M861) recognizing TNFR2 and CpG ODN more potently inhibited mouse CT26 colon tumor development and induced greater tumor regression in syngeneic mice, resulting in greater long-term tumor-free survival of the mice. In addition, in a mouse 4T1 breast cancer model, a better antitumor effect was observed by simultaneously blocking TNFR2 and CD25. Furthermore, tumor antigen-specific immunity developed in the mice that survived CT26 tumor graftment; they completely (over the time course of the experiment) and selectively resisted a rechallenge by CT26, but not 4T1, tumor cells. This effect was associated with a decrease in the proportion of Treg cells among tumor-infiltrating leukocytes, a reduced surface abundance of TNFR2 on Treg cells, and increased tumor infiltration of interferon-γ (IFN-γ)–producing CD8+ T cells. Thus, the combination of TNFR2 antagonism and immunotherapy may be a promising cancer treatment strategy.

RESULTS

M861 inhibits the stimulatory effect of TNF on Treg cells

Previously, we showed that treatment with TNF preferentially promoted the proliferative expansion of Treg cells, accompanied by increased abundance of TNFR2 on Treg cells in vitro and in lipopolysaccharide (LPS)–treated mice (18, 34). In CD4+ T cells cultured with interleukin-2 (IL-2), treatment with the TNFR2 antibody M861 significantly inhibited TNF-induced proliferation (Fig. 1A) and expansion (Fig. 1B) of Treg cells. M861 also significantly blocked TNF-induced increases in the cell surface abundance of TNFR2 on Treg cells (Fig. 1C). In LPS-challenged mice, although administration of M861 failed to reduce the number of Treg cells in the spleens and lymph nodes within 24 hours, the proportion of TNFR2+ Treg cells was significantly reduced by 64% and the abundance of TNFR2 on splenic Treg cells was significantly reduced by >56% (Fig. 1D). The decrease in TNFR2+ Treg cells was not due to cell death (fig. S1). Therefore, unlike two other antibodies recognizing human TNFR2 described in a recent study (29), M861 was not a Treg-depleting antibody. Furthermore, its capacity to reduce TNFR2 abundance appears to be more potent than its inhibition of the proliferative expansion of Treg cells induced by LPS treatment (Fig. 1). Overall, our data favor the idea that M861 is a blocking antibody that inhibits ligand-induced TNFR2 signaling.

Combination therapy with TNFR2-blocking antibody and CpG ODN potently inhibits the growth of CT26 tumors and generated tumor-specific immunity

To examine the effect of TNFR2 blockade on the efficacy of tumor immunotherapy, we treated female CT26 tumor-bearing Balb/c mice with M861 and CpG ODN or various controls (Fig. 2A). Treatment was started when the tumor reached 5 to 6 mm in diameter (day 0). CpG ODNs were administered by intratumoral injection, which was previously reported to achieve an optimal antitumor effect (39). To reveal the beneficial effect of combination therapy, we administered M861 with a suboptimal dose of CpG ODN, neither of which markedly inhibited tumor growth alone (Fig. 2B). The combination of M861 and CpG ODN potently inhibited the growth of primary CT26 tumors (Fig. 2B). Eighty percent of mice were tumor-free and survived up to the end of the experiment at 60 days, whereas mice in other groups died from tumor burden within 50 days after tumor inoculation (Fig. 2C). The individual tumor growth curves varied; although a few mice had slow tumor growth with phosphate-buffered saline (PBS), CpG, or M861 alone, the antitumor effect of the M861 and CpG ODN combination is clear (Fig. 2, D to G).

To investigate whether the tumor-free mice developed long-term CT26 tumor-specific immunity, the surviving mice were reinoculated subcutaneously with CT26 tumor cells into the right flanks, and 4T1 tumor cells were inoculated into their left flanks. As controls, both 4T1 tumor cells and CT26 tumor cells were inoculated into naïve mice at the same manner, and as expected, both tumors developed in all naïve mice (Fig. 2H). Whereas all of the CT26 (intratumoral)–surviving mice developed measurable 4T1 tumors by day 26 after inoculation, none of these mice developed CT26 colon tumors (Fig. 2I). These results indicate that the treatment with combination of M861 and CpG ODN induced the development of long-term tumor antigen-specific immunity.

M861 did not induce the death of TNFR2+ Treg cells (fig. S1), indicating that its effect was caused by binding and blocking TNFR2 signaling. The possibility that this antibody also binds and neutralizes soluble shed TNFR2 should be addressed in a future study. It was recently reported that antibodies recognizing human TNFR2, in addition to eliminating Treg cells, could also directly act on TNFR2-expressing tumor cells (29). A considerable proportion of CT26 cancer cells are TNFR2+ cells (fig. S2A). However, treatment with M861 (up to 20 μg/ml) did not inhibit the growth of CT26 cells (fig. S2B), indicating that the in vivo inhibitory effect of this antibody on CT26 tumor was not due to any direct effect on tumor cells.

Combination therapy with TNFR2-blocking antibody and CpG ODN reduces the proportion of tumor-infiltrating TNFR2+ Treg cells and increases IFN-γ+ CD8+ CTLs

Because combination therapy resulted in inhibited CT26 tumor growth and marked tumor regression in many of the mice, we were unable to examine the immune cell profile present in the tumor environment. To obtain a tumor mass for further study, we delayed the treatment until tumors reached ~10 mm in diameter, and the effect of treatments on tumor-infiltrating Treg cells was examined by fluorescence-activated cell sorting (FACS) analysis (Fig. 3, A to C). The proportion of intratumoral Treg cells was increased to ~20% by treatment with CpG ODN alone (Fig. 3D). Furthermore, CpG ODN treatment also significantly increased the amount of TNFR2 on Treg cells by ~20% (Fig. 3E). These results suggest that, in addition to stimulating the expansion of Treg cells as previously reported (41, 42), CpG ODN can also increase the function of Treg cells, as indicated by the increased abundance of TNFR2 (10, 45). Both the proportion of TNFR2+ Treg cells and the abundance of TNFR2 in Treg cells (measured as per cell) were markedly reduced by treatment with the TNFR2-blocking antibody (Fig. 3, E and F). Notably, the CpG ODN–induced expansion of Treg cells and increased abundance of TNFR2 on Treg cells were completely abrogated by M861 treatment (Fig. 3, D and E).

The combination therapy markedly increased the production of IFN-γ by CD8+ CTLs, and the proportion of IFN-γ–producing CD8+ T cells was greater than threefold as compared with CpG ODN treatment alone (Fig. 3, C and G). Thus, our data indicate that combination therapy reduced Treg activity and promoted the induction of potent type 1 helper T cell antitumor immune responses.
Fig. 1. The in vitro and in vivo effects of an antibody recognizing TNFR2 (M861) on Treg cells. (A to C) Magnetic-activated cell sorting–purified CD4+ T cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE). The cells were cultured with interleukin-2 (IL-2; 10 ng/ml) alone or with tumor necrosis factor (TNF; 20 ng/ml) and M861 (10 μg/ml), as indicated, for 72 hours. Proliferation of regulatory T (Treg) cells shown by CFSE dilution (A), the proportion of Treg cells in CD4+ cell cultures (B), and the surface abundance of TNF receptor type II (TNFR2) on Treg cells (C) were analyzed with fluorescence-activated cell sorting (FACS), gating for Foxp3+ staining (A and C) or not gated (total cells; B). (D) Wild-type Balb/c mice were injected intraperitoneally with phosphate-buffered saline (PBS), lipopolysaccharide (LPS; 200 μg), M861 (100 μg), mouse immunoglobulin G (muIgG), or a combination thereof, as indicated, for 24 hours. The abundance of TNFR2 on the surface of Treg cells in the spleen was analyzed by FACS, gating for CD4+Foxp3+ staining. FACS plots are representative of three independent experiments. Data are means ± SEM of n = 5 mice. Number in each FACS plot indicates the percentage of gated cells. *P < 0.05, **P < 0.01, ***P < 0.001 by Student’s t test (A to D).
Combination of TNFR2 antagonistic antibody and CD25 antagonistic antibody inhibits the development of mouse 4T1 breast tumors

CD25, the IL-2 receptor α chain, was the first identified surface marker of T<sub>reg</sub> cells (46), and targeting of CD25 has been a conventional approach for T<sub>reg</sub> elimination (47, 48). PC61 is a CD25-recognizing monoclonal antibody that blocks IL-2 binding to the receptor (49), and it was shown that pretreatment with PC61 led to tumor rejection in mouse tumor models (50, 51). We thus compared the effect of TNFR2 antagonistic antibody and CD25 antagonistic antibody on the tumor development. To this end, TR75-54.7, a well-characterized functional blocking TNFR2 antibody (52), and PC61 were administered separately or in combination into mice. Three days later, the mice were inoculated with 4T1 tumor cells (Fig. 4A). The results showed that TR75-54.7 more potently inhibited the development of 4T1 tumor than PC61 (Fig. 4, B and C). Although the difference in tumor size was not statistically different between mice treated with two antibodies (Fig. 4B), only TR75-54.7 markedly enhanced the survival of tumor-bearing mice (Fig. 4C), whereas PC61 did not have such effect (Fig. 4C). Moreover, the combination of TR75-54.7 and PC61 was superior to the monotherapy in the inhibition of tumor growth (Fig. 4B). Consequently, the median survival of tumor-bearing mice was longer after combination therapy (38 days), as compared with monotherapy of TR75-54.7 (34 days) or PC61 (27 days) (Fig. 4, C and D; median survival of PBS control group was 24 days). Therefore, TR75-54.7 has more potent antitumor effect than PC61 in mouse 4T1 breast cancer model, whereas the combination of both resulted in the optimal antitumor effect.

DISCUSSION

Our data presented in this study showed that blockade of TNFR2 potently enhanced the effect of immunotherapy with CpG ODN against CT26 tumors, which was attributable to the reduction in T<sub>reg</sub> activity and consequent mobilization of CD8<sup>+</sup> CTLs. Because of the paucity of T<sub>reg</sub> cells recovered from tumor tissue, we could not determine the functionality of the tumor-infiltrating T<sub>reg</sub> cells in this study directly. Nevertheless, the marked reduction in TNFR2 abundance on T<sub>reg</sub> cells, which correlates with T<sub>reg</sub> function, suggests that M861 reduced the suppressive capacity of T<sub>reg</sub> cells in the tumor environment. This effect of M861 treatment appears to be specific for tumor-infiltrating T<sub>reg</sub> cells, given that the proportion of T<sub>reg</sub> cells, the abundance of TNFR2 on T<sub>reg</sub> cells, and the suppressive function of T<sub>reg</sub> cells present in the peripheral lymphoid tissues were not decreased by treatment with the TNFR2-blocking antibody. Although this antibody did not attenuate TNFR2 signaling in unstimulated mice, it potently inhibited the increased abundance of TNFR2 induced by LPS (Fig. 1), suggesting that increased TNFR2 abundance both in the tumor environment and in LPS-challenged mice is similarly induced by the interaction of TNF and TNFFR2.

CD25 has been used as an exclusive target for physical depletion of T<sub>reg</sub> cells to elicit antitumor immunity in both murine cancer models (47) and human cancer patients (48). Depletion of T<sub>reg</sub> cells by administration of CD25-blocking antibody (PC61) before tumor challenge provokes effective immune response to syngeneic tumors in otherwise unresponsive mice (50, 51). Our previous study showed that TNFR2 was more closely associated with the suppressive function of T<sub>reg</sub> cells and the phenotype of tumor-infiltrating T<sub>reg</sub> cells, as compared with CD25 (10, 20, 45). Thus, we directly compared the antitumor effect of a better characterized and commercially available...
TNFR2 antagonistic antibody (for example, TR75-54.7) (52) and PC61 on mouse 4T1 breast cancer model. The results show that pretreatment with TR75-54.7 is superior to PC61 in the inhibition of tumor growth (Fig. 4). The simultaneous blockade of TNFR2 and CD25 appears to have better effect as compared with monotherapy (Fig. 4). Nevertheless, the proportion of MDSCs in the spleen and tumor of CT26 tumor-bearing mice was not decreased by M861 treatment in our study. This intriguingly aligned well with our previous observation that, in mice, the sequence of suppressive capacity of Treg subsets is TNFR2 + CD25 + > TNFR2 + CD25 − > TNFR2 − CD25 + (Fig. 4). Activation and expansion of Treg cells through TNF-TNFR2 interaction represent a general negative feedback loop triggered by many immunological stimulators, including immunotherapeutic agents (56). For example, in addition to CpG ODN, in vivo treatment with the TLR4 ligand LPS (34) or the TLR2 ligand Pam3CSK4 (57) also promotes the activation and expansion of Treg cells, accompanied by the increased abundance of TNFR2 on Treg cells (34). Furthermore, cancer immunotherapy with IL-2, a cytokine that increases the abundance of MDSCs also require TNF-TNFR2 signaling (53, 54). Furthermore, CpG ODNs reportedly reduce the number of MDSCs in tumor-bearing mice (55). Nevertheless, the proportion of MDSCs in the spleen and tumor of CT26 tumor-bearing mice was not decreased by M861 treatment in our study.
Further investigation is needed to directly compare the efficacy of targeting of TNFR2 with that of other Treg checkpoint inhibitors.

Because of their clear therapeutic value in cancer treatment, strategies have been developed to deplete Treg cells or reduce their suppressive functions by targeting CD25 or other immune checkpoint proteins that are preferentially present on tumor-infiltrating Treg cells (such as CTLA-4, TIM-3, LAG-3, PD-1, and GITR) (62, 63). Because a large abundance of TNFR2 is associated with the maximally suppressive Treg cells in tumor-infiltrating lymphocytes (10, 19–21), targeting of TNFR2 may have an advantage in eliminating the more functional Treg cells. This idea is supported by studies of ovarian cancer and AML that show that patients treated with reagents that reduce TNFR2 abundance on Treg cells have benefitted from antitumor effects (21, 23, 24, 29).

Further investigation is needed to directly compare the efficacy of targeting of TNFR2 with that of other Treg checkpoint inhibitors.

Together, our findings reveal that an antibody blocking ligand-induced activation of TNFR2 markedly enhances the antitumor efficacy of immunotherapies with CpG ODN in mouse models of colon and breast cancer by reducing the number of tumor-infiltrating TNFR2+ Treg cells while increasing the number of IFN-γ+ CD8+ CTLs. Thus, combining a TNFR2 antagonist with an immunostimulant may represent a novel and more effective treatment strategy for patients with various cancers.

MATERIALS AND METHODS

Mice

Female wild-type 8- to 12-week-old Balb/c mice were obtained from the Animal Production Area of the National Cancer Institute at Frederick (NCI-Frederick). NCI-Frederick is accredited by the American Association for the Accreditation of Laboratory Animal Care International and follows the Public Health Service Policy for the Care and Use of Laboratory Animals. The animal study was approved by Institutional Animal Care and Use Committee of NCI-Frederick. Animal care was provided in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals (National Research Council, revised 1996).

Cells and reagents

The CT26 colon cancer and 4T1 breast cancer cell lines were purchased from the American Type Culture Collection and examined with Molecular Testing of Biological Materials by Animal Health Diagnostic Laboratory (NCI-Frederick) and Luminescence Mycoplasma Test by Animal Molecular Diagnostics Laboratory (NCI-Frederick). Cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and 2 mM glutamine at 37°C in a humidified incubator with 5% CO2. CpG ODN 1668 was purchased from InvivoGen. Anti-mouse antibodies, including anti-mouse CD45, CD4, CD8a, and TNFR2 antibodies, were purchased from BD Biosciences. LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit was purchased from Thermo Fisher Scientific. An antibody recognizing mouse TNFR2 (M861), as well as its control immunoglobulin G (IgG) (mouse IgG1), was a gift from Amgen Inc. Anti-mouse TNFR2 antibody TR75-54.7 and anti-mouse CD25 antibody PC61 were gifts from G. Trinchieri [NCI, National Institutes of Health (NIH)].

CT26 tumor cell inoculation and separation of tumor-infiltrating leukocytes

CT26 tumor cells were subcutaneously injected into the right flank of recipient mice in single-cell suspension with $2 \times 10^6$ cells in 0.2 ml of PBS per mouse. After indicated times, tumors were excised, minced, and digested in RPMI 1640 supplemented with collagenase IV (1 mg/ml) and deoxyribonuclease I (0.1 mg/ml). The fragments were pushed through a 70-um pore size cell strainer to create a single-cell suspension. In some experiments, tumor-free mice 8 weeks after anti-TNFR2 and CpG ODN treatment were reinoculated with CT26 cells ($2 \times 10^7$) into the right flank, and the same number of 4T1 cells was injected subcutaneously into the left flank. Tumor size was calculated by the following formula: $(\text{length} \times \text{width}^2)/2$. "Survival" represents the time to develop a 4-cm³ tumor or a moribund state, a humane end point that triggers euthanasia. Mice were monitored daily and were euthanized when signs of morbidity from metastatic disease burden became evident.
Treatment of mouse model of CT26 colon cancer
When the diameter of tumor reached 5 to 6 mm, mice were treated with the following dose schedule: An antibody recognizing TNF2R (M861) or mouse IgG1 was administered at days 1, 5, and 9 intraperitoneally at 100 μg in 0.2 ml of PBS. CpG ODN or control ODN was administered intratumorally at days 2 and 4 at 20 μg in 0.1 ml of PBS. The same quantity of PBS was administered intraperitoneally or intratumorally as a control. In some experiments, the treatment was started when the diameter of tumor reached 10 mm. One day after the last treatment, mice were sacrificed and tumor and lymphoid tissues were harvested for study.

Treatment of mouse 4T1 breast cancer model
An antibody recognizing TNF2R (TR75-54.7) and/or an antibody recognizing CD25 (PC61) were administered at 200 μg in 0.2 ml of PBS 3 days before the inoculation of 1 × 10^5 4T1 breast tumor cells in 0.1 ml of PBS per mouse subcutaneously injected into right mammary fat pads (thoracic No. 2 mammary glands) of recipient female Balb/c mice.

In vitro culture of CD4⁺ T cells
CD4⁺ cells were purified from lymphocytes with Mouse CD4 (L3T4) MicroBeads and LS Columns (Miltenyi Biotec). CD4⁺ cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and cultured with 4 × 10⁷ cells per well in a 96-well plate with medium (12) alone or IL-2 (10 ng/ml), or with or without TNF (20 ng/ml), or in the presence of M861 (10 μg/ml). After 72 hours, the proliferation of Treg cells (as indicated by CFSE dilution), the proportion of Treg cells, and TNF2R abundance on Treg cells were analyzed by FACS, gating for Foxp3⁺ cells.

In vivo treatment with LPS and M861
Normal Balb/c mice were intraperitoneally injected with 200 μg of LPS (Sigma-Aldrich, catalog no. L9764) in PBS. Some mice were injected intraperitoneally with 200 μg of M861 or control Mu IgG1 1 hour before injection of LPS. Mouse spleens and mesenteric lymph nodes were harvested at 24 hours after injection for the FACS analysis of phenotype.

In vitro proliferation of CT26 and 4T1 tumor cells
CT26 and 4T1 tumor cells were seeded into 96-well plate at 5 × 10⁴ cells per well. The cells were cultured with media alone or with M861 (10 or 20 μg/ml). After 72 hours, cells were treated with pulsed 1 mCi [³H]thymidine (PerkinElmer Life Sciences) per well for the last 6 hours of the culture period. The proliferation was evaluated by [³H]thymidine incorporation.

Flow cytometry
After blocking FcR, cells were incubated with appropriately diluted antibodies. The acquisition was performed using a Fortessa cytometer (BD Biosciences), and data analysis was conducted using FlowJo software (Tree Star Inc.). FACS analysis was gated on the live cells only by using a LIVE/DEAD Fixable Dead Cell Stain kit.

Statistical analysis
Two-tailed Student’s t test was used for the comparison of two indicated groups. One-way analysis of variance (ANOVA) test was used for the comparison of tumor growth between groups at the same day shown in Figs. 2B and 4B. Log-rank test was used for the comparison of survival shown in Figs. 2C and 4C. All statistical analysis was performed with GraphPad Prism 7.0.

**SUPPLEMENTARY MATERIALS**

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Fig. S1. M861 does not induce the death of Treg cells in LPS-treated mice.

Fig. S2. M861 does not inhibit the proliferation of TNF2R-expressing CT26 tumor cells.

**REFERENCES AND NOTES**


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Taking aim at regulatory T cells

Cancer immunotherapy attempts to stimulate the patient's own immune system against a tumor, but despite its potential, the clinical efficacy of immunotherapy has been extremely limited. In some cases, the patient's own immune system can counteract those efforts, such as through T cell exhaustion or the action of naturally suppressive regulatory T (T_{reg}) cells. Nie et al. show that co-inhibiting a receptor for tumor necrosis factor (TNF) reduced T_{reg} cell activity and proliferation, stimulated antitumor immune memory, and slowed the growth of—or even shrank—colon and breast tumors in mice that were unresponsive to common single-agent immunotherapies. The findings suggest that the addition of anti-TNF therapeutics may help increase and broaden the efficacy of immunotherapy for cancer patients.