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ORIGINAL ARTICLE

TNFR2 antagonistic antibody induces the death of tumor infiltrating CD4$^+$Foxp3$^+$ regulatory T cells

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Abstract

Background TNFR2 expression is a characteristic of highly potent immunosuppressive tumor infiltrating CD4$^+$Foxp3$^+$ regulatory T cells (Tregs). There is compelling evidence that TNF through TNFR2 preferentially stimulates the activation and expansion of Tregs. We and others, therefore, proposed that targeting TNFR2 may provide a novel strategy in cancer immunotherapy. Several studies have shown the effect of TNFR2 antagonistic antibodies in different tumor models. However, the exact action of the TNFR2 antibody on Tregs remained understood.

Method TY101, an anti-murine TNFR2 antibody, was used to examine the effect of TNFR2 blockade on Treg proliferation and viability in vitro. The role of TNFR2 on Treg viability was further validated by TNFR2 knockout mice and in the TY101 antagonistic antibody-treated mouse tumor model.

Results In this study, we found that an anti-mouse TNFR2 antibody TY101 could inhibit TNF-induced proliferative expansion of Tregs, indicative of an antagonistic property. To examine the effect of TY101 antagonistic antibody on Treg viability, we treated unfractionated lymph node (L.N.) cells with Dexamethasone (Dex) which was known to induce T cell death. The result showed that TY101 antagonistic antibody treatment further promoted Treg death in the presence of Dex. This led us to find that TNFR2 expression was crucial for the survival of Tregs. In the mouse EG7 lymphoma model, treatment with TY101 antagonistic antibody potently inhibited tumor growth, resulting in complete regression of the tumor in 60% of mice. The treatment with TY101 antagonistic antibody elicited potent antitumor immune responses in this model, accompanied by enhanced death of Tregs.

Conclusion This study, therefore, provides clear experimental evidence that TNFR2 antagonistic antibody, TY101, can promote the death of Tregs, and this effect may be attributable to the antitumor effect of TNFR2 antagonistic antibody.

Keywords TNFR2 · TNFR2 antagonistic antibody · CD4$^+$Foxp3$^+$ regulatory T cells (Tregs) · Cell death · Lymphoma

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1 Introduction

There is now compelling evidence that TNF-TNFR2 interaction plays a decisive role in the activation, expansion, phenotypic stability, and function of Tregs [1, 2]. Moreover, in mouse models and human patients, maximally immunosuppressive TNFR2-expressing Tregs accumulated in the tumor environment [3, 4]. TNFR2$^+$ Tregs in tumor tissue potently inhibit the antitumor immune responses [5]. In human patients, TNFR2 expression levels on Tregs correlated with clinical pathology and disease progression [6–9]. The presence of TNFR2-expressing Tregs represents a primary cellular mechanism underlying immune evasion of tumors. Therefore, we and others proposed that targeting TNFR2 is a novel strategy in tumor immunotherapy [10, 11].

Currently, TNFR2 antagonists are investigated as potential tumor therapeutic agents[12]. For the first time, we reported that targeting TNFR2 with an antagonistic antibody (M861) in combination with CpG-ODN or anti-CD25 antibody markedly inhibited the growth of colon and mammary carcinoma in mice [13]. Furthermore, TY101, an anti-murine TNFR2 antibody, induced tumor regression in CT26 and MC38 mouse colon carcinoma models [14]. Moreover, TY101, combined with anti-PD-1 antibodies or R848 (TLR7/8 agonists, activating dendritic cells), potently inhibited tumor growth [14, 15]. These results also indicated that eliminating tumor-infiltrating Tregs by TNFR2-targeting antibody potently enhanced the antitumor immune responses. Several studies also showed that anti-human TNFR2 antagonistic antibodies also promoted the death of Tregs and TNFR2$^+$ tumor cells [16, 17], indicating the therapeutic potential of anti-TNFR2 therapeutics in the treatment of human cancer.

In this study, we examined the effect of TY101 antagonistic antibody on the Treg survival in different experimental system. The results showed that the treatment of TY101 antagonistic antibody inhibited TNF-induced Treg proliferation, but TY101 antagonistic antibody alone did not affect Treg viability. However, TY101 antagonistic antibody further impaired the Treg viability in the presence of Dex, a known agent that could induce the death of T cells [18]. Additionally, TNFR2-deficient Tregs were more vulnerable to Dex-induced cell death, while the activation of the TNF-TNFR2 axis protected Tregs from Dex-induced cell death. In vivo administration of TY101 antagonistic antibody into EG7 lymphoma-bearing C57BL/6Jmice resulted in complete tumor regression in 60% of mice. The antitumor effect was attributable to decreased number of tumor-infiltrating Tregs and increased number of infiltrating IFN-γ-expressing CD8 cytotoxic T lymphocytes (CTLs). Our results indicate that induction of Treg cell death is a mechanism underlying the antitumor effect of the TNFR2 antagonistic antibody.

2 Materials and methods

2.1 Mice and reagents

Female wild-type (W.T.) C57BL/6J, B6.129S2-Tnfrsf1^{btm1Mwm}/J (TNFR2$^{-/-}$ mice) and BALB/c mice (8–12 weeks old) were provided by the Animal Facility of University of Macau. The animal study protocol was approved by Animal Research Ethics Committee of University of Macau. Anti-murine TNFR2 antibody (TY101) was a gift from Dr. Denise Faustman's group (Massachusetts General Hospital & Harvard Medical School) [14]. RPMI-1640 (1×), fetal bovine serum (F.B.S.), trypsin-EDTA (0.25%) phosphate-buffered saline (1×), Pen Strep (100×), non-essential amino acids solution (NEAA, 100×), HEPES (1 M) and Trypan Blue Stain (0.4%), 2-mercaptoethanol (2-Me) and LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit and eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set were purchased from ThermoFisher Scientific (U.S.A.). Red blood cell lysis buffer was purchased from Beyotime Biotechnology (C.N.). Mouse CD4 (L3T4) microbeads and L.S. columns were purchased from Miltenyi Biotech. Antibodies were purchased from BD Pharmingen (San Diego, CA) consisted of PerCP-Cy5.5 anti-mouse TCR- β (H57-597), PE anti-mouse CD120b/TNFR2 (TR75-89), APC anti-mouse CD8 (53 – 6.7) and PE anti-mouse IFN-γ (XMG1.2). Antibodies purchased from eBioscience include eFluor 450 anti-mouse CD45 (30F11), APC anti-mouse CD4 (RM4-5), PE-Cy7 anti-mouse CD4 (GK1.5) and APC anti-mouse/rat Foxp3 staining set (FJK-16s). Recombinant mouse IL-2 and TNF were obtained from BD Pharmingen. 1-monothioglycerol (M6135), dexamethasone (D4902) and dimethyl sulfoxide (DMSO) were obtained from the Sigma-Aldrich (St. Louis, MO, U.S.A.).

2.2 Cell line and cell culture

EG7 and B16-F10 cell line were purchased from American Type Culture Collection (Manassas, U.S.A.). EG7 cells were cultured in Roswell Park Memorial Institute 1640 Medium (RPMI-1640) supplemented with 10% F.B.S., 1% Pen Strep, 1% L-glutamine and 50 μM monothioglycerol. B16-F10 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% F.B.S. The incubation was carried out at 37℃ in humidified air containing 5% CO_2 in a tissue culture flask.

2.3 Cell purification and *in vitro* cell culture

Wild-type (W.T.) C57BL/6J or BALB/c mice were euthanized by CO_2 inhalation, then the spleens and L.N.s in the axillary, inguinal and mesenteric regions were harvested and

pooled for the preparation of single-cell suspension. CD4 T cells were purified with Mouse CD4 (L3T4) microbeads and L.S. columns (Miltenyi Biotech).

Unfractionated lymph node cells or MACS-purified CD4 T cells isolated from W.T. C57BL/6J mice or $TNFR2^{-/-}$ mice were cultured in RPMI-1640 supplemented with 10% F.B.S., 1% Pen Strep, 5×10^{-5} M 2-Me, 1% non-essential amino acids with IL-2 (10 ng/ml), or with TNF (20 ng/ml), in the presence of TY101 (10 μg/ml) or Dexamethasone (10 nM). The cells were incubated at 37℃ in humidified air containing 5% CO_2. About 5×10^5 isolated CD4 T cells or unfractionated lymph node cells were cultured in a 96-well U-type culture plates. The proportion and cell viability of $CD4^+Foxp3^+$ Tregs were analyzed by FCM (Flow cytometry).

2.4 Tumor cell inoculation and separation

2×10^5 EG7 cells in 0.2 ml of PBS were subcutaneously injected into the right flank of recipient mice. After indicated times, the mice were euthanized by CO_2 inhalation. Mouse 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 after TY101 antagonistic antibody treatment were reinoculated with 2×10^5 EG7 cells into the right flank, and the same number of B16-F10 cells were injected subcutaneously into the left flank. Tumor size was calculated by the following formula: (length × width ^ 2) / 2. "Survival" represented the time to when a 4 cm^3 tumor developed or mice became moribund. A humane end point that triggered euthanasia. Mice were monitored daily and were euthanized when signs defined of morbidity from metastatic disease burden became evident.

2.5 Flow cytometry (FCM)

eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set was used for cell fixation and permeabilization. About 1×10^6 cells were stained with appropriately diluted antibodies in FACS buffer. Antibodies used in FCM analysis included PerCP-Cy5.5 anti-mouse TCR β antibody (1:500 diluted), PE anti-mouse CD120b/TNFR2 (1:250 diluted), APC anti-mouse CD8 (1:500 diluted). PE anti-mouse IFN-γ (1:250 diluted), eFluor 450 anti-mouse CD45 (1:500 diluted), APC anti-mouse CD4 (1:500 diluted), PE-Cy7 anti-mouse CD4 (1:500 diluted) and APC anti-mouse/rat Foxp3 staining set (1:250 diluted). The acquisition was performed using a Fortessa cytometer (B.D. Biosciences), and data analysis was conducted using FlowJo software (Tree Star Inc.).

2.6 Statistical analysis

Two-tailed Student's t test was used for the comparison of two indicated groups. one-way analysis of variance (ANOVA) with Tukey's post-test was used for the comparison of differences among groups as described in our previous study. Log-rank test was used for the comparison of survival shown in Fig. 4D. All statistical analysis was performed with GraphPad Prism 7.0.

3 Results

3.1 TY101 antagonistic antibody abrogates TNF-induced proliferation of Tregs

Previously, we and others reported that, in the presence of low levels of IL-2, TNF preferentially stimulates the proliferation of Tregs present in cultured CD4 cells [4, 19]. We thus used this assay to characterize the TY101 function in modulating TNFR2 function. To this end, MACS-purified mouse CD4 T cells isolated from C57BL/6J mice or BALB/c mice were cultured with IL-2 and stimulated with TNF. As expected, TNF treatment increased the proportion of Foxp3-expressing Tregs and the absolute number of Tregs in the cultured CD4 cells by 51.7% and 150%, respectively (Fig. 1A-C, $P<0.01$). However, treatment with TY101 antagonistic antibody (10 μg/ml) alone did not change the proportion of Tregs in CD4 cells cultured with IL-2 alone, while it completely blocked the expansion of Tregs induced by TNF (Fig. 1A-C, $P<0.01$). Therefore, just like M861 [13], TY101 is an antagonistic anti-mouse TNFR2 antibody that inhibited TNF-induced proliferative expansion of Tregs.

3.2 TY101 treatment increases the death of Tregs in the presence of Dex (Dexamethasone)

It was reported that anti-TNFR2 antibody was able to induce the death of Tregs in purified CD4 T cells [17, 20]; we thus used LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit to assess the effect of TY101 antagonistic antibody on Treg viability. However, TY101 alone did not affect Treg viability (Fig. S1A-B, $P>0.05$). To further determine the role of the TNF-TNFR2 axis on Treg viability, we used Dexamethasone (Dex), an anti-inflammatory agent that induces Treg cell death [18]. To this end, unfractionated lymph node cells from normal C57BL/6J mice were cultured with Dex plus IL-2 (10 ng/ml), Dex plus IL-2 and TNF (20 ng/ml), or Dex plus IL-2, TNF, and TY101 antagonistic antibody (10 μg/ml) or IL-2 alone for 72 h. As shown in Fig. 2, IL-2 alone partially maintained the survival of Tregs, while the addition of TNF was able to almost completely abrogate Treg death induced by Dex (Fig. 2C-D,

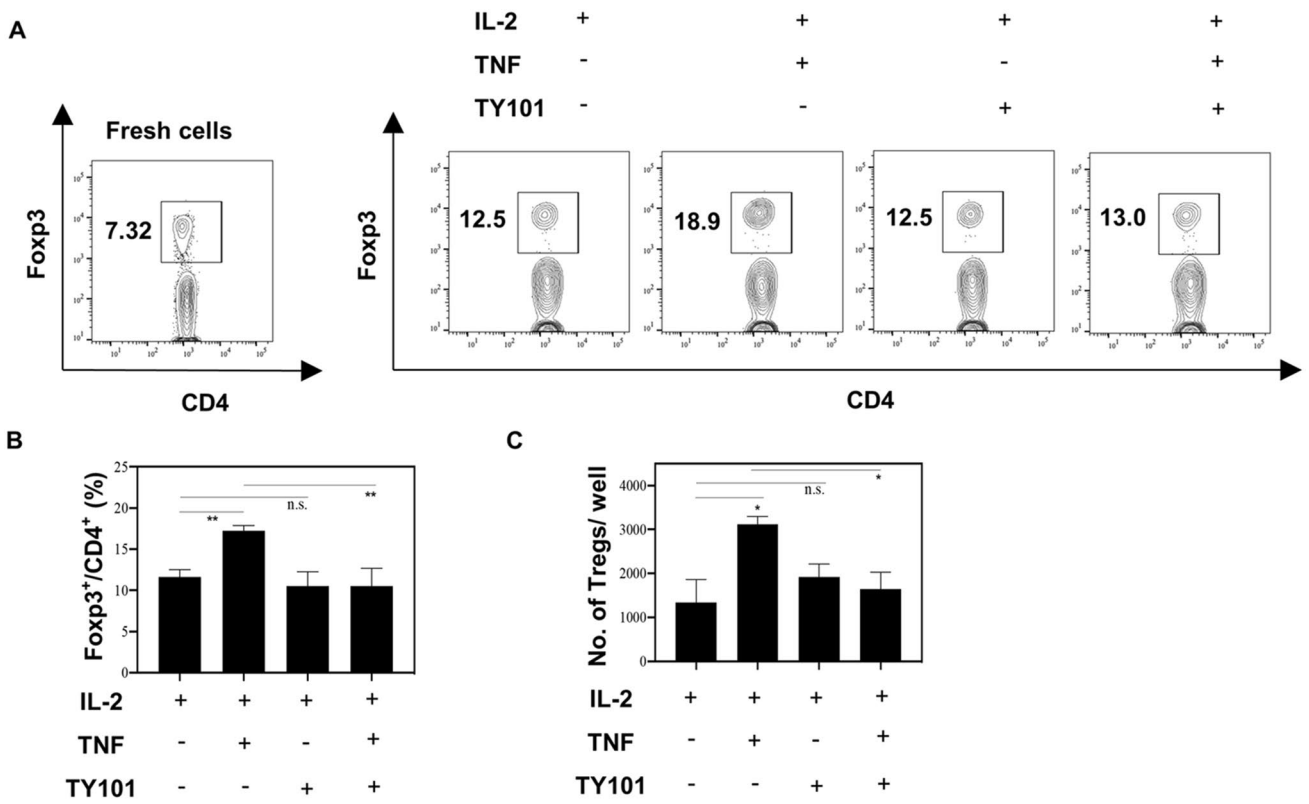

Fig. 1 TY101 antagonistic antibody abrogated TNF-induced Treg proliferation. CD4 T cells were isolated from L.N. and spleen of W.T. C57BL/6J or BALB/c mice with MACS. The cells were cultured with IL-2, in the presence or absence of TNF, with or without TY101 for 72 h. The proportion of Foxp3$^+$ Tregs was analyzed by FCM. **A** Typical FCM plots. The number indicates the proportion of Tregs in CD4 T cells. **B** and **C** Summary of the proportion and the absolute number of Tregs. Data (Mean ± S.E.M., n = 3) shown are representatives of three separate experiments with similar results. Comparison of indicated groups. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. N.S., no significance

$P < 0.01$). Furthermore, TNF treatment resulted in a marked increase in the proportion of Foxp3$^+$ Tregs in the cultured CD4 cells (Fig. 2A-B, $P < 0.001$), which was attributable to the proliferative expansion of Tregs even in the presence of Dex, as we reported previously [18, 21]. These effects of TNF on the expansion of Tregs and the survival of Tregs were inhibited by the treatment of TY101 antagonistic antibody.

3.3 TNFR2 signaling dampens death induction by Dex on Tregs

To further clarify that TY101 antagonistic antibody inhibited Treg survival mediated by the blockade of TNFR2, we examined whether TNFR2 deficiency impairs Treg viability in vitro and in vivo. To this end, in the presence or absence of Dex, unfractionated lymph node cells isolated from wild-type (W.T.) C57BL/6J mice and from TNFR2$^{-/-}$ mice were cultured with IL-2, with or without TNF. The viability of Tregs was determined by LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit by gating on Foxp3$^+$ Tregs. The result showed that, when unfractionated lymph node cells were cultured in IL-2 alone, the viability of W.T. Tregs and TNFR2-deficient Tregs were comparable (Fig. S2A-B, $P > 0.05$). Thus, genetic ablation of TNFR2 did not enhance the death of Tregs in the presence of IL-2 alone. Nevertheless, TNF-TNFR2 interaction markedly protected Tregs from Dex-induced cell death, as evidenced by the fact that TNF markedly enhanced the proportion and viability of Dex-treated W.T. Tregs (Fig. 3A-D, $P < 0.01$), but not Dex-treated TNFR2-deficient Tregs (Fig. 3A-D, $P > 0.05$). Interestingly, TNF treatment did not enhance the viability of W.T. Teffs (Fig. S3A-B, $P > 0.05$), which could be attributable to the relatively lower TNFR2 expression on Teffs.

To verify the in vivo effect of TNFR2 expression on Treg viability, Dex (5 mg/kg/d) was i.p. injected into the W.T. C57BL/6J or TNFR2$^{-/-}$ mice three times as described previously [21]. One day after the last treatment, the mice were sacrificed, and the proportion of CD4$^+$Foxp3$^+$ Tregs

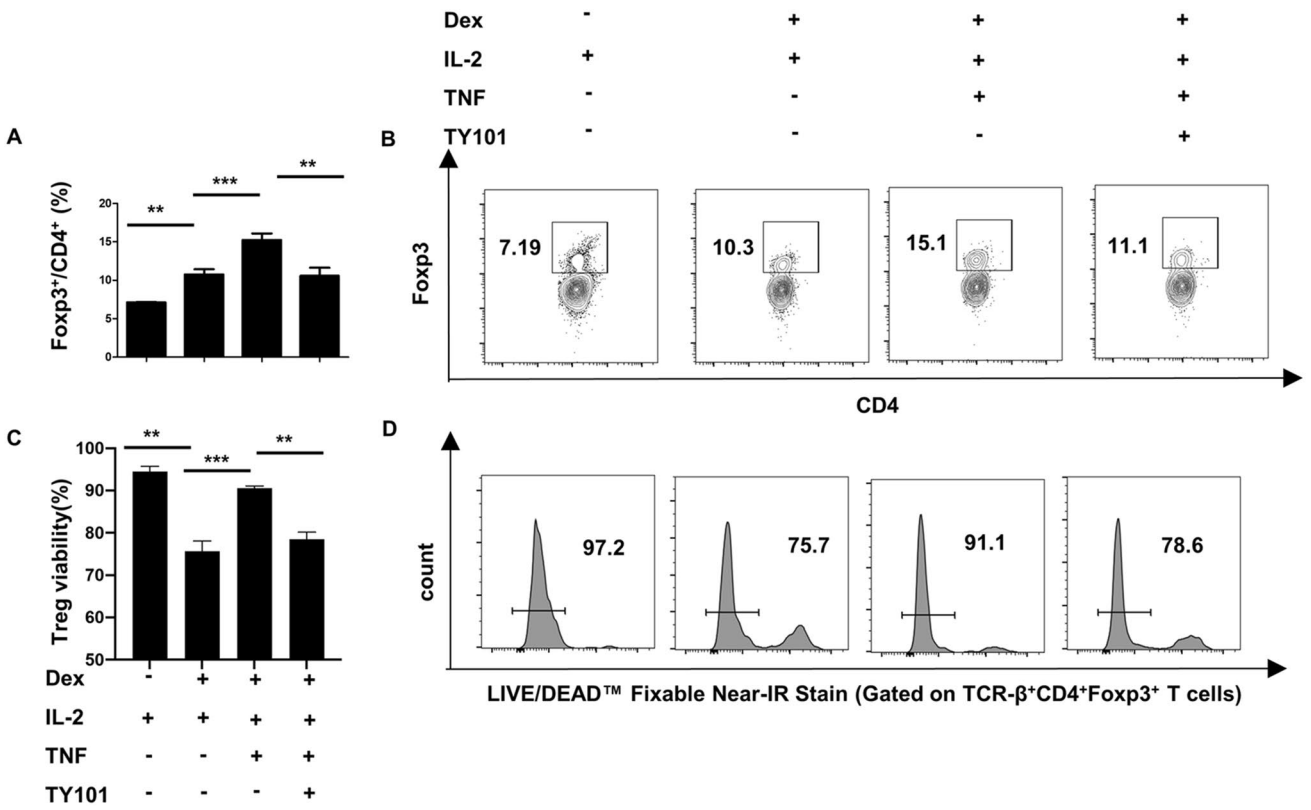

Fig. 2 Effect of TY101 antagonistic antibody on Treg cell survival. Unfractionated L.N. cells from W.T. C57BL/6J mice were treated with Dex, in the presence or absence of IL-2, or TNF, or TY101, for 72 h. The proportion of Tregs (**A** and **B**) and the proportion of living Tregs (**C** and **D**) were analyzed by FCM, gating on Foxp3$^+$ cells. **A** and **C** Representative FCM plots. The number indicates the proportion of Foxp3$^+$ cells in CD4 T cells. **B** and **D** Summary of results. Data (Mean ± S.E.M., n = 3) shown are representatives of three separate experiments with similar results. Comparison of indicated groups. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. N.S., no significance

in the spleen and lymph nodes was analyzed by FCM, and the number of Tregs were determined. The gating strategy for CD4$^+$Foxp3$^+$ Tregs was shown in Fig. S4. The results showed the proportion of Tregs decreased markedly in spleens and lymph nodes in Dex-treated W.T. mice and TNFR2$^{-/-}$ mice (Fig. 3F-G, $P < 0.001$ and Fig. S5). The absolute number of splenic Tregs decreased by 50.3% (Fig. 3F) in the TNFR2$^{-/-}$ mice group. In contrast, the splenic Treg percentage decreased by 30.7% in W.T. mice (Fig. 3G, $P < 0.01$). The results indicated that TNFR2-deficient Tregs were more susceptible to Dex-induced cell death in vivo (Fig. 3H). Therefore, the in vivo and in vitro data supported that TNF promoted Treg viability in a TNFR2-dependent manner.

3.4 TY101 antagonistic antibody potently inhibits the growth of EG7 lymphoma in mice

To investigate whether TY101 enhances antitumor responses as previously reported [14, 15], we examined the in vivo antitumor effect of TY101 in the mouse EG7 lymphoma model. As shown in the schematic diagram in Fig. 4A, EG7 lymphoma cells were inoculated subcutaneously in W.T. C57BL/6J or BALB/c mice. Treatment started on day 7 after tumor inoculation (tumor diameter reached about 10 mm). TY101, or isotype-matched IgG control, was injected into the tumor-bearing mice (200 μg, i.p.) twice a week for up to five times. The results showed that the administration of TY101 antagonistic antibody markedly inhibited the growth of EG7 lymphoma (Fig. 4B-C, $P < 0.001$), and complete regression of EG7 lymphoma was observed in 60% of the mice at the end of the experiment (50 days after tumor inoculation). In contrast, the tumor grew rapidly in the mice treated with control IgG, and all mice died from the tumor burden by day 30 (Fig. 4D).

To investigate whether TY101 could induce long-term tumor-specific immunity, treated mice with complete tumor regression were reinoculated with EG7 lymphoma into the right flank 8 weeks after becoming tumor-free. In

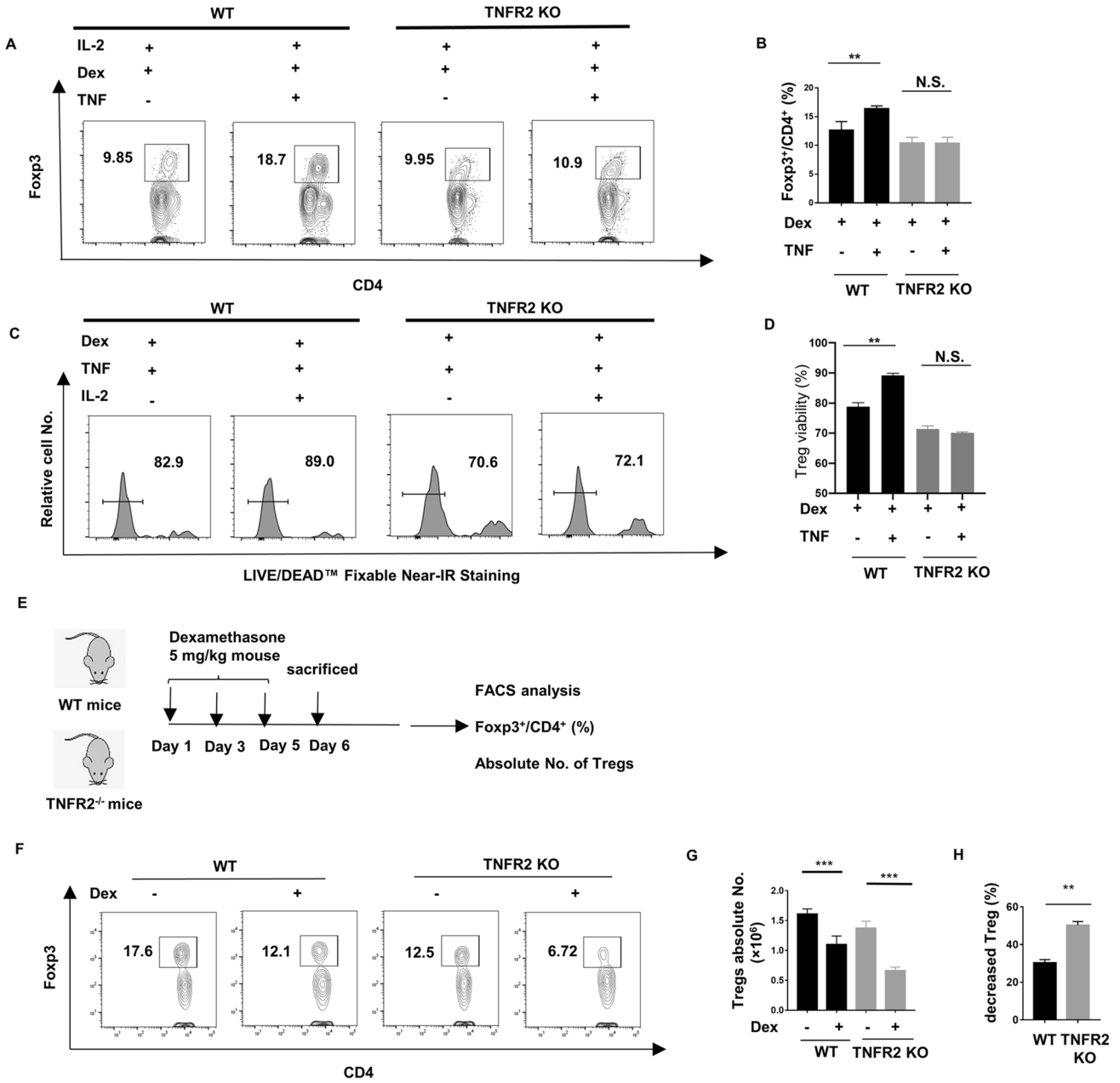

Fig. 3 The effect of TNF on the viability of W.T. Tregs and TNFR2 deficient Tregs. **A**-**D** Unfractionated LN cells isolated from W.T. C57BL/6J mice and TNFR2$^{-/-}$ mice were treated with Dex, in the presence or absence of IL-2, or TNF, for 72 h. The proportion of Tregs (**A** and **B**) and the proportion of living Tregs (**C** and **D**) were analyzed by FCM, gating on Foxp3$^+$ cells. **A** and **C** Representative FCM plots. The number indicates the proportion of Foxp3$^+$ cells in CD4 T cells. **B** and **D** Summary of results. Data (Mean ± S.E.M., n = 3) shown are representatives of five separate experiments with similar results. **E**-**H** W.T. or TNFR2 KO mice were treated with Dex once every two days for three times. One day after the last treatment, the mice were sacrificed. The proportion of Tregs was analyzed by FCM and the absolute number of Tregs in spleen were determined. **E** The schematic diagram of the experiment procedure. **F** The representative FCM plots, the number indicate the proportion of CD4$^+$Foxp3$^+$ Treg in total CD4 T cells. **G** The absolute number of Tregs in spleen. **H** The percent decrease of Tregs. Data (Mean ± S.E.M., n = 6) shown are representatives of two separate experiments with similar results. Comparison of indicated groups. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, N.S., no significance

comparison, irrelevant B16-F10 melanoma cells were inoculated into the left flank. As another control, normal mice were also inoculated with EG7 lymphoma into the right flank and B16-F10 melanoma into the left flank in the same manner. As a result, none of the mice with complete tumor regression developed EG7 lymphoma (0/3), while all developed B16-F10 melanoma (3/3). In contrast, all the normal control mice developed B16-F10 melanoma and EG7 lymphoma (3/3),

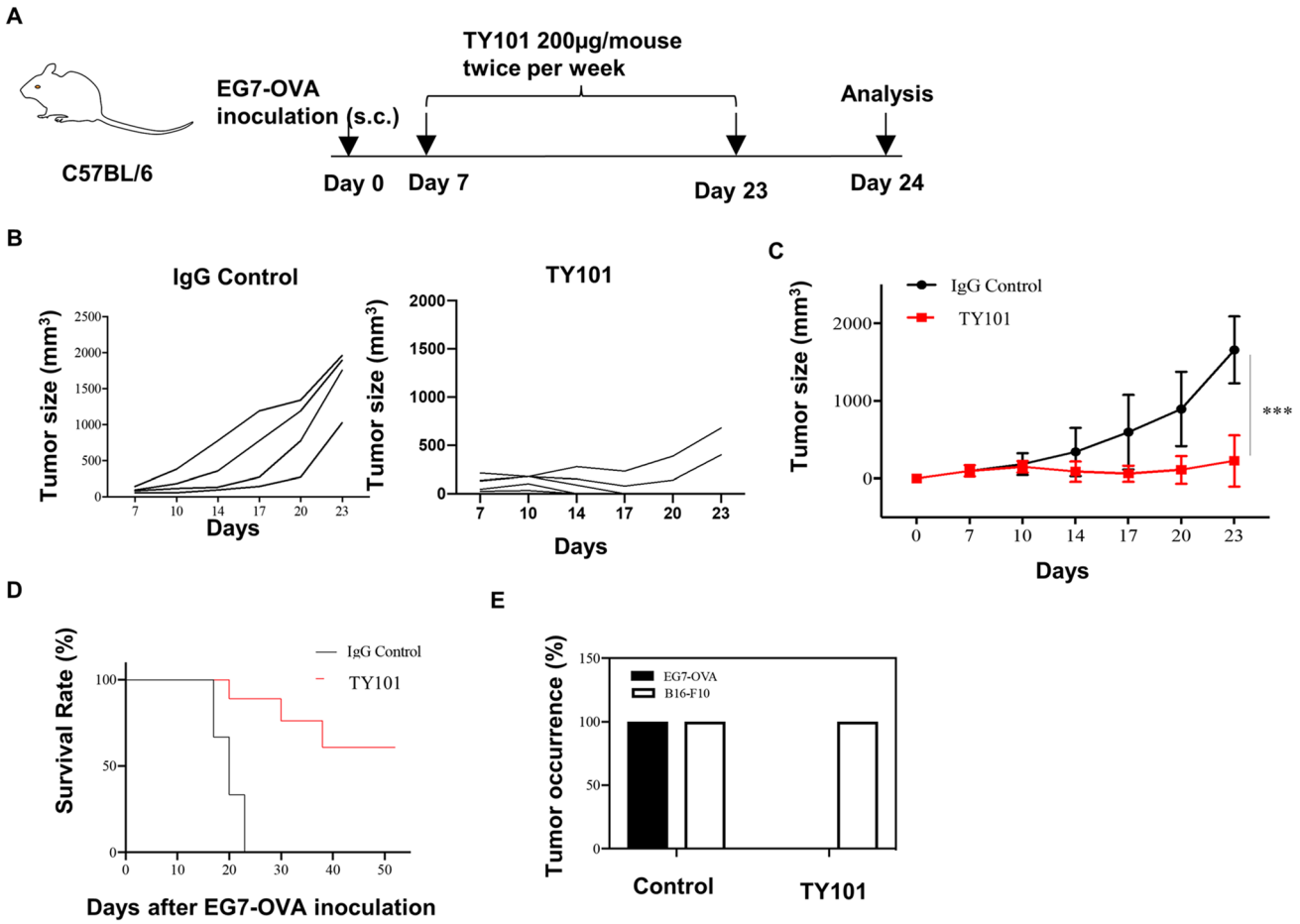

Fig. 4 *In vivo* effect of TY101 antagonistic antibody on tumor growth in mouse EG7 lymphoma model. **A**-**D** C57BL/6J or BALB/c Mice were treated with TY101 or IgG control twice a week for five times, starting on the day 7 after inoculation. **A** Schematic diagram of the experimental procedure. **B** EG7 tumor growth curves for each individual EG7 tumor-bearing mouse treated with TY101 or IgG control. **C** Mean growth curves of EG7 lymphoma, data are means ± S.E.M. of 5 mice. **D** Survival curves of the EG-7 tumor-bearing mice treated as described in A. **E** The mice with complete regression of tumor were reinoculated with EG7 tumor cells into the right flank and B16-F10 tumor cells into the left flank 8 weeks after the mice became tumor-free. As a control, normal mice were inoculated with EG7 tumor cells into the right flank and B16-F10 tumor cells into the left flank in the same manner. Data (n = 3 mice) show the percentage of tumor incidence of normal mice and surviving mice on day 26 after (re)challenge. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, N.S., no significance

Fig. 4E). Thus, this result indicated that TY101 treatment induced long-term tumor-specific immune memory.

3.5 TY101 antagonistic antibody treatment promotes tumor-infiltrating Treg cell death and enhances the antitumor responses

To determine whether TNFR2 antagonistic antibody elicits antitumor immune responses by promoting intratumoral Treg cell death, we started the treatment after the tumor size reached about 10 mm in diameter in W.T. C57BL/6J or BALB/C mice. The tumor-infiltrating Tregs were analyzed by FCM. As shown in Fig. 5A-B, over 95% of Tregs were viable in the IgG-treated control group, while TY101 treatment significantly reduced the Treg viability in tumors (Fig. 5A-B, $P < 0.01$). Moreover, administration of TY101 decreased the proportion of tumor-infiltrating CD4$^+$ Foxp3$^+$ Tregs by about 50% in mouse EG7 tumors (Fig. 5C-D, $P < 0.05$). The absolute number of tumor-infiltrating Tregs also decreased significantly in the TY101 treated mice group (Fig. 5E, $P < 0.05$). In addition, the percentage of IFNγ-expressing CD8 T cells was increased by about 50% compared to control (Fig. 5F-G, $P < 0.01$). Furthermore, as we used same number of cells (1×10^6 cells/tube) when analyzing the Treg and CD8 T cells from tumor tissue, we thus calculated the ratio of CD8 T cells to Tregs, an important indicator of clinical outcome of different cancer types [22, 23]. The results showed that TY101 significantly increase the ratio of CD8 T cells to Tregs (Fig. 5H, $P < 0.01$). Therefore, TY101 could enhance the antitumor immune responses by reducing intratumoral Treg viability.

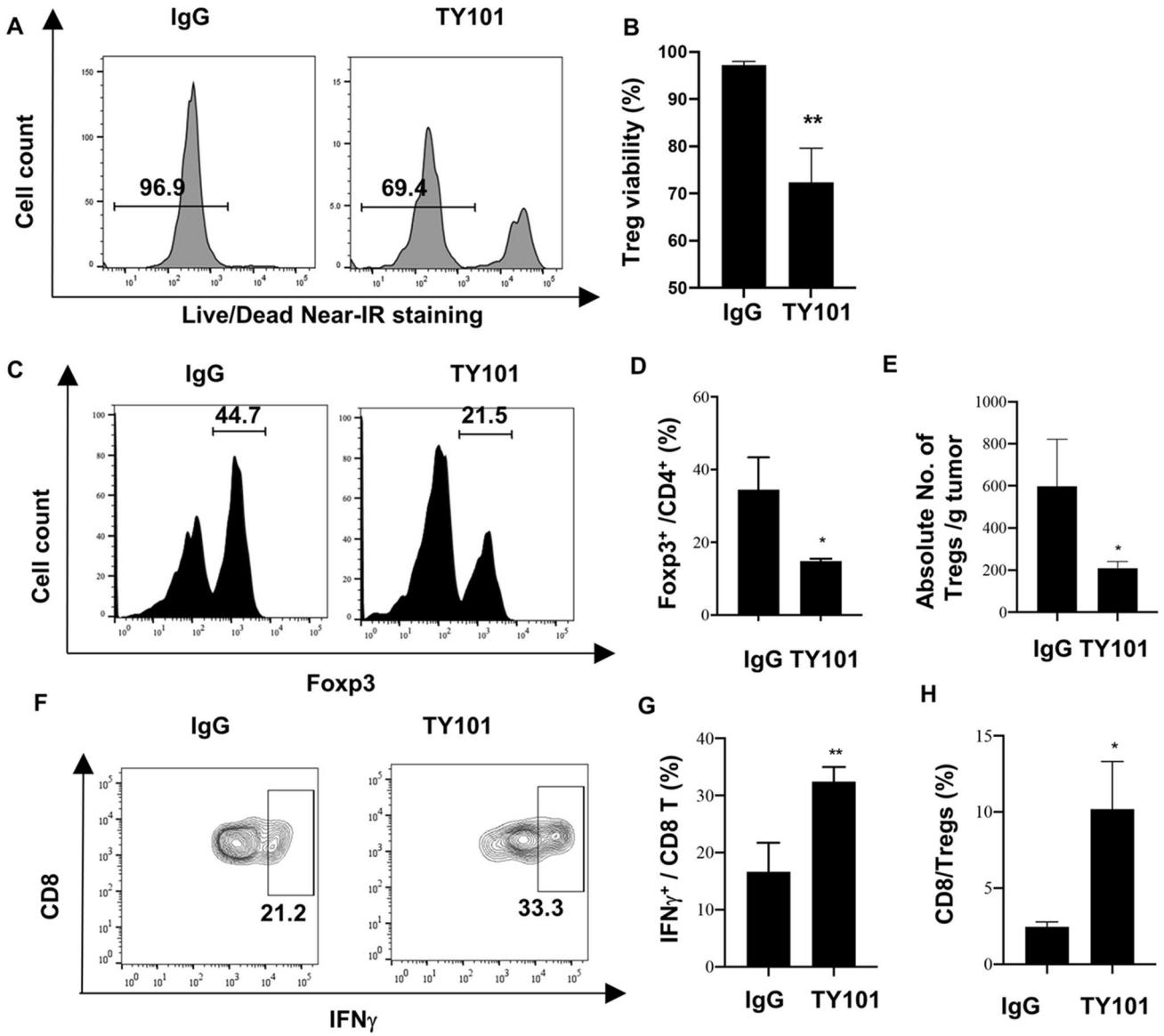

Fig. 5 Effect of TY101 on tumor infiltrating Tregs and IFN-γ^+ CD8 CTLs. **A**-**H** Treatment started when the tumor reached tumor size reach about 10 mm in diameter, then the tumor tissue was cleaved into single cell for flow cytometry (FCM). The Treg viability were detected by LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit. **A**-**D** The percentage of viable Tregs in total Tregs and the proportion of Tregs in CD4 T cells. **A** and **C** Representative FCM plots. **B** and **D** The summary data. **E** The absolute number of Tregs per gram tumor tissue. **F** Representative FCM analysis of IFN-γ^+ cells in $CD8^+$ cells from mice described in (**A**) and the summary data (**G**). The number indicates the percentage of interferon–positive (IFN-γ^+) cells in total $CD8^+$ cells. **H** The percentage of CD8 IFN-γ^+ cells to Tregs. Data (n = 4 mice) showed the percentage of Treg proportion and Treg viability, as well as the proportion of IFN-γ^+ CTLs and the ratio of CD8/Tregs. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, N.S., no significance

4 Discussion

In this study, we showed a possible mechanism of the antitumor effect of TNFR2 antagonists. Genetic or pharmacological inhibition of TNFR2 promoted Treg cell death, while activation of TNFR2 protected Tregs from cell death. We further found that in vivo treatment of TY101antagonistic antibody reduced the viability of the immunosuppressive TNFR2-expressing Tregs in the tumor environment and, as a result, mobilized and activated antitumor immune responses, resulting in the complete regression of EG7 lymphoma in the majority of mice.

There is compelling evidence that TNF promotes activation, function, proliferation, and phenotypic stability of Tregs [1, 2, 19, 24]. Recent studies have shed light on the mechanism of the role of TNFR2 in Treg activation and proliferation. It was shown that specific deletion of TNFR2 in Treg significantly reduced the expression of *Myb* [25],

a cell cycle regulator important for controlling Treg differentiation and function [26]. Moreover, TNFR2 costimulation enhanced NF-κB signaling pathway in Tregs, which could be associated with TNF-TNFR2-induced Treg proliferation [27]. Upregulation of the pro-survival gene is also a characteristic of the transcriptomic profile of TNFR2 agonist-treated Tregs [27]. Previous results from Dr. Faustmann and her colleagues showed antagonistic anti-TNFR2 antibodies, in addition to inhibiting the proliferation of human Tregs, could induce the death of TNFR2-expressing Tregs [14, 16, 17]. They showed that TNFR2 antagonistic antibody alone decreased Treg ratio or Treg number in purified CD4 T cells, which was interpreted that the blockade of TNFR2 induced Treg cell death. However, our previous results showed M861, another anti-murine TNFR2 antagonistic antibody, did not affect Treg viability in spleen and lymph nodes [13]. In this study, we confirmed that TNFR2 signaling was dispensable for Treg viability in normal culture conditions. Interestingly, blockade of TNFR2 promoted Treg cell death in the presence of Dexamethasone, an anti-inflammatory agent that induces Treg cell death. A study further supported the pro-survival effect of TNF and showed that TNF-treated Tregs are more resistant to oxidative stress-induced cell death [28]. Therefore, activation of the TNF-TNFR2 signaling pathway could be essential for maintaining the Treg viability in some conditions.

We further showed that TNFR2 antagonistic antibody promoted the death of intratumoral Treg cells. Hypoxia, increased R.O.S. (reactive oxidative species) and low-PH value (high concentration of lactic acid) are the characteristics of the tumor microenvironment. These factors suppress the function of CTLs but favor the accumulation of Treg and other immunosuppressive subsets [29]. Several recent studies showed that metabolic adaptation is vital for tumor-infiltrating Tregs' immunosuppressive function and viability. Compared with effector T cells, tumor-infiltrating Tregs are less dependent on glycolysis but use lactate to fuel their activities [30]. Moreover, upregulation of fat metabolism was also found in mouse tumor-infiltrating Tregs [31]. It was reported that the specific deletion of FASN (fatty acid synthase) in Treg significantly inhibits mouse tumor growth [32]. The role of TNF-TNFRs signal in metabolic regulation has been long investigated in different cell types, however, its role in T cells remains largely unknown [33]. Recently, it was reported that TNFR2 stimulation promotes glycolysis and glutamine metabolism in Tregs [34, 35]. However, the in vivo effect of TNF-TNFR2 on Treg metabolism remains unknown. Therefore, whether TNFR2 blockade disrupts intratumoral Treg metabolism, and induces tumor-infiltrating Treg cell death, should be further studied.

Interestingly, pharmaceutical companies are developing TNFR2 antagonistic antibodies but also TNFR2 agonistic antibodies for tumor treatment. Theoretically, as previously reported, TNFR2 agonistic antibody would boost Treg proliferation [36], which may impair the antitumor immune responses. However, one study found that TNFR2 agonistic antibody promoted antitumor immune responses and inhibited tumor growth [37]. This study also found that TNFR2 agonistic antibody did not increase infiltration of Tregs but promoted CD8 T cell infiltration in mouse tumors. In comparison with Tregs, although Teffs and CTLs express a lower level of TNFR2, Teffs' activation and pro-inflammatory effect were also dependent TNF-TNFR2 axis [38, 39]. These studies suggested that a high dose of TNFR2 agonist may also elicit the antitumor responses of Teffs and CTLs. However, the role of TNFR2 expression by tumor-infiltrating Teffs or CTLs has not been well studied. Mouse specifically lacking TNFR2 expression in Tregs has been developed and used to study autoimmune inflammatory diseases of the central nervous system [25]. This mouse strain will also be useful in clarifying the role of TNF-TNFR2 in modulating Teffs, CTLs, and other immune subsets in the tumor and, consequently, advance the understanding of the mechanism of action of anti-TNFR2 therapeutics.

Taken together, in this study, we showed the activation of TNF-TNFR2 promoted Treg viability, and induction of the death of Tregs represented an underlying mechanism of the antitumor effect of TNFR2 antagonistic antibodies.

Abbreviations *TNF*: Tumor necrosis factor; *TNFR2*: Tumor necrosis factor receptor 2; *Treg*: Regulatory T cells; *Dex*: Dexamethasone; *RPMI-1640*: Roswell Park Memorial Institute Medium,; *Pen*: Penicillin; *Strep*: Streptomycin; *HEPES*: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; *F.B.S.*: Fetal bovine serum; *2-Me*: 2-Mercaptoethanol; *FcR*: Fc receptor; *MACS*: Magnetic-activated cell sorting; *Teffs*: Effect T cells; *FCM*: Flow cytometry; *FACS*: Fluorescence-activated Cell Sorting; *CTL*: Cytotoxic T lymphocyte

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Data availability The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Declarations

Consent for publication All authors have read and agreed to the published version of the manuscript.

Ethical approval and consent to participate The animal study was reviewed and approved by Animal Research Ethics Committee of the University of Macau.

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

1. X. Chen et al., Interaction of TNF with TNF receptor type 2 promotes expansion and function of mouse CD4 + CD25 + T regulatory cells. J. Immunol **179**(1), 154–161 (2007)
2. X. Chen et al., TNFR2 is critical for the stabilization of the CD4 + Foxp3 + regulatory T cell phenotype in the inflammatory environment. J. Immunol **190**(3), 1076–1084 (2013)
3. X. Chen et al., Effective chemoimmunotherapy with anti-TGFβ antibody and cyclophosphamide in a mouse model of breast cancer. PLoS ONE **9**(1), e85398 (2014)
4. X. Chen et al., Cutting edge: Expression of TNFR2 defines a maximally suppressive subset of mouse CD4 + CD25 + Foxp3 + T regulatory cells: Applicability to tumor-infiltrating T regulatory cells. J. Immunol **180**(10), 6467 (2008)
5. C. Govindaraj et al., Impaired Th1 immunity in ovarian cancer patients is mediated by TNFR2 + Tregs within the tumor microenvironment. Clin. Immunol **149**(1), 97–110 (2013)
6. M. Wang et al., Increased regulatory T cells in peripheral blood of acute myeloid leukemia patients rely on tumor necrosis factor (TNF)-α-TNF receptor-2 pathway. Front. Immunol **9**, 1274–1274 (2018)
7. F. Yan et al., Expression of TNFR2 by regulatory T cells in peripheral blood is correlated with clinical pathology of lung cancer patients. Cancer Immunol. Immunother **64**(11), 1475–1485 (2015)
8. N.C. Kampan et al., Interleukin 6 present in inflammatory ascites from advanced epithelial ovarian cancer patients promotes tumor necrosis factor receptor 2-expressing regulatory T cells. Front. Immunol. **8**(1482) (2017)
9. L.-L. Ye et al., Accumulation of TNFR2-expressing regulatory T cells in malignant pleural effusion of lung cancer patients is associated with poor prognosis. Ann. Transl. Med. **8**(24), 1647 (2020)
10. X. Chen, J.J. Oppenheim, Targeting TNFR2, an immune checkpoint stimulator and oncoprotein, is a promising treatment for cancer. Sci. Signal **10**(462), eaal2328 (2017)
11. ÉS. Vanamee, D.L. Faustman, TNFR2: A novel target for cancer immunotherapy. Trends Mol. Med **23**(11), 1037–1046 (2017)
12. H. Zou et al., Modulation of regulatory T cell activity by TNF receptor type II-targeting pharmacological agents. Front. Immunol. **9** (2018)
13. Y. Nie et al., Blockade of TNFR2 signaling enhances the immunotherapeutic effect of CpG ODN in a mouse model of colon cancer. Sci. Signal **11**(511), eaan0790 (2018)
14. K. Case et al., TNFR2 blockade alone or in combination with PD-1 blockade shows therapeutic efficacy in murine cancer models. J. Leukoc. Biol **107**(6), 981–991 (2020)
15. M. Jiang et al., A TNFR2 antibody by countering immunosuppression cooperates with HMGN1 and R848 immune stimulants to inhibit murine colon cancer. Int. Immunopharmacol. **101**(Pt A), 108345 (2021)
16. H. Torrey et al., Targeted killing of TNFR2-expressing tumor cells and Tregs by TNFR2 antagonistic antibodies in advanced Sézary syndrome. Leukemia (2018)
17. H. Torrey et al., Targeting TNFR2 with antagonistic antibodies inhibits proliferation of ovarian cancer cells and tumor-associated Tregs. Sci. Signal **10**(462), eaaf8608 (2017)
18. X. Chen et al., Differential response of murine CD4 + CD25 + and CD4 + CD25– T cells to dexamethasone-induced cell death. Eur. J. Immunol **34**(3), 859–869 (2004)
19. K.N. Atretkhany et al., Intrinsic TNFR2 signaling in T regulatory cells provides protection in CNS autoimmunity. Proc. Natl. Acad. Sci. U. S. A. **115**(51), 13051–13056 (2018)
20. H. Torrey et al., Targeted killing of TNFR2-expressing tumor cells and Tregs by TNFR2 antagonistic antibodies in advanced Sézary syndrome. Leukemia **33**(5), 1206–1218 (2019)
21. X. Chen et al., Glucocorticoid amplifies IL-2-dependent expansion of functional FoxP3 + CD4 + CD25 + T regulatory cells in vivo and enhances their capacity to suppress EAE. Eur. J. Immunol **36**(8), 2139–2149 (2006)
22. C.C. Preston et al., The ratios of CD8 + T cells to CD4 + CD25 + FOXP3 + and FOXP3- T cells correlate with poor clinical outcome in human serous ovarian cancer. PLoS ONE **8**(11), e80063 (2013)
23. A.S. Baras et al., The ratio of CD8 to Treg tumor-infiltrating lymphocytes is associated with response to cisplatin-based neoadjuvant chemotherapy in patients with muscle invasive urothelial carcinoma of the bladder. Oncoimmunology **5**(5), e1134412 (2016)
24. B. Zaragoza et al., Suppressive activity of human regulatory T cells is maintained in the presence of TNF. Nat. Med **22**(1), 16–17 (2016)
25. E. Ronin et al., Tissue-restricted control of established central nervous system autoimmunity by TNF receptor 2-expressing Treg cells. Proc. Natl. Acad. Sci. U. S. A. **118**(13) (2021)
26. S. Dias et al., Effector regulatory T cell differentiation and immune homeostasis depend on the transcription factor Myb. Immunity **46**(1), 78–91 (2017)
27. M. di LubranoRicco et al., Tumor necrosis factor receptor family costimulation increases regulatory T-cell activation and function via NF-κB. Eur. J. Immunol **50**(7), 972–985 (2020)
28. D. Mougiakakos et al., Increased thioredoxin-1 production in human naturally occurring regulatory T cells confers enhanced tolerance to oxidative stress. Blood **117**(3), 857–861 (2011)
29. F. Chalmin et al., Regulation of T cell antitumor immune response by tumor induced metabolic stress. Cell. stress **3**(1), 9–18 (2018)
30. M.J. Watson et al., Metabolic support of tumour-infiltrating regulatory T cells by lactic acid. Nature **591**(7851), 645–651 (2021)
31. I. Pacella et al., *Fatty acid metabolism complements glycolysis in the selective regulatory T cell expansion during tumor growth* Proceedings of the National Academy of Sciences, 2018. **115**(28): p. E6546-E6555
32. S.A. Lim et al., Lipid signalling enforces functional specialization of T(reg) cells in tumours. Nature **591**(7849), 306–311 (2021)
33. J.K. Sethi, G.S. Hotamisligil, Metabolic messengers: tumour necrosis factor. Nat. Metab. **3**(10), 1302–1312 (2021)

34. M. Mensink et al., TNFR2 costimulation differentially impacts regulatory and conventional CD4 + T-cell metabolism. Front. Immunol. **13** (2022)
35. S. de Kivit et al., Stable human regulatory T cells switch to glycolysis following TNF receptor 2 costimulation. Nat. Metab. **2**(10), 1046–1061 (2020)
36. M. Chopra et al., Exogenous TNFR2 activation protects from acute GvHD via host T reg cell expansion. J. Exp. Med **213**(9), 1881–1900 (2016)
37. E.M. Tam et al., Antibody-mediated targeting of TNFR2 activates CD8(+) T cells in mice and promotes antitumor immunity. Sci. Transl. Med. **11**(512) (2019)
38. X. Chen et al., TNFR2 expression by CD4 effector T cells is required to induce full-fledged experimental colitis. Sci. Rep **6**, 32834 (2016)
39. M.S. Alam et al., TNF plays a crucial role in inflammation by signaling via T cell TNFR2. Proc. Natl. Acad. Sci. **118**(50), e2109972118 (2021)