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A TNFR2 antibody by countering immunosuppression cooperates with HMGN1 and R848 immune stimulants to inhibit murine colon cancer

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ABSTRACT

Immunosuppressive $CD4^+Foxp3^+$ regulatory T cells (Tregs) promote tumor immune evasion and thus targeting of Tregs has become an strategy in cancer immunotherapy. Tumor necrosis factor receptor 2 (TNFR2) is highly expressed and important for the immunosuppressive function of Tregs in humans and mice. Thus, the benefit of targeting TNFR2 in cancer immunotherapy merits more investigation. A previous report identified a new murine monoclonal anti-TNFR2 antibody (designated TY101), which showed therapeutic efficacy in murine cancer models, but its mechanism of action was less understood. In this study, the capacity of a combination of immunostimulants to enhance the effect of this inhibitor of Tregs was investigated. We examined the efficacy of TY101 as an anti-tumor immune reagent combined with HMGN1 (N1, a dendritic cell activating TLR4 agonist) and R848 (a synthetic TLR7/8 agonist). This immunotherapeutic combination exerted synergistic antitumor effects as compared with any single treatment. The antitumor response was mainly mediated by the depletion of Tregs and stimulation of cytotoxic CD8 T cell activation. The result also suggested that the effect of TY101 was similar to that of anti-PD-L1 when used in combination with these immunostimulants. Therefore, we propose that treatment strategies of antagonizing TNFR2 on Tregs would behave as potent checkpoint inhibitors and can potentially be utilized to develop a novel antitumor immunotherapy.

1. Introduction

There is accumulating evidence that $CD4^+Foxp3^+$ regulatory T cells (Tregs) are highly enriched in the tumor microenvironment (TME) and play crucial roles in promoting tumor progression [1,2]. To date, a large increase in intratumoral Tregs has been demonstrated in melanomas, colorectal cancers and gastric cancers. Furthermore, the accumulation of tumor-infiltrating Tregs was also widely associated with poor prognosis [3–5]. Tregs inhibit not only effector T cells, but also innate immune dendritic cells (DCs) and natural killer cells [6,7]. Consequently, targeting of Tregs has become a successful strategy in the treatment of human cancers [8–10].

We were the first group to report that tumor necrosis factor (TNF) could promote activation and expansion of Tregs by interacting with TNF receptor type 2 (TNFR2) [11]. Moreover, our research and subsequent studies have confirmed that TNFR2 was preferentially expressed on Treg cells, and high expression of TNFR2 identified the maximally immunosuppressive subset of Tregs in humans and mice [12–14]. In

Abbreviations: CFSE, Carboxyfluorescein succinimidyl ester; CTL, Cytotoxic T lymphocyte; DCs, Dendritic cells; dLNs, draining lymph nodes; MSCs, Mesenchymal stem cells; MDSCs, Myeloid-derived suppressor cells; Tregs, $CD4^+Foxp3^+$ regulatory T cells; TME, Tumor microenvironment; TNF, Tumor necrosis factor; TNFR2, Tumor necrosis factor receptor type 2; TLRs, Toll-like receptors.

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contrast, as a result of TNFR2 knock-down or knock-out, Treg cells only showed minimal or no suppressive activity by disturbing Foxp3 expression [15]. Overall, the evidence clearly demonstrates that TNFR2 plays a key role in maintaining and promoting the immunosuppressive function of Tregs. Thus, targeting of TNFR2 may provide a therapeutic approach in cancer immunotherapy.

Tumor infiltrating dendritic cells express a tolerogenic immature phenotype in the immunosuppressive tumor microenvironment and fail to promote anti-tumor response. Stimulant of Toll-like receptors (TLRs) expressed by DCs plays crucial roles in inducing their maturation and activation [16]. Currently, HMGN1 (N1), a TLR4 ligand which was identified as an alarmin that activates DCs [17], and R848, a synthetic TLR7/8 agonist that also activates DCs [18], together induce therapeutic antitumor responses. The combination of N1 and R848 have been reported to synergistically promote the activation of dendritic cells and mobilize antitumor immune responses in mice [19].

We have used a murine anti-TNFR2 antibody (TY101, isotype is Rat IgG), which has the capacity to block the binding of TNF to CRD3 and CRD4 regions of TNFR2 receptor, with the ability to inhibit Treg activities and promote anti-tumor immune responses [20]. In order to enhance its efficacy in promoting anti-tumor immune responses, TY101 was given together with N1 and R848 to promote the generation of antitumor immune responses by triggering the maturation of tumor-infiltrating dendritic cells and their subsequent homing to draining lymph nodes [21]. Given the effective therapy of CT26-bearing mice by the combination of N1 + R848 + anti-PD-L1 or N1 + R848 + TY101 (present study), it is presumed that tumor-infiltrating dendritic cells and macrophages express TLR4, TLR7 and PD-L1. In fact, it has been documented since 2003 that antigen presenting cells (including DCs and macrophages) in the TME of both humans and mice expressing functional TLR4 and TLR7 receptors [22].

Furthermore, we also compared the effect of TY101 with anti-PD-L1 treatment given together with N1 and R848. This study demonstrates that the combination of TY101 with N1 and R848 can exert robust antitumor effects based on depletion of TNFR2-expressing tumor infiltrating Tregs and increased activity of cytotoxic CD8 T cells. The antitumor effect of TY101 equaled the effect of anti-PD-L1 antibody. Therefore, combination therapy of TY101 with other immunostimulants can be considered a promising cancer treatment strategy.

2. Materials and methods

2.1. Mice and therapeutic drugs

Female wild-type Balb/c or C57BL/6 mice, 6–8 weeks old were purchased from The Jackson Laboratory. The NCI as authorized by American association for the accreditation of laboratory animal care (AAALAC) follows the public health service policy for the care and use of laboratory animals. Animal care was provided according to the procedures and guidelines for care and use of laboratory animals (National research council, Washington, DC). All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of NCI.

Anti-TNFR2 antibody (TY101, 10.90 mg/ml) was generated and purified as described previously [20]. Anti-mouse PD-L1 (B7H1) antibody (Clone 10F.9G2, cat# BE0101) was purchased from Bio X Cell. Recombinant HMGN1 (N1) was generated and purified as previously reported [17]. R848 (Cat# 144875-48-9) was purchased from InvivoGen. Recombinant murine IL2 (Cat# 212-12) and TNF-α (Cat# 315-01A) were purchased from PeproTech, USA.

2.2. Cell isolation and culture

$CD4^+CD25^+$ T cells were purified from spleen and lymph nodes (including inguinal, axillary and mesenteric regions) of C57BL/6 or Balb/c mice, using the mouse $CD4^+CD25^+$ Regulatory T Cell Isolation Kit (Cat# 130091041, Miltenyi Biotec), yielding a purity of 90% for $CD4^+FoxP3^+$ Tregs. The purified cells were cultured in 96-well round bottom plates in medium of RPMI-1640 (Corning, VA, USA) containing 10% heat inactivated fetal bovine serum (FBS, BioWhittaker, USA), 2 mM L-glutamine, 10 mM HEPES buffer, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 1% penicillin (100 U/ml)/streptomycin (100 mg/ml), and 50 μM 2-Methylmercaptoethanol, at 37℃ with 5% CO_2 incubator.

2.3. Reagents and flow cytometry

Antibodies purchased from BioLegend included Percp/cy5.5 anti-mouse CD45 (Clone I3/23), PE anti-mouse CD120b (TNFR Type II / p75) (clone TR75-89); antibodies purchased from BD Pharmingen included FITC Rat anti-mouse CD4 (clone RM4-5); antibodies purchased from eBioscience (Invitrogen) included eFlour 450 anti-Hu/Mo CD44 (clone IM7), APC anti-mouse CD62L (clone MEL-14), eFlour 450 anti-mouse CD8a (clone 53-6.7), eFlour 660 anti-mouse CD107a (clone eBiol D4B), and Percp/Cyanine 5.5 anti-mouse CD4 (clone RM4-5). Besides, APC anti-Mo/Rt Foxp3 (clone FJK-16s) for intracellular staining of Foxp3 was purchased from Invitrogen. Cells were resuspended in FACS buffer (PBS + 2% FBS + 0.05% NaN3), and cell surface staining was conducted at room temperature for 30 min. For intracellular staining of Foxp3, cells were fixed and permeabilized using Transcription Factor Staining Buffer Kit (Cat# TND-0607-KIT, TONBO biosciences), following the manufacturer's instructions. After blocking FcR, cells were incubated with appropriately diluted antibodies. Flow cytometry was performed on LSR II (BD FACSCalibur), FACS analysis was gated on the live cells only by using a LIVE/DEAD Fixable Dead Cell Stain kit, then analyzed by FlowJo software (version 10.6.1).

2.4. Effects of anti-TNFR2 antibody (TY101) on Tregs

$CD4^+CD25^+$ T cells were purified and seeded in a round bottom 96-well plate with 1×10^5 cells/well. Cells were cultured with IL-2 (10 ng/ml) and/or TNF (20 ng/ml) to maintain and activate their survival and expansion. To examine the effect of anti-TNFR2 antibody (TY101) on Tregs, the cells were treated with increasing concentrations of TY101 (0, 0.625, 1.25, 2.5 μg/ml) in the culture medium containing IL2 and TNF for 72 h. After the cells were collected and fixed/ permeabilized, then stained with specific antibodies of PercP/cy5.5 anti-mouse CD4, PE anti-mouse CD120b (TNFR2) and APC anti-mouse FoxP3, the relative isotype was used as an internal control, for FACS analysis. The CellTrace CFSE (carboxyfluorescein succinimidyl ester) Cell Proliferation Kit was used for labeling of cells to detect cell proliferation.

2.5. In vivo effect of TY101 on murine colon cancer models

Thirty BALB/c or C57BL/6 mice were randomly divided into six groups. All of the mice were subcutaneously (s.c.) injected with 2×10^5 CT26 or MC38 tumor cells suspended in PBS on the right flank. Once the tumors reached 50 to 100 mm^3 (usually 7 to 10 days after tumor inoculation), the tumor-bearing mice were intratumorally (i.t.) injected with either hamster IgG (10 μg), or anti-TNFR2 antibody (TY101) (10 μg), or i.t. injections of (N1 (10 μg) + R848 (10 μg)), or with TY101 (10 μg) + (N1 (10 μg) + R848 (10 μg)) or anti-PD-L1 (10 μg) + (N1 (10 μg) + R848 (10 μg)) twice per week for a total of 5 doses. The survival, tumor size and body weight of tumor bearing mice were monitored twice weekly. The calculating formula to determine tumor volume was V = length × width × width/2. Animals reached an endpoint and were euthanized when tumor volume reached 2000 mm^3, exhibited large necrotic areas on the skin or the mice had a 20% weight loss.

2.6. Isolation of tumor-infiltrating lymphocytes and immunostaining

Following treatments, the tumors and draining lymph nodes (dLNs) of mice were surgically dissected and dissociated using Miltenyi

Dissociation Kit (Miltenyi, MA, USA). After dissociation, 1 × ACK lysing buffer (Invitrogen, ThermoFisher, USA) was used to further lyse the contaminating red blood cells (RBCs). Cells were washed twice using FACS buffer and centrifuged at 300*g* for 5 min. Finally, cell suspensions were stained with anti-CD45/PerCP-Cy5.5, anti-CD4/FITC, anti-CD8/APC-Cy7 for the detection of CD4 and CD8 T cells; anti-CD62L/APC and anti-CD44/Pacific Blue for the detection of effector T cells; anti-TNFR2/PE and anti-FoxP3/APC for Tregs. Flow cytometry analysis was performed on LSRII (BD FACSCalibur), and data analyzed by FlowJo software (version 10.6.1).

2.7. Cytotoxic T lymphocyte (CTL) assay for immune function

A monolayer of CT26 cells was prepared using a 48-well plate by seeding 5 × 10^4 cells/well and incubating at 37℃ with 5% CO_2 incubator overnight. The dLNs cells were added to the plate at a ratio of 10:1 (dLNs: CT26). After 24 h incubation, cells were harvested and stained with anti-CD8-eFluor450 and anti-CD107a-eFluor660. Flow cytometry was conducted using BD LSRII, and the result was analyzed by FlowJo software.

2.8. Statistical analysis

The statistical significance between two groups were analyzed by two tailed student's *t*-test, and the multiple groups comparison were analyzed by one-way ANOVA and Tukey's post hoc test using GraphPad Prism 8.3 (GraphPad, San Diego, CA, USA). The results data were represented as mean ± standard deviation (SD) of multiple experiments. Statistical significance was determined by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

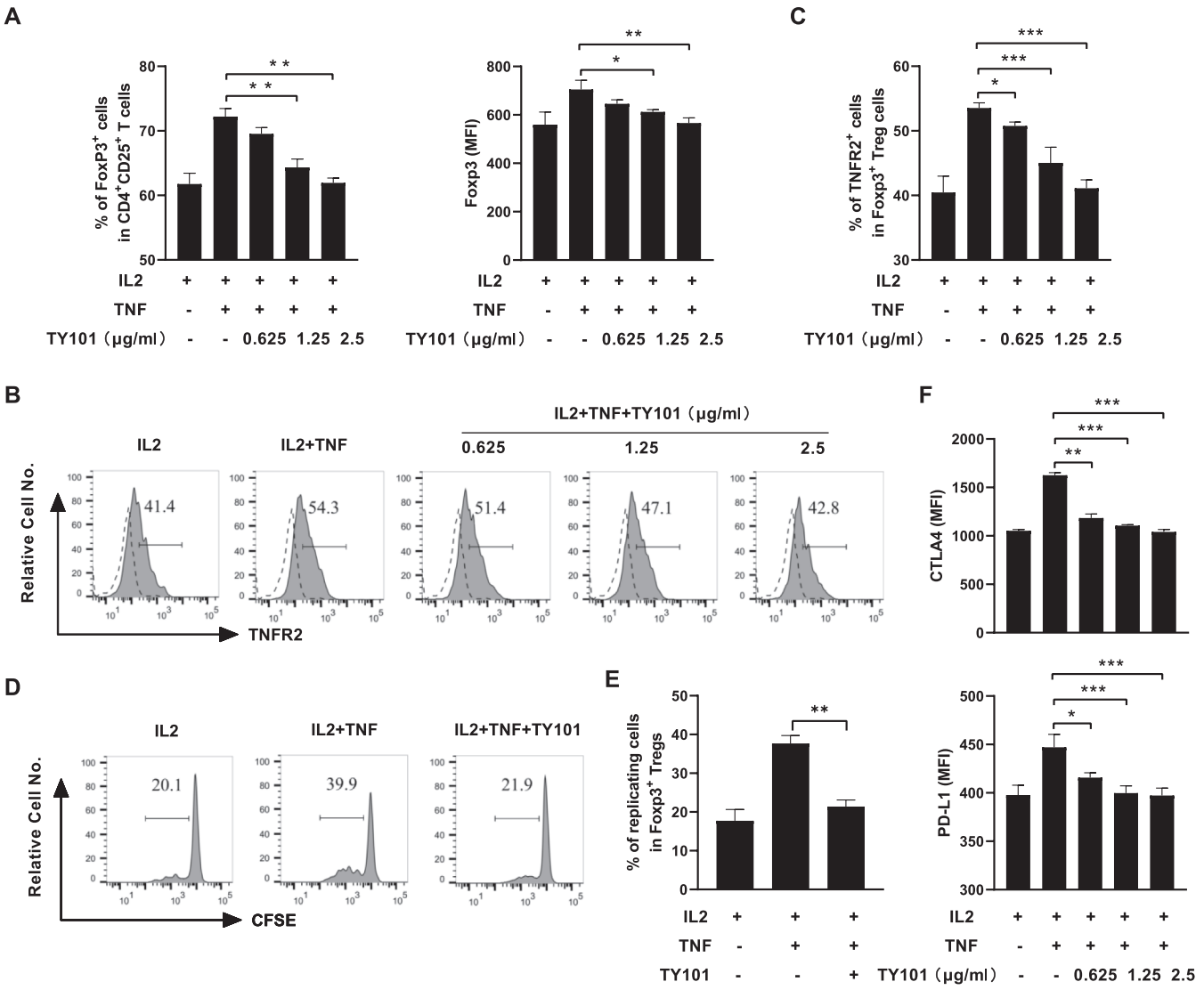

Fig. 1. Effect of *in vitro* treatment with TY101 on the proportion and proliferation of Tregs and their TNFR2 expression. $CD4^+CD25^+$ T cells were purified and treated with different concentrations of anti-TNFR2 antibody TY101 (0, 0.625, 1.25, 2.5 μg/ml) in the presence of 10 ng/ml IL-2 with or without 20 ng/ml TNF in RPMI1640 complete medium for 3 days. A, Summary of percentage of Foxp3-expressing cells and mean fluorescence intensity (MFI) of Foxp3 expression present in the $CD4^+CD25^+$ T cell subsets ($N = 3$, means ± SEM); B, Flow cytometric plot of TNFR2-expressing cells in Tregs. Number shows the proportion of gated cells. C, summary of the proportion of TNFR2-expressing cells present in $CD4^+FoxP3^+$ Tregs ($N = 3$, means ± SEM); D, CellTrace CFSE stained $CD4^+CD25^+$ T cells were treated with 2.5 μg/ml TY101 in the presence of 10 ng/ml IL-2 with or without 20 ng/ml TNF were cultured for 3 days, the proportion of proliferating cells is indicated in the histogram; E, the summarized statistical data of proliferating Treg cells are shown ($N = 3$, means ± SEM). F, The mean fluorescence intensity (MFI) of CTLA4 and PD-L1 expression by Tregs. All data shown are representative of three independent experiments with similar results. The statistical significance was analyzed using one-way ANOVA by comparison with IL-2 plus TNF group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3. Results and discussion

3.1. Effect of TY101 on the proportion, proliferation and TNFR2 expression of Tregs in vitro

Mouse $CD4^+CD25^+$ T cell subsets purified using a $CD4^+CD25^+$ Regulatory T Cell Isolation Kit, yielded greater than 90% pure of $CD4^+FoxP3^+$ Tregs as previously reported [23]. TNF could activate Tregs and promote their proliferation and expansion by interacting with TNFR2. To confirm the effect of anti-TNFR2 antibody (TY101) on Tregs, purified $CD4^+CD25^+$ T cells were cultured in a medium containing IL-2 (10 ng/ml) and TNF (20 ng/ml). The cells were treated with increasing concentrations of TY101 (0.625–2.5 μg/ml). The results showed that in the presence of IL-2 (required for *in vitro* survival of Tregs), TNF could increase the proportion of $Foxp3^+$ Tregs and TNFR2 expression by Tregs (~1.2-fold and ~1.4-fold, respectively), which may reflect the effect of TNF on the maintenance of Foxp3 expression by *in vitro* cultured Tregs. However, treatment with TY101 reduced the proportion of $Foxp3^+$ Tregs (from 72.3% to 61.4%) as well as Foxp3 expression (Fig. 1A) and down-regulated TNFR2 expression by Tregs (~0.4-fold) (Fig. 1B and C) compared to TNF treatment Tregs, in a dose-dependent manner ($p < 0.05$). Based on cell viability assays, the proportion of live cells in $CD4^+CD25^+$ T cells treated with TY101 was equal to those in the control cells (data not shown), indicating TY101 was not cytotoxic for Tregs.

To determine the effect of TY101 on the proliferation of Tregs, Tregs stained with CFSE were cultured in the absence or presence of TNF and TY101. After 3 days of incubation with IL-2, 20.1% cells were proliferative (Fig. 1D left panels). In the presence of IL-2, TNF promoted the

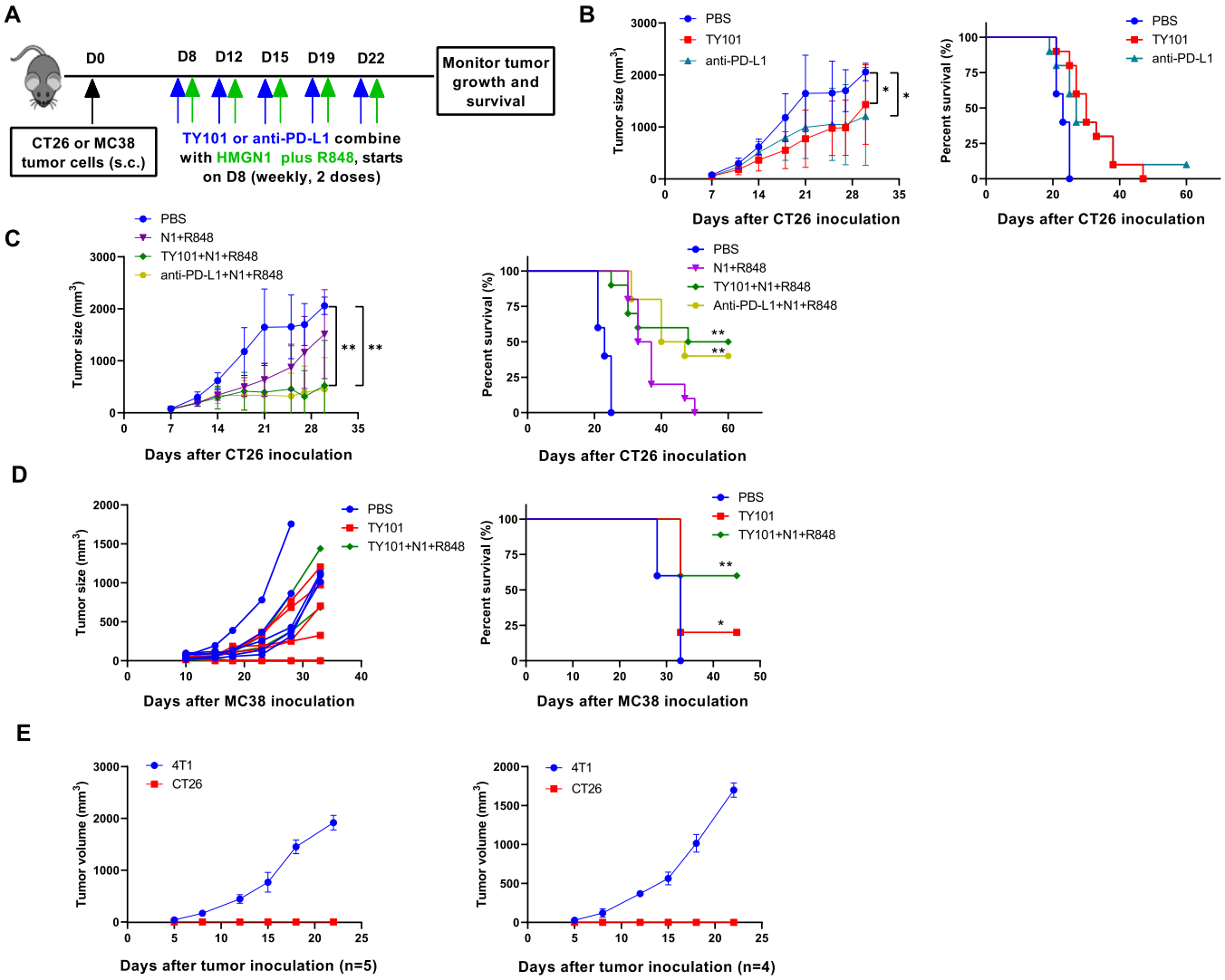

Fig. 2. Therapeutic effect of TY101 therapy alone or in combination with N1 and R848 on mouse colon cancer models. 2×10^5 CT26 or MC38 colon cancer cells were subcutaneously (s.c.) injected into Balb/c or C57BL/6J mice on day 0, respectively. At day 8, when tumor size reached 50–100 mm^3, TY101, anti-PD-L1, or HMGN1 plus R848, alone or in combinations of TY101 or anti-PD-L1 together with HMGN1 plus R848 were injected intratumorally (i.t.) for 5 doses biweekly. A, shows the overall therapeutic procedural design scheme. Blue arrow represents TY101 or anti-PD-L1 injection, green arrow represents HMGN1 plus R848 injection; B, CT26 tumor growth in individual mice treated with TY101 or anti-PD-L1 (n = 10, respectively) or control (PBS) group (n = 10) is shown (means ± SEM), and the survival curves for each group are also indicated; C, Effect on CT26 tumor growth in individual mice being treated with TY101 or anti-PD-L1 combined with N1 and R848 group (n = 10, each group) is shown (means ± SEM), and the survival curves for each group are also indicated; D, Effect on MC38 tumor growth in individual mice of TY101 treatment alone (n = 5) or combined with N1 and R848 (n = 5), the tumor growth for each mouse is shown and the survival curves for each group are also indicated; E: Tumor free mice in TY101 combination group (n = 5) and anti-PD-L1 combination group (n = 4) were subcutaneously injected with 2×10^5/mouse of CT26 cells in the right flank and 4T1 cells in left flank and the tumor growth curves were represented, data are summarized as means ± SEM. Red line represents CT26, Blue line represents 4T1. Statistically significant difference was analyzed using one-way ANOVA in comparison with PBS group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

proliferation of Tregs up to 39.9%, however, when treated with TY101, the proportion of replicating cells decreased back to 21.6% (Fig. 1D right panels). Therefore, TY101 was an antagonistic antibody to TNFR2 and completely inhibited the proliferation of Tregs induced by TNF ($p < 0.01$, Fig. 1E). Moreover, to explore whether TY101 interferes with other inhibitory markers of Tregs by blocking TNF-TNFR2 axis, we assessed the effect on the expression of CTLA4 and PD-L1. The expression of theses inhibitory markers was decreased markedly (Fig. 1F), which indicated the important role of TY101 in interfering the suppressive phenotype of Tregs.

3.2. *Effect of TY101 in combination with N1 and R848 on the growth of murine colon tumors*

To determine the *in vivo* effect of TY101, CT26 or MC38 tumor-bearing mice were treated with TY101 or anti-PD-L1, combined with or without N1 and R848 for up to 5 doses (twice a week), starting from day 8 after tumor reached 50–100 mm^3 in size, as schematically shown in Fig. 2A. Mice receiving a single treatment of TY101 showed partial inhibition of tumor growth, and prolonged the survival of tumor bearing mice, the potency of TY101 was comparable to anti-PD-L1 treatment (Fig. 2B). However, none of the CT26 tumor bearing mice was cured by their treatment. We previously documented that N1 and R848 synergistically activated DCs and promoted antitumor responses. As a result, intratumoral delivery of N1 plus R848 with therapeutics capable of countering tumor immunosuppression (e.g., Cytoxan, anti-PD-L1), the mouse tumors could be cooperatively suppressed to a much great extent [19]. We therefore examined whether N1 and R848 could enhance the antitumor effect of anti-TNFR2 antibody. Although treatment of HMGN1 and R848 could not induce complete regression of tumors, the combination of TY101 with N1 plus R848 resulted in a cure rate of 50% (5/10). Moreover, to compare the effect of this combination with anti-

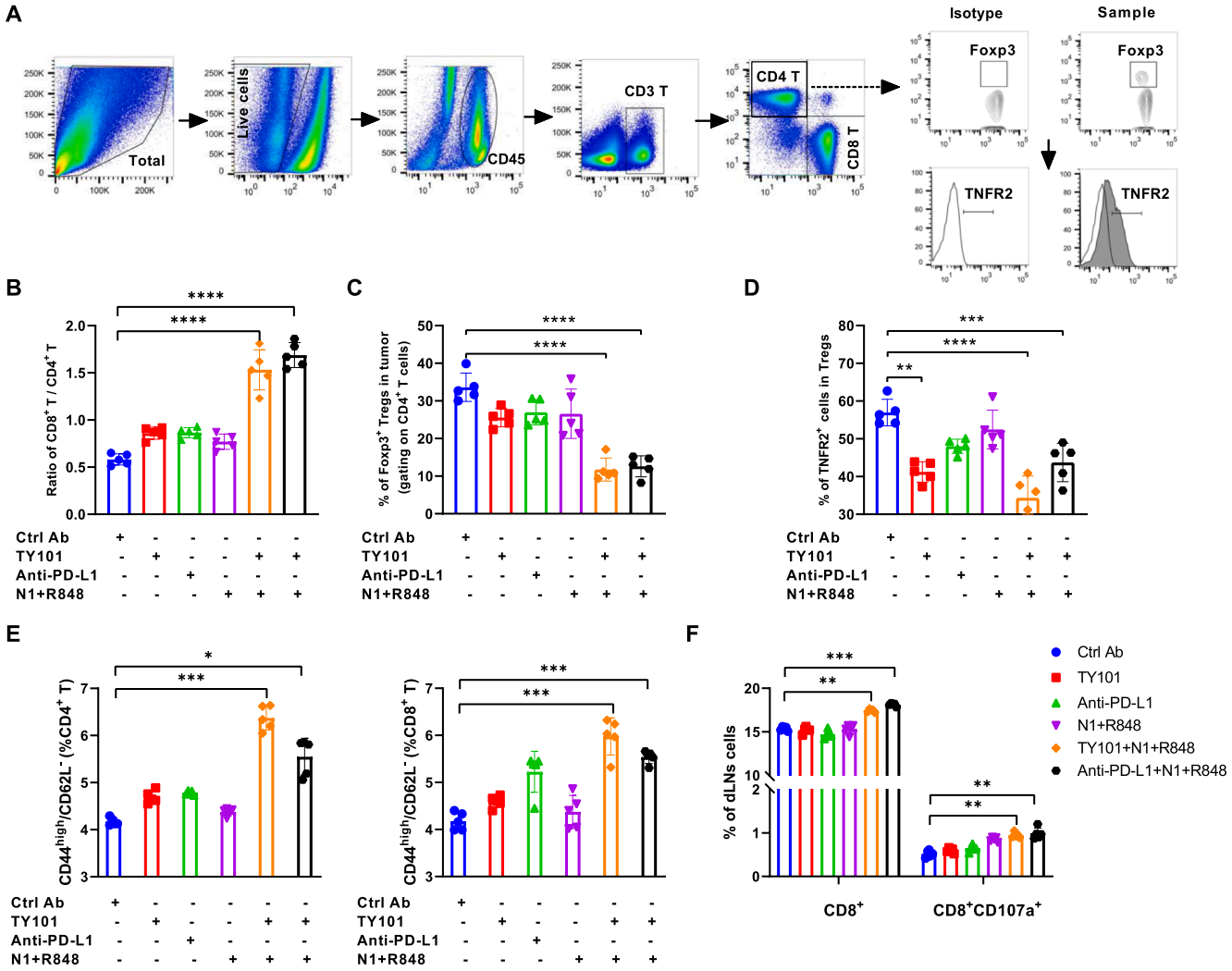

Fig. 3. Effect of TY101 combined with N1 and R848 on the tumor infiltration by Tregs and TNFR2 expression, and on effector CD4 and CD8 T cells and CTL. After treatment by TY101, or anti-PD-L1, or N1 plus R848, and their respective combinations for 3 doses, the immune cells in tumors and draining lymph nodes (dLNs) were detected by flow cytometry. To examine the cytotoxic CD8 T lymphocytes (CTL) function, the draining lymph nodes (dLNs) from each treatment group were cocultured with CT26 tumor cells at a ratio of 100:1, then flow cytometry was performed to detect the proportion of CTL. A, The gating strategies of CD4 T, CD8 T, Tregs and $TNFR2^+$ Tregs in tumor tissue by flow cytometry; B, summary of the ratio of $CD8^+$ versus $CD4^+$ T cells (n = 5, means ± SEM) in individual mouse from each group treated with Ctrl Ab, TY101, anti-PD-L1, N1 plus R848, or a combination of TY101 or anti-PD-L1 with N1 plus R848; C, summary of the proportion of $CD4^+FoxP3^+$ Tregs in $CD4^+$ T cells in tumor tissues from each group (n = 5), data shown are means ± SEM; D, summary of the percentage of TNFR2-expressing cells in Treg cells from each group (n = 5), data shown are means ± SEM; E, summary of the percentage of effector CD4 T ($CD4^+CD44^{high}/CD62L^-$) cells and CD8 T ($CD8^+CD44^{high}/CD62L^-$) cells in dLNs from each group (n = 5), data shown are means ± SEM; F, summary of the percentage of $CD8^+$ T cells and CT26-specific cytotoxic $CD8^+$ T ($CD8^+CD107a^+$) cells in dLNs from each group (n = 5), data shown are means ± SEM. Statistically significant difference was analyzed using one-way ANOVA by compared to Ctrl Ab group. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, **** $p < 0.0001$.

PD-L1 inhibitor, we found the combination of anti-PD-L1 with N1 and R848 also resulted in a cure rate of 40% (4/10). The overall survival of mice in both combination groups was also markedly prolonged (Fig. 2C). Similarly, in MC38 tumor bearing mice, TY101 treatment alone could induce a cure rate of 20%, whereas the combination of TY101 with N1 and R848 resulted in 60% of mice being cured. Consequently, the long-term survival of tumor bearing mice was also markedly improved (Fig. 2D).

To investigate whether tumor free mice could generate persistent tumor-specific immune responses, CT26 and murine mammary carcinoma 4T1 cells were injected into tumor-free mice cured by TY101 combined with N1 and R848 (n = 5) or by anti-PD-L1 combined with N1 and R848 (n = 4) treatment, on their contralateral flanks three weeks later after the completion of first treatment. We then monitor the tumor growth for another month. All of the cured mice in both groups developed 4T1 tumors, whereas none of the cured mice formed CT26 tumors (Fig. 2E). This indicates that the cured mice have developed tumor-specific immunity.

3.3. Effect of TNFR2 antagonism in combination with N1 and R848 on the levels of CD4 and CD8 T cells intratumorally and Tregs and in draining lymph nodes

To demonstrate the effects of immunotherapy on immune cell content in CT26 tumor bearing mice, we profiled the tumor infiltrating leukocytes and immune cells present in tumor tissue and in draining lymph nodes by flow cytometry. The flow cytometry gating strategies as shown in Fig. 3A. TY101 treatment alone or in combination with N1 and R848 increased the ratio of intratumoral CD8/CD4 T cells equally as did a single treatment of anti-PD-L1 or in combination with N1 and R848 (Fig. 3B). Moreover, after treatment with TY101 or anti-PD-L1 in combination with N1 plus R848, the numbers of $CD4^+FoxP3^+$ Tregs in the tumor microenvironment were markedly decreased (Fig. 3C). Since TY101 acted as an TNFR2 antagonist, we further examined the expression of TNFR2 by Tregs, which revealed that treatment with TY101 alone or when combined with N1 and R848 markedly decreased the expression of TNFR2 by Tregs in tumor tissue (Fig. 3D). Therefore, TY101 has the capacity to reduce Tregs in tumor bearing mice.

Furthermore, the combination of TY101 or anti-PD-L1 together with N1 and R848 resulted in greater increases in effector CD4 ($CD4^+CD44^{high}/CD62L^-$) and effector CD8 ($CD8^+CD44^{high}/CD62L^-$) T cells (Fig. 3E) as compared with control groups. Moreover, based on coculture of dLNs with CT26 tumor cells to examine the cytotoxic function of CD8 T cells, combination immunotherapy also resulted in the highest levels of CD8 T cells as well as tumor-specific effector CD8 ($CD8^+CD107a^+$) T cells present in draining lymph nodes (Fig. 3F). Overall, these data indicate that combination therapy by TY101 with N1 and R848 exerted its antitumor effects based on depletion of Tregs and resultant stimulation of cytotoxic CD8 T cells.

In this study, monotherapy with TY101 moderately reduced the proportion of Tregs and more profoundly decreased the expression of TNFR2 by tumor infiltrating Tregs. The combination therapy with N1 and R848 more dramatically reduced the proportion of Tregs in tumor, presumably resulting from the restriction of Tregs, and from the expansion of Teffs though the activated DCs induced by N1 and R848 treatment. The infiltration of CD8 T cells in tumor microenvironment by combination therapy was also markedly increased.

In addition to Tregs, recent studies have shown that TNFR2 is also expressed by other immunosuppressive cells, such as mesenchymal stem cells (MSCs) and myeloid-derived suppressor cells (MDSCs) [24,25]. Thus, the immune suppressive effect of these immunosuppressive cells was likely inhibited by TY101 treatment as well. Moreover, endothelial progenitor cells (EPCs) also express TNFR2 [26]. Since EPCs play important roles in the formation of new blood vessels to support tumor growth [26], the inhibition of TNFR2 may mitigate the tumor-promoting effect of EPCs. Global inhibition of all lineages of TNFR2-expressing cells may be attributed collaboratively to the overall anti-tumor effect of TY101. However, most likely that the inhibition of Tregs, may play a key role. To further identify additional relevant cellular targets of TNFR2 blocking antibodies merits further investigation.

As previously reported, a number of tumor cells also express TNFR2, and it was shown that TY101 had a moderate cytotoxic effect on TNFR2-expressing CT26 and MC38 tumor cells, as determined by trypan blue staining [21]. To further confirm its effects on tumor cells, we examined the proliferation of tumor cells in the presence of different concentrations of TY101 with a cell counting kit-8 (CCK-8) assay. However, we did not detect any significant inhibitory effect of TY101 on the proliferation of tumor cells (data not shown). Although the discrepant results may be caused by the different assays, we favor the idea that the major anti-tumor effect of TY101 was mediated by reduction of TNFR2-expressing Tregs, rather than any direct inhibition on tumor cells.

CD8 T cells also express TNFR2, however, the proportion of TNFR2-expressing cells in CD8 subset was ~12 folds lower than that of Tregs (data not shown). The role of TNFR2 expressed by CD8 T cells is highly controversial and there are plenty of publications showing that TNFR2 negatively regulates CD8 cell function. For example, it was reported that TNFR2-expressing CD8 T cells are exhausted cells or immunosuppressive $CD8^+$ Treg cells [27]. Intriguingly, it was shown that TNFR2-deficient memory CD8 T cells provided superior protection against tumor cell growth [28], presumably due to the fact that CD8 cells deficient in TNFR2 were more resistant to activation-induced cell death (AICD) [29]. Therefore, it is possible that blockade of TNFR2 by TY101 could inhibit the activation of CD4 Tregs, while reversing the exhaustive phenotype of CD8 T cells, promoting the survival of CD8 CTLs and enhancing their anti-tumor activity. This possibility should be experimentally studied in the future.

It should be noted that a previous report showed intraperitoneal (i.p) treatment by TY101 alone of CT26 tumor bearing mice resulted in a ~55% cure rate, while the combination of TY101 with anti-PD-1 therapy could induce a cure rate of up to ~63% [20]. However, in our report, none of the CT26 tumor bearing mice was cured by the treatment with TY101 by itself and had only a ~20% cure rate in MC38 tumor bearing mice. The reason for this discrepancy may be attributable to the method of administration and dosage of TY101, because the intratumorally administration in our study did not result in a more potent immune responses in comparison with the reported intraperitoneal injection of higher doses of TY101. However, the comparatively lower doses used in our study would be advantageous. Furthermore, the high expression of TNFR2 on tumor cells and/or abundance of tumor infiltrated $TNFR2^+$ Tregs in tumors would be more susceptible to anti-TNFR2 therapy.

Unlike in previous studies, our approach endeavored to reduce or eliminate the immunosuppressive environment to improve the effector T cells activation. In this study, TY101 by selective blocking TNFR2, might have prevented downstream signaling by NF-kB and PI3K/Akt [30], which is necessary for Tregs survival and expansion. TY101 exhibited potent *in vitro* and *in vivo* efficacy in decreasing the numbers of TNFR2-expressing Tregs and reducing their suppressive function. Though N1 plus R848 did not have any direct antitumor effects, they could markedly activate the maturation of DCs in the tumor microenvironment. Stimulation by this combination resulted in the production of pro-inflammatory cytokines and antigen-presenting capacities of DCs. Therefore, the combination of anti-TNFR2 antibody with N1 and R848 by dual-targeting of immunosuppressive Tregs and immature DCs, resulted in greater promotion of presentation of tumor-specific antigens to CTL effectors, thus promoting greater antitumor immune responses. Furthermore, combination therapy of TY101 with N1 plus R848 equaled the anti-tumor effects of anti-PD-L1 together with N1 and R848, indicating that anti-TNFR2 antibody could be developed as a novel treatment option for cancer therapy.

In conclusion, our study clearly shows that combination therapy by

anti-TNFR2 antibody with N1 and R848 may represent a novel treatment for solid tumors. The mechanism of TNFR2 antagonism is presumably based on blocking TNF-TNFR2 signaling of Tregs in the tumor microenvironment. Recently, multiple humanized anti-TNFR2 antibodies have been developed for cancer immunotherapy, and our results suggest the efficacy and safety of anti-TNFR2 merit clinical investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Contributions

Mengmeng Jiang and Jia Liu performed and completed the experiments. De Yang, Xin Chen and Joost J. Oppenheim designed the experiments. Mengmeng Jiang drafted the manuscript. Debra Tross, Ping Li, Fengyang Chen and Md Masud Alam helped to review and editing the manuscript. Denise L. Faustman contributed to provide drug analysis. Joost J. Oppenheim, De Yang and Xin Chen revised the manuscript. All authors agreed to submit the manuscript.

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