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

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Research Article

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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 now compelling evidence that TNF through TNFR2 preferentially stimulates the activation and expansion of Tregs. We and others, therefore, proposed that targeting of TNFR2 may provide a novel strategy in cancer immunotherapy. Several studies have showed the anti-tumor effect of TNFR2 antagonistic antibody in different tumor model. However, the exact action of TNFR2 antibody on Tregs remained fully understood.

Method

TY101, an anti-murine TNFR2 antibody, was used for examining the effect of TNFR2 blockade on Treg proliferation and viability in vitro. The role of TNFR2 on Treg viability were further validated by TNFR2 konckout mice and in TY101-treated mouse tumor model.

Results

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

Conclusion

This study therefore provides clear experimental evidence that the anti-tumor effect of TNFR2 antagonistic antibody can promote the death of Tregs, and this effect may be attributerable to the anti-tumor effect of TNFR2 antagonistic antibody.

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, maximally immunosuppressive TNFR2-expressing Tregs accumulated in the tumor environment in mouse models and human patients [3, 4]. TNFR2+ Tregs in tumor tissue potently inhibit the anti-tumor immune responses [5]. In human patients,
TNFR2 expression levels on Tregs correlated with the clinical pathology and disease progression [6–11]. The presence of TNFR2-expressing Tregs represent a major cellular mechanism underlying immune evasion of tumor. We and others therefore proposed that targeting of TNFR2 represents a novel strategy in the tumor immunotherapy [12, 13].

Currently, TNFR2 antagonists are under investigation as potential tumor therapeutic agents[14]. We firstly reported that targeting TNFR2 with antagonistic antibody (M861) in combination with CpG-ODN or anti-CD25 antibody was reported to inhibit the growth of colon carcinoma and mammary carcinoma in mice [15]. TY101, an anti-murine TNFR2 antibody, was shown to induce the regression of tumor in CT26 and MC38 mouse colon carcinoma models [16]. Moreover, TY101 in combined with anti-PD1 antibodies or R848 (TLR7/8 agonists, activating dendritic cells) potently inhibit tumor growth[16, 17]. These results also indicated that the elimination of tumor-infiltrating Treg by TNFR2-targeting antibody potently enhanced the antitumor immune responses. Several studies also showed that targeting anti-human TNFR2 antibody also inhibit human treg and promote Treg and TNFR2+ tumor cell death[18, 19], indicating the therapeutic potential of anti-TNFR2 therapeutics in the treatment of human cancer.

In this study, we investigated the effect of TY101 on Treg. Treatment of TY101 inhibit TNF-induced Treg proliferation but TY101 alone did not affect Treg viability. However, TY101 could impair the Treg viability in the presence of Dex. We further compare the viability of TNFR2 deficient Tregs with WT Tregs. TNFR2 deficient Treg are more sensitive to Dex-induced cell death while activation of TNF-TNFR2 axis protect Treg from Dex induced cell death. In addition, we also tested the antitumor effect of TY101. In vivo administration of TY101 into EG7 lymphoma-bearing C57BL/6 mice resulted in complete regression of tumor in 60% of mice. The antitumor effect could be attributable to the decreased tumor infiltrating Treg and increased infiltration of IFN-γ cytotoxic T cells (CTLs). Our results indicated that TNFR2 antagonistic antibody promote Treg cell death, which could be related with the antitumor effect of TNFR2 antagonistic antibody.

Materials And Methods

Mice and reagents

Female wildtype (WT) C57BL/6J 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. Mouse TY101 was a gift from Dr. Denise Faustman's group (Massachusetts General Hospital & Harvard Medical School)[20]. RPMI-1640 (1×), Fetal Bovine serum (FBS), trypsin-EDTA (0.25%) phosphate-buffered saline (1×), Pen Strep (100×), HEPES (1M) and Trypan Blue Stain (0.4%), and LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit, were purchased from ThermoFisher Scientific (USA). Dexamethasone were purchased by Sigma. Antibodies were purchased from BD Pharmingen (San Diego, CA) consisted of PerCP-Cy5.5 anti-mouse TCR (H57-597), PE anti-mouse CD120b/TNFR2 (TR75-89), APC anti-mouse CD8 (53-6.7) and PE anti-mouse IFN (XMG1.2) and Propidium iodide. Antibodies purchased from eBioscience include eFluor 450 anti-mouse CD45 (30F11), FITC anti-mouse BCL-2(10C4), 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. Dimethyl sulfoxide (DMSO) were obtained from the Sigma-Aldrich (St. Louis, MO, USA).

Cell line and cell culture

EG7-OVA cell line was purchased from American Type Culture Collection (Manassas, USA). EG7-OVA cells were cultured in RPMI-1640 supplemented with 10% FBS 1% Pentrip, 1% L-glutamine and 50μM monothioglycerol, the incubation was carried out at 37°C in humidified air containing 5% CO₂ in a tissue culture flask.

Cell purification and in vitro cell culture

Mouse lymphocytes were harvested from spleens, axillary lymph nodes, inguinal lymph nodes and mesenteric lymph nodes. CD4⁺ T cells were purified from lymphocytes using Mouse CD4 (L3T4) MicroBeads and LS Columns (Miltenyi Biotec).

The lymphocytes or CD4 T cells isolated from WT C57BL/6 mice or TNFR2 knock-out were cultured in RPMI-1640 supplemented with 10% FBS 1% Pentrip, 5x10⁻⁵ M 2-Me, 1% No Essential Amino Acid with alone or IL-2 (10 ng/ml), or with or without TNF (20 ng/ml), in the presence of TY101 (10 μg/ml) or Dexamethasone (10 nM), the incubation was carried out at 37°C in humidified air containing 5% CO₂ in a 96-well U-type culture plate. The proportion of Treg cells, the viability rate of Treg, and TNFR2 expression on Treg cells was analyzed by FCM (Flow cytometry), gating for Foxp3⁺ cells.

Tumor cell inoculation and separation

EG7-OVA was subcutaneously injected into the right flank of recipient mice as a single-cell suspension with 2 x 10⁵ 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 treatment with anti-TNFR2 were reinoculated with EG7-OVA cells (2 x 10⁵) into the right flank, and the same number of B16-F10 cells was injected subcutaneously into the left flank. Tumor size was calculated by the following formula: (length x width ^ 2) / 2. “Survival” represents the time to when a 4 cm³ tumor develops or mice become moribund. A humane end points that trigger euthanasia. Mice were monitored daily and were euthanized when signs of morbidity from metastatic disease burden became evident.

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 were conducted using FlowJo software (Tree Star Inc.).
Statistical analysis

Two-tailed Student’s t test was used for the comparison of two indicated groups. One-way analysis of variance test was used for the comparison of tumor growth, Treg percentage, Treg viability among groups. Log-rank test was used for the comparison of survival shown in Fig 5D. All statistical analysis was performed with GraphPad Prism 7.0.

Results

1. TY101 abrogates TNF-induced proliferation of Treg cells

Previously, we and others reported that, in the presence of low levels of IL-2, TNF can preferentially stimulate the proliferation of Tregs present in cultured CD4 cells [4]. We thus used this assay to examine the effect of an anti-TNFR2 antibody TY101 on TNF-induced Treg proliferation. To this end, MACS-purified mouse CD4 T cells were cultured with IL-2 and stimulated with TNF. As expected, TNF treatment increased the proportion of Foxp3-expressing Tregs as well as the absolute number of Tregs in the cultured CD4 cells by 51.7% and 150%, respectively (Fig 1A-C, p<0.01). Treatment with TY101 (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 [15], TY101 is an antagonistic anti-mouse TNFR2 antibody that inhibited TNF-induced proliferative expansion of Tregs.

2. TY101 treatment increases the death of Treg cells 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 [21, 22], we thus used LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit to assess the effect of TY101 on Treg viability. However, TY101 alone did not affect Treg viability (Fig S1A-B). To further determine the role of TNF-TNFR2 axis on Treg viability, we examined the effect of TY101 on dexamethasone (Dex)-induce the death of Treg cells. To this end, lymphocytes from normal mice were cultured with Dex (1×10^{-8} M), or Dex plus IL-2 (10 ng/ml), or Dex plus IL-2 and TNF (20 ng/ml), or Dex plus IL-2, TNF and TY101 (10 µg/ml) or IL-2 alone for 72 hours. The result showed that dexamethasone alone markedly reduced the proportion of Foxp3-expressing Tregs and a decreased viability of Tregs (Fig 2A-B) and (Fig 2C-D). IL-2 alone partially maintained the survival of Tregs, while addition of TNF was able to almost completely abrogated Treg death induced by Dex (Fig 2C-D). Furthermore, TNF treatment resulted in a marked increase of proportion of Foxp3^{+} Tregs in the cultured CD4 cells (Fig 2A-B), which was attributable to the proliferative expansion of Tregs even in the presence of Dex as we reported previously [23, 24]. In compare with IL-2 treated group, IL-2 plus TNF treated group can further increase Treg viability (from 77.2% to 91.5%). These effects of TNF on the expansion of Tregs and on the survival of Tregs was largely inhibited by the treatment of TY101 (Fig 2C-D).

3. TNFR2 signaling dampens death induction by Dex on Tregs
To further clarify if the effect of TY101 on Treg survival was mediated by the blockade of TNFR2, we determined the role of TNFR2 on Treg survival. To this end, lymphocytes were isolated from wild type mice or from TNFR2−/− mice. The cells were cultured with IL-2, with or without TNF, in the presence or absence of Dex. The viability of Tregs was determined by LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit, by gating on Foxp3+ Tregs. The result show that, when cells were cultured in IL-2 alone, the viability of both WT Tregs and TNFR2-deficient Tregs were comparable (Fig S2 A-B). Thus, genetic ablation of TNFR2 did not enhance the death of Treg cells in the presence of IL-2 alone. Nevertheless, TNF-TNFR2 interaction markedly protected the death of Tregs induced by Dex, as evidenced by the fact that TNF markedly enhanced proportion and viability of Dex-treated WT Tregs (p<0.001), but not Dex-treated TNFR2-deficient Tregs (P>0.05, Fig 3A-D). Interestingly, TNF treatment did not enhance the viability of WT Teffs (Fig S3A-B), 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 WT or TNFR2−/− mice for three times as described previously [37]. One day after the last treatment, the mice were sacrificed and the ratio and number of CD4+Foxp3+ Tregs in the spleen and LNs were analyzed by flow cytometry. The results showed the absolute number of Treg cells decreased significantly after the Dex-treated WT mice and TNFR2−/− mice (p<0.001, Fig 3F-G). The absolute number of Treg cells decreased by 50.3% (Fig 3F) in TNFR2−/− mice group. In contrast, the percentage of Tregs decreased by 30.7% in WT mice (Fig 3G). There results indicated TNFR2 deficient Treg were more susceptible to DEX-induced cell death in vivo (Fig 3H). Therefore, the both *in-vivo* and *in-vitro* data support the pro-survival effect of TNFR2.

4. TY101 potently inhibits the growth of EG7 lymphoma in mice

To investigate whether TY101 enhance antitumor responses as previously reported [16, 25], we next examined the *in vivo* anti-tumor effect of TY101 in the mouse EG7 lymphoma model. As shown in the schematic diagram in Fig. 4A, C57BL/6 mice were inoculated subcutaneously with EG7 lymphoma. Treatment was started on day 7 after tumor inoculation (tumor diameter ~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 5 times. One day after the last treatment (Day 24), the mice were sacrificed. The results show that the administration of TY101 markedly inhibited the growth of EG7 lymphoma (p<0.001, Fig. 4B-C), and complete regression of EG7 lymphoma was observed in 60% of the mice at the end of the experiment (50 days after tumor inoculation, FIG 4D). 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 completely tumor regression were re-inoculated with EG7 lymphoma into the right flank 8 weeks after becoming tumor-free. In 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. None of the mice with complete regression of tumor developed EG7 lymphoma (0/3), while all of them developed B16-F10 melanoma (3/3). In contrast,
all the normal control mice developed both B16-F10 melanoma and EG7 lymphoma (3/3), FIG 4E). Thus, this result clearly indicated that TY101 treatment induced long-term tumor specific immune memory.

5. TY101 treatment promote tumor infiltrating Treg cell death and enhance the antitumor responses

To determine whether TNFR2 antagonistic antibody elicit antitumor immune response by promoting intratumoral Treg cell death, we started the treatment after the tumor size reach about 10 mm in diameter, then the tumor-infiltrating Tregs were analyzed by flow cytometry. As shown in Fig 5A-B, over 95% of Treg cells were viable in IgG-treated control group, while TY101 significantly reduced the Treg viability in tumors (From 97.2% to 77.6%). Moreover, administration of TY101 decreased proportion of tumor infiltrating CD4⁺ Foxp3⁺ T cells by about 50% in mouse EG7-OVA tumor (FIG 5C-D). The absolute number of tumor-infiltrating Tregs also decreased significantly in the TY101 treated mice group (FIG 5E), which was accompanied with a significant reduction of TNFR2 expression on Tregs as well (FIG 5F-G). Percentage of IFN-gamma expressed CD8 T cells were increased by about 50% as compare with control (FIG 5H-I). Moreover, the ratio of CD8 T cells to Treg was significantly increased after treatment with TY101 (FIG 5J). Therefore, the TY101 could enhance the antitumor immune response by reducing intratumoral Treg viability.

Discussion

In this study, we showed a possible mechanism of the antitumor effect of TNFR2 antagonist. Genetic or pharmacological inhibition of TNFR2 promote Treg cell death, while activation of TNFR2 protected Treg from cell death. We further found that TY101 treatment reduce the viability of the suppressive TNFR2-expressing Tregs in the tumor environment, and as a result, mobilized and activated anti-tumor immune responses, resulting in the complete regression of EG7 lymphoma in the majority of mice.

Upregulation of pro-survival genes is one of the characteristics in the transcriptomic profile of TNFR2 agonist-treated Tregs[26]. Previous results from Dr. Faustmann and her colleagues showed antagonistic anti-TNFR2 antibodies, in addition to inhibiting proliferation of human Tregs, could induce the death of TNFR2-expressing Tregs [19, 22, 27]. They showed that TNFR2 antagonist antibody alone decreased Treg ratio or Treg number in purified CD4 T cells, which was interpreted that blockade of TNFR2 induce Treg cell death. However, in this study we provided a direct evidence that TNFR2 inhibition cannot induce Foxp3⁺Treg cell death in the normal culture condition, indicating that TNFR2 alone decreased the Treg ratio could depend on other mechanisms. Interestingly, blockade of TNFR2 promote Treg cell death in the presence of Dexamethasone, an anti-inflammatory agents induced Treg cell death. The pro-survival effect of TNF was further supported by a previous study showed that TNF-treated Tregs are more resistant to oxidative stress-induced cell death[28]. Therefore, activation of TNF-TNFR2 signaling pathway could be important for protecting the Treg from cellular stress-induced cell death.

Tumor environment is a stressful cellular environment, which can be characterized as hypoxia, increased ROS (reactive oxidative species) and low-PH value (high concentration of lactic acid). These factors
inhibit antitumor response and promote the accumulation of immunosuppressive subsets including Tregs. That Treg cells are more adaptive to tumor microenvironments could be one of the explanation that Foxp3+ Tregs were enriched in tumor microenvironment. In this study, we showed that targeting TNFR2 promote Treg cell death, indicating that TNF-TNFR2 activation promote intratumoral Treg viability. However, the mechanism that TNFR2 promote Treg viability remains unknown, which should be investigated in future studies.

Take it all, in this study we showed the activation of TNF-TNFR2 promote Treg viability and this effect could represent a underlying mechanism of the antitumor effect of TNFR2 antagonistic antibodies. However, TNFR2 antibodies may also decrease the intratumoral Treg infiltration through FcγR-mediated antibody cell-mediated cytotoxicity (ADCC) or antibody-mediated phagocytosis (ADCP). Our results indicated that in vivo effect of TNFR2 antagonistic antibody to Treg could be more complicated and which should be further investigated.

**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
FBS
Fetal bovine serum
2-Me
2-Mercaptoethanol
FcR
Fc receptor
MACS
Magnetic-activated cell sorting
Declarations

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J.J.M, X.C, designed the study and reviewed the manuscript. T.Z.H, Y.B.C, M.S.I, C.K.C, J.R.L, performed the experiments and wrote the manuscript. J.J.M, X.C were involved in the conceptualization, supervision, project administration and funding acquisition. All authors have read and agreed to the published version of the manuscript.

Consent for publication

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

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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.

Data Availability Statement

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

References


Figures
TY101 abrogated TNF induced Treg proliferation

CD4 T cells were isolated from normal mouse LN and spleen with MACS. The cells were cultured with IL-2, in the presence or absence of TNF, with or without TNFR2 Ab for 72 hours. The proportion of Foxp3+ Tregs was analyzed by FCM. (A) Typical FCM plots. Number in FCM plot indicates the proportion of gated Tregs. (B-C) Summary of the proportion (B) and absolute number (C) of Treg cells. Data (Mean ± SEM, 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.
Figure 2

Effect of TY101 on Treg cell survival

Mice lymphocytes were isolated and cultured with IL-2 alone, Dex alone, or Dex plus IL2, or Dex Plus IL2 and TNF, or Dex plus IL2, TNF and TY101 for 72 hours. A). The representative FCM analysis data. B). Summary data of A. Number in FCM plot indicates the proportion of gated Tregs. C). Representative FCM data of Treg viability. D). Summary data of C. Number in FCM plot dicates the proportion of viable Tregs. Data (Mean ± SEM, 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.

Figure 3

The effect of TNF on viability of WT Treg and TNFR2 deficient Treg

Lymphocyte isolated from WT mice and TNFR2⁻/⁻ mice were cultured with Dex plus IL-2 or Dex plus IL-2 and TNF for 72 hours. A). Representative data of Treg percentage. B). The summary data of A. Number in FCM plot indicates the proportion of gated Tregs. C). The representative FCM analysis data of Treg viability. D). Summary data of C. Number in FCM plot indicates the proportion of the viable Treg. Data (Mean ± SEM, n=3) shown are representatives of five separate experiments with similar results. Comparison of indicated groups. E-H). Mice received Dex treatment once per two days for three time, in the day after the last treatment mice were sacrificed for analysis. E). The schematic diagram of Dex treatment on WT mice and TNFR2 KO mice. F). The representative FCM analysis data. G). Absolute
number of Treg H). The percentage of decreased Treg. Number in FCM plot indicates the proportion of gated Tregs. Data (Mean ± SEM, 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.

Figure 4

Effect of TNFR2 Ab EG7-OVA on tumor growth.

A-D). 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). Mean growth curves of EG7 lymphoma. Data are means ± SEM of 5 mice. (C). EG7 tumor growth curves for each individual EG7 tumor-bearing mouse treated with TY101 or IgG control. D). Survival curves of the EG7 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 also 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

Figure 5

Effect of TY101 on tumor infiltrating Treg and IFN-Y CD8 CTLs.

A-J). Treatment start when the tumor reach tumor size reach about 10mm in diameter, then the tumor tissue was cleaved into single cell for fluorescence-activated cell sorting (FCM) analysis. The Treg viability were detected by LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit. A to B). Representative FCM analysis result of viability of intratumoral Treg (A) and and the summary data (B) from (A). The number indicate the percentage of viable Treg. Cto E). Representative FCM analysis result (C) and the summary date (D)of tumor infiltrating Treg, the number indicates the percentage of Treg cells in CD4 T cells. E). the absolute number of Treg per gram tumor tissue. F to G). Representative FCM analysis of TNFR2+ cells and summary data in Treg cells from mice described in (FIG.6.C). The number indicates the percentage of TNFR2+ cells in CD4+Foxp3+ cells. H to I) Representative FCM analysis of IFN-γ+ cells in CD8+ cells from mice described in (C). The number indicates the percentage of interferon–positive (IFN-γ+) cells in CD8+...
cells. J). the ratio between CD8 IFN-γ+ cells to Treg, the number indicate the percentage of CD8/Tregs. Data (n = 4 mice) showed the percentage of Treg proportion and Treg viability, as well as the proportion of CTLs and the ratio of CD8/Tregs. *P < 0.05, **P < 0.01, ***P < 0.001

Figure 6

Legend not included with this version

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