

MiR-125b-5p modulates the function of regulatory T cells in tumor microenvironment by targeting TNFR2

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ABSTRACT

Background Tumor necrosis factor receptor type 2 (TNFR2) is primarily expressed by CD4⁺FoxP3⁺ regulatory T cells (Tregs), especially those present in tumor microenvironment. There is compelling evidence that TNFR2 plays a crucial role in the activation, expansion, and phenotypic stability of Tregs and promotes tumor immune evasion. Understanding of epigenetic regulation of TNFR2 expression in Tregs may help devise a novel strategy in cancer immunotherapy.

Methods MiR-125b-5p-overexpressing or knockdown murine CD4 T cells and Tregs were constructed, and the effect of miR-125b-5p on Tregs proliferation, suppressive function and TNFR2 expression were examined. In vivo antitumor efficacy of Ago-125b-5p (miR-125b-5p agomir) was evaluated in MC38 tumor bearing mice, and tumor-infiltrating Tregs and CD8⁺ cytotoxic T lymphocytes (CTLs) were analyzed. RNA-seq analysis was applied to reveal the genes and signaling pathways regulated by miR-125b-5p in Tregs.

Results In this study, we found that TNFR2 was a direct target of miR-125b-5p. Overexpression of miR-125b-5p decreased the proportion of Tregs and their expression of TNFR2 and consequently inhibited its proliferation and suppressive function by regulating the metabolism-related signaling pathways. Moreover, in colon cancer bearing mice, the administration of Ago-125b-5p markedly inhibited the tumor growth, which was associated with reduction of Tregs and increase of IFN γ ⁺CD8⁺ T cells in tumor environment. Furthermore, in human colon adenocarcinoma patients, we verified that miR-125b-5p expression was downregulated, and low levels of miR-125b-5p were associated with poor prognosis. Interestingly, the expression of miR-125b-5p and TNFR2 were negatively correlated.

Conclusions Our study for the first time found that the expression of TNFR2 by Tregs was regulated by miR-125b-5p. Our results showed that miR-125b-5p had the capacity to inhibit the expression of TNFR2 and immunosuppressive activity of Tregs and consequently enhanced the antitumor efficacy. This property of miR-125b-5p may be therapeutically harnessed in the treatment of human cancers.

BACKGROUND

Overcoming the immunosuppressive tumor microenvironment (TME) is a prerequisite for an effective immunotherapy against tumor.¹

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Tumor necrosis factor receptor type 2 (TNFR2), one of two receptors transducing TNF biological function, preferentially expressed by the most highly suppressive CD4⁺FoxP3⁺ Tregs, including those in the tumor environment. TNFR2 signals play decisive role in the activation, expansion, function and phenotypical stability of Tregs. There is compelling evidence that targeting of TNFR2 represents a novel strategy in enhancing the efficacy of tumor immunotherapy by eliminating Treg activity.

WHAT THIS STUDY ADDS

⇒ We found TNFR2 was a direct target of miR-125b-5p in Tregs. Overexpression of miR-125b-5p markedly inhibited TNFR2 expression and suppressive function of Tregs. Furthermore, in vivo treatment with miR-125b-5p agomir showed the capacity to inhibit tumor growth in mouse models, accompanied by the decrease of Tregs and increase of CD8 cytotoxic T lymphocytes (CTLs) in tumor tissues.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ These findings for the first time provide experimental evidence that miR-125b-5p could directly regulate TNFR2 expression and suppressive function of Tregs, and this property of miR-125b-5p may be harnessed to design novel cancer immunotherapy.

The accumulation of CD4⁺FoxP3⁺ regulatory T cells (Tregs) is largely attributable to the maintenance of immunosuppressive environment in tumor tissue,^{2–4} and consequently, the abundance of Tregs is associated with poor prognosis of broad spectrum of human cancers, including colorectal cancer,⁵ lung cancer,⁶ and breast cancer.⁷ Targeting of Tregs has become a strategy to enhance the efficacy of cancer immunotherapy.

MicroRNAs (MiRNAs) are a series of highly conserved single-stranded nucleotide (20–25 nt) non-coding RNAs. The function of miRNAs is mediated by binding

of RNA-induced silencing complex to complementary sequences of 3' untranslated region (3'UTR) in the target mRNA, then inhibit the gene expression at post-transcriptional level.^{8–10} Through its regulation, miRNAs have been shown to act as oncogenes or tumor suppressors in cancer development and play critical roles in the regulation of immune responses.^{11,12} Increasing evidence demonstrated that miRNAs could regulate the development and suppressive function of Tregs. For example, miR-142-3p could impair the differentiation of Tregs and reduce Foxp3 stability in models of type 1 diabetes by directly downregulating TET2 expression.¹³ miR-21, a key regulator of Treg stability, is more highly expressed by Treg cells and can regulate Foxp3 expression.^{14,15}

Our group for the first time found and reported that TNF potently promotes the activation and expansion of Tregs by interacting with TNFR2, one of TNF receptors that expressed by highly suppressive subset of Tregs.^{16,17} We also reported that tumor-infiltrating Tregs express markedly higher levels of TNFR2 as compared with peripheral Tregs.¹⁸ A recent single-cell RNA-sequencing study showed that in patients with metastatic melanoma, TNFR2 is one of the most upregulated genes in Tregs.¹⁹ Upregulation or downregulation of TNFR2, therefore, can modulate Treg activity for therapeutic purpose.

Recent studies showed that TNFR2 could be regulated by miRNAs. For example, protumor TNFR2 signal in gastric cancer tissue may be regulated by miR-19a and miR-103a.²⁰ Downregulation of TNFR2 by Let-7f-5p was reported to be the molecular mechanism underlying the osteoprotective effects of *plastrum testudinis* extracts,²¹ while miR-125a-5p was purported to be a positive regulator of osteoclast formation by targeting TNFR2.²² However, the epigenetic regulation of TNFR2 expression in Tregs remains elusive.

In this study, we first predicted several potential miRNAs with pseudo binding sites in the 3'UTR of TNFR2 and found that TNFR2 was a direct target of miR-125b-5p. Through transfection of miR-125b-5p mimic or inhibitor (anti-miR-125b-5p), the effects of miR-125b-5p on TNFR2 expression by Tregs and on the proliferation and inhibitory function of Tregs were examined. Moreover, the antitumor efficacy of chemically synthesized miR-125b-5p analog (Ago-125b-5p) was determined in murine tumor models. The results of our study indicate that miR-125b-5p reduced the function of Treg cells by downregulation of TNFR2. This property of miR-125b-5p may be therapeutically harnessed in the treatment of human cancers.

MATERIALS AND METHODS

Clinical samples

A total of 31 pairs of colon adenocarcinoma (COAD) tissues and matched adjacent normal tissues were acquired from Tianjin Medical University Cancer Institute and Hospital (Tianjin, China). The patients were clinically and histopathologically diagnosed as COAD according to

WHO criteria. None of the patients have received radiotherapy and chemotherapy before enterectomy.

Mouse, cells and reagents

Female wide type C57BL/6J mice (6–8 weeks old) were provided by the Animal Facility of University of Macau. The mouse colon cancer cell lines of CT26 and MC38 were purchased from American Type Culture Collection (ATCC). The flow cytometry fluorescent conjugated antibodies of FITC anti-mouse CD45 (30-F11), and PE-Cyanine7 anti-mouse CD4 (GK1.5) were purchased from eBioscience; PerCP-Cy5.5 anti-mouse TCR β /CD3 (H57-597), PE anti-mouse IFN- γ (XMG1.2), and APC anti-mouse CD8a (53–6.7) were purchased from BD Pharmingen; PE anti-mouse CD120b/TNFR2 (TR75-89) was purchased from BioLegend, and APC anti-mouse Foxp3 (FJK-16s) for intracellular staining of Foxp3 was purchased from Invitrogen. Recombinant mouse IL-2 (200 μ g/mL) and TNF (200 μ g/mL) were obtained from BD Pharmingen. The miRNeasy Mini kit (Cat# 217004) was purchased from QIAGEN, and the PrimeScript RT reagent kit (Cat# RR047A.) was purchased from TAKARA.

Bioinformatic analysis

The online software of Targetscan (<http://www.targetscan.org/>), miRDB (<http://mirdb.org/>), miRWalk (<http://mirwalk.umm.uni-heidelberg.de/>) and Starbase V.3.0 (<http://starbase.sysu.edu.cn/>) were used to predict potential miRNAs that target TNFR2 and the binding sites of miRNA in TNFR2 mRNA.

T cell purification and in vitro culture

Mouse CD4⁺ T cells were purified from spleen and lymph nodes using CD4 (L3T4) microbeads. CD4⁺CD25⁺ T cells were purified using the mouse CD4⁺CD25⁺ Regulatory T Cell Isolation Kit (Cat# 130091041, Miltenyi Biotec), yielding a purity of ~90% for CD4⁺FoxP3⁺ Treg cells. The purified cells were cultured in a U-bottom 96 well plate in a medium of RPMI1640 supplemented with 10% FBS, 2mM L-glutamine, 10mM HEPES buffer, 0.1mM non-essential amino acids, 1mM sodium pyruvate, 1% penicillin (100 U/mL)/streptomycin (100 mg/mL), and 50 μ M 2-Methylmercaptoethanol, at 37°C in a humidified incubator with 5% CO₂. For T cells culture, IL-2 (10 ng/mL, BD Pharmingen) was added into the culture medium to maintain the survival of CD4⁺ T cells, and TNF (10 ng/mL, BD Pharmingen) was added to activate the Tregs expansion and proliferation.

MiRNA transfection

The mimics of miR-125b-5p, let-7a-5p, let-7b-5p, let-7c-5p, let-7d-5p, let-7k, and anti-miR-125b-5p and their relative negative control (NC) were chemically synthesized by RiboBio (Guangzhou, China). MiR-125b-5p, anti-miR-125b-5p or five other miRNAs and their negative controls (miR-NC, anti-miR-NC) were transfected into CD4⁺ T cells or CD4⁺CD25⁺ T cells by using riboFECT transfection reagent at a working concentration of 100 nM, following the manufacturer's instructions. The lentiviral

vector GV369 expressing mouse miR-125b-5p (LV-miR-125b-5p) and its empty vector (LV-miR-NC) were generated by Genechem (Shanghai, China). The CD4⁺CD25⁺ T cells were transfected with lentivirus at a multiplicity of infection (MOI) of 30 according to the manual protocol. All cells were prepared for analysis after 48 to 72 hours of transfection.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA and miRNA were extracted and purified with miRNeasy Kit (Qiagen), and 0.5 µg of RNA was transcribed into cDNA using PrimeScript RT reagent kit with DNA erase, according to the manufacturer's instructions. The Bulge-loop miRNA qRT-PCR primer sets for the quantification of miR-125b-5p and other miRNAs were designed and synthesized by RiboBio. The quantitative RT-PCR detection for mRNA and miRNA using TB Green Premix Ex Taq II (Takara). β-actin and U6 were used as the normalization control for mRNA and miRNA. Primers used in this study were as follows: β-actin (forward: 5'-GCTTCTTTGCAGCTCCTTCGT-3' and reverse: 5'-CCTTCTGACCCATTCCCACC-3'); TNFR2 (forward: 5'-ACAGTGCCCCCAGGTTGTCTTG-3' and reverse: 5'-GAAATGTTTCACATATTGGCCAGGAGG-3'); Foxp3 (forward: 5'-CTTATCCGATGGGCCATCTG-3' and reverse: 5'-GTGGAAACCTCACTTCTTGTC-3'). Data analysis were performed using the 2^{-ΔΔC_T} methods.

Dual-luciferase reporter assay

293T cells (2×10⁵) were seeded in a 24 well plate, then transfected with wildtype pGL3-TNFR2 3'-UTR (WT) or mutant pGL3-TNFR2 3'-UTR (MUT) and pRL-TK renilla luciferase plasmid with miR-125b-5p mimic, or miR-NC using the lipofectamine 3000 reagent (Invitrogen) according to the manufacturer's instructions. After 48-hour culture, the relative luciferase activity was determined by the dual-luciferase reporter assay system (Promega); the renilla luciferase value was used as the normalization for firefly luciferase value.

Cell proliferation assay

For in vitro assays of suppression of proliferation by Tregs, CD4⁺FoxP3⁺ Tregs (0.5~1×10⁵ cells/well) were seeded in a U-bottom 96-well plate, cultured in complete RPMI-1640 medium with 10 ng/mL IL-2 and 10 ng/mL TNF, then transfected with lentivirus of miR-125b-5p (LV-miR-125b-5p) or scramble control (LV-miR-NC) for 2 days. CFSE-labeled CD4⁺CD25⁻ effector T cells (Teff, 5×10⁴ cells/well) were cocultured with 2×10⁵ irradiated (3000Rad) APCs (Antigen-presenting cells, T lymphocytes depleted splenocytes) and 0.5 µg/mL of soluble antimouse CD3 antibody, then Tregs were added to the wells at a desired ratio (Teff: Treg=1:1, 2:1, 4:1, 8:1). After 48~72 hours, CFSE dilution was determined by flow cytometry. The percentage of suppression by Teff/ Treg ratio was calculated by the following formula: [Y (Teffs alone) - Y (Teffs+Tregs)] / Y (Teffs alone) × 100. The Y value represents the average number of divided Teff cells.

RNA sequencing (RNA-seq)

Purified CD4⁺CD25⁺ Treg cells were cultured in RPMI1640 complete medium containing 10 ng/mL IL2 and 10 ng/mL TNF, then infected with GFP labeled lentivirus of miR-125b-5p (LV-miR-125b-5p) or scramble control (LV-miR-NC), respectively. After 2 days, FACS-sorted CD4⁺FoxP3⁺GFP⁺ Tregs and total RNA were also isolated. RNA sequencing libraries preparation and sequencing were conducted at Shanghai Genechem Co, Ltd. RNA libraries were generated using NEBNext UltraTM RNA Library Prep Kit for Illumina (NEB, USA) following manufacturer's recommendations, and sequencing were performed on an Illumina Novaseq platform. For transcriptome sequencing, FPKM can be used as gene expression data.

Treatment of MC38 mouse model and tumor tissue processing

C57BL/6J mice (female, 6~8 weeks old) were subcutaneously implanted with 0.1 mL PBS containing 5×10⁵ MC38 tumor cells in the right flank. When tumor size reached 50~100 mm³, the tumor-bearing mice were randomly divided into two groups (n=5 mice/group), then intratumorally injected with chemical synthetic analogs of miR-125b-5p (Ago-125b-5p) or its negative control Ago-NC (2.5 nmol/mice, respectively) twice a week for a total of five doses. The tumor size (length and width) and body-weight of tumor-bearing mice were monitored every 3 days. The tumor size was calculated by the formula: (length×width²)/2. After indicated treatments, the tumors were excised, minced and digested in RPMI1640 medium containing collagenase IV (100 U/mL) and 0.1 mg/mL DNase, and draining lymph nodes (dLNs) or spleen were minced and pushed into a 70 µm cell strainer to create single cell suspensions. Finally, cells were washed using PBS and stained with specific diluted antibodies to determine the contribution of lymphocytes to the development of antitumor immune defense.

Inflammation cytokines assay

Mouse inflammation cytokines were detected using the mouse inflammation kit by BD Cytometric Bead Array, including mouse IL-6, IL-10, MCP-1, IFN-γ, TNF and IL-12p70. For cytokines staining, 10 µL of mouse serum from treated mice were added 10 µL of each mouse inflammation capture bead, 10 µL PE detection reagent and 20 µL PBS, then incubated at room temperature and prevented light for 2 hours. Reconstitute mouse inflammation standards by serial dilutions were used to make a standard curve. Finally, flow cytometry analysis was performed to detect the cytokines level, then convert to concentrations according to the calibration curve.

Quantitation of soluble TNFR2 by ELISA assay

A mouse TNFR2/TNFRSF1B ELISA kit was obtained from SinoBiological Inc (Cat# KIT50128, Beijing, China) for the quantitative detection of mouse soluble TNFR2 in peripheral blood serum from mice treated with Ago-125b-5p or Ago-NC. It is based on a Sandwich assay

principle and carried out according to the manufacturer's specifications. The concentration of mouse TNFR2 was calibrated according to the standard curve. All samples and standards were assayed in duplicate.

Intracellular staining and flow cytometry

After blocking non-specific FcR, cells were incubated with appropriately diluted antibodies according to the manufacturer's instructions. For intracellular staining of Foxp3, the cells were resuspended in fixation/permeabilization buffer; for IFN- γ staining, the cells were cultured and stimulated by PMA (phorbol 12-myristate 13-acetate) and ionomycin, then resuspended in fixation/permeabilization buffer. All cells acquisition was performed using a BD Fortessa cytometer (BD Biosciences), and data were analyzed by FlowJo software (V.11.0). FACS analysis was gated on the live cells only by using a LIVE/DEAD Fixable Dead Cell Stain kit.

Statistical analysis

GraphPad Prism software (V.8.3.0) was used for data analysis. The statistical significance between two groups was analyzed by Student's t-test, and the multiple groups' comparison was analyzed by one-way analysis of variance. The results data were represented as mean \pm SD (SEM) of multiple experiments. Statistical significance was determined by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

RESULTS

TNFR2 is a direct target of miR-125b-5p

To determine the effect of miR-125b-5p on TNFR2, online resource in websites including Targetscan, miRDB, miRWalk and Starbase V.3.0 were used to predict the putative binding of miRNAs to 3' untranslated region (3'UTR) of TNFR2. There are six common potential miRNAs obtained from the analysis of four databases, as summarized in [figure 1A](#). The results suggested that several miRNAs might regulate TNFR2 expression. To verify the indeed miRNA that responsible for targeting TNFR2 expression, we examined the expression of miRNAs in CD4⁺ T cells. As shown in [figure 1B](#), the expression of miR-125b-5p by CD4⁺ T cells was significantly lower as compared with five other miRNAs. Then we synthesized and transfected these six miRNAs mimic into CD4⁺ T cells and found that the proportion of TNFR2⁺ Tregs were markedly reduced. Interestingly, the overexpression of miR-125b-5p was much more potent in the inhibition of TNFR2 expression, in comparison with overexpression of five others ([figure 1C](#), $p < 0.01$). The 3'UTR of TNFR2 containing the complementary binding sites within miR-125b-5p was shown in [figure 1D](#). In addition, the luciferase activities were tested in 293T cells transfected with miR-125b-5p mimic, including wildtype (WT) or Mutant (MUT) version of predicted TNFR2 3'UTR binding sites of miR-125b-5p. We observed that the overexpression of miR-125b-5p reduced the luciferase activity in WT 3'UTR of TNFR2 but not mutated type ([figure 1E](#)).

These data suggested that TNFR2 could be directly targeted by miR-125b-5p.

miR-125b-5p downregulates TNFR2 expression on Tregs

Previously, we and others showed that TNFR2 plays a crucial role in the phenotypical and functional stability of Tregs, and TNF-TNFR2 signal stimulates the activation of Tregs and enhances their suppressive activity.^{23 24} Thus, miR-125b-5p may reduce the stability of Tregs through downregulation of TNFR2 expression. To test this idea, CD4⁺ T cells were cultured in the medium containing IL-2 with or without TNF (10 ng/mL each). The result showed that TNF could markedly increase the proportion of FoxP3⁺ Tregs and TNFR2 expression by Tregs (online supplemental figure S1A,B $p < 0.001$), as expected. Then, miR-125b-5p expression in CD4 T cells was overexpressed or suppressed by transfecting with miR-125b-5p mimic or anti-miR-125b-5p, respectively (the transfection efficiency was shown in online supplemental figure S1C). The result showed that the overexpression of miR-125b-5p in CD4 T cells markedly reduced the proportion of FoxP3⁺ Treg cells by 40%, and downregulated their TNFR2 expression by ~40% ([figure 2A,B](#), $p < 0.001$). In sharp contrast, silence of miR-125b-5p expression resulted in a 30% increase in the proportion of Tregs in CD4 T cells and an increase of ~25% TNFR2 expression by Tregs ([figure 2C,D](#), $p < 0.01$), which might be explained by the low levels of miR-125b-5b expression. Interestingly, the transcriptional levels of TNFR2 and FoxP3 expression were also markedly reduced in miR-125b-5p overexpressing Tregs ([figure 2E](#), $p < 0.0001$), while their expression were increased by the treatment with anti-miR-125b-5p ([figure 2F](#), $p < 0.01$).

Furthermore, overexpression of miR-125b-5p also markedly reduced the proportion of FoxP3⁺ Tregs in TNFR1^{-/-} CD4⁺ T cells by 37%, but it only resulted in a 25% reduction of Tregs in TNFR2^{-/-} CD4⁺ T cells (online supplemental figure S2A,B $p < 0.01$), which suggesting that miR-125b-5p may also downregulate FoxP3 expression in a TNFR2-independent manners. Moreover, overexpression of miR-125b-5p markedly decreased the expression of TNFR2 by TNFR1^{-/-} Treg cells (online supplemental figure S2C,D $p < 0.001$). Therefore, our data clearly indicated that miR-125b-5p could significantly reduce TNFR2 and FoxP3 expression in Tregs.

miR-125b-5p inhibits the proliferation and attenuates the suppressive activity of Tregs

There is compelling evidence that TNFR2 expression by Tregs is required for their immunosuppressive function.^{25 26} Thus, decreased expression of TNFR2 by miR125b-5p may attenuate Treg function. To test this possibility, Treg cells from normal C57BL/6J mice were MACS-purified based on surface expression of CD4 and CD25 and the purity of FoxP3⁺ Tregs >90% ([figure 3A](#)). The proliferation of Tregs was induced by stimulating with IL-2 and TNF as we previously reported.²⁷ Results showed that the overexpression of miR-125b-5p reduced the proliferation of Tregs by 40% ([figure 3B,C](#), $p < 0.01$). In contrast, the proportion of

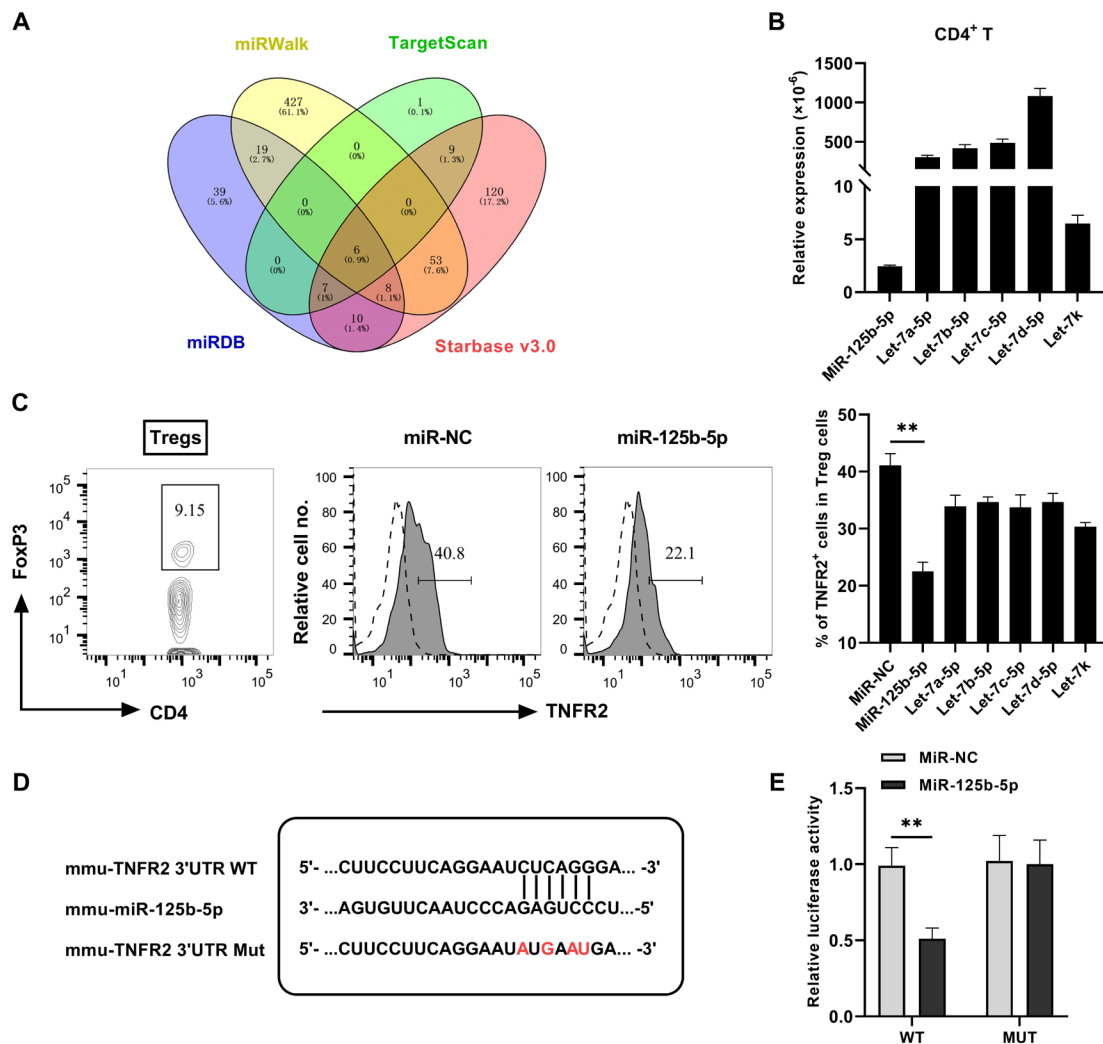


Figure 1 TNFR2 is a direct target of miR-125b-5p. (A) Bioinformatics search through four public databases of miRWalk, TargetScan, miRDB and Starbase V.3.0 predicted six potential microRNAs (let-7a-5p, let-7b-5p, let-7c-5p, let-7d-5p, let-7k, miR-125b-5p) that target TNFR2, as shown by Venn diagram. (B) The relative expression of miRNAs expression in CD4⁺ T cells were examined by qRT-PCR. (C) Flow cytometric plot of the proportion of Tregs in CD4 T cells and TNFR2 expression by Tregs, and summary of the proportion of TNFR2⁺ Tregs after transfected with indicated miRNAs mimic in CD4 T cells. Representative FACS plot on the left, and the quantitative data on the right were presented as mean±SEM of three independent experiments. (D) Schematic presentation of miR-125b-5p binding sites in the 3'UTR regions of TNFR2. Sequences below indicate the mutant form of this site (red mark). (E) Dual luciferase reporter assay in 293T cells showed miR-125b-5p inhibit the relative luciferase activity of TNFR2 vector-3'UTR (WT) compared with negative control, while miR-125b-5p had no obvious effect on mutant TNFR2 vector-3'UTR (MUT) (n=3, mean±SEM). Comparison of indicated groups, *p<0.05, **p<0.01 (Student's t-test). TNFR2, tumor necrosis factor receptor type 2; WT, wildtype.

proliferating Tregs was increased by 30% after the silence of miR-125b-5p (figure 3D,E, p<0.01). However, there was no difference in cellular viability between miR-125b-5p or anti-miR-125b-5p treatment and its controls (online supplemental figure S3). Therefore, we assumed that altered proliferative expansion may result in an increase or decrease in the number of Tregs, which will affect its function. Besides, transfection of miR-125b-5p or anti-miR-125b-5p did not alter the activation and proliferation of CD4⁺CD25⁻ Teff cells (online supplemental figure S4A–E) and even CD8 T cells (data not shown).

To examine the effect of miR-125b-5p on the suppressive function of Tregs, CD4⁺CD25⁺ Treg cells were infected with LV-miR-125b-5p and cocultured with

CFSE-labeled Teff cells. The result showed that the suppressive activity of miR-125b-5p-overexpressing Treg cells was markedly reduced (figure 3E,G, p<0.05), indicating that miR-125b-5p could regulate the function of Tregs which is at least partially resulted from the down-regulation of TNFR2. Moreover, consistent with previous report, TNF treatment markedly reduced PD-1 expression but increased CTLA4 expression in Tregs (online supplemental figure S5A p<0.05). However, overexpression of miR-125b-5p markedly reduced CTLA4 expression, but increased PD-1 expression in Tregs (online supplemental figure S5B, p<0.01). In contrast, silence of miR-125b-5p showed an opposite effect (online supplemental figure S5C, p<0.05). Therefore, miR-125b-5p may

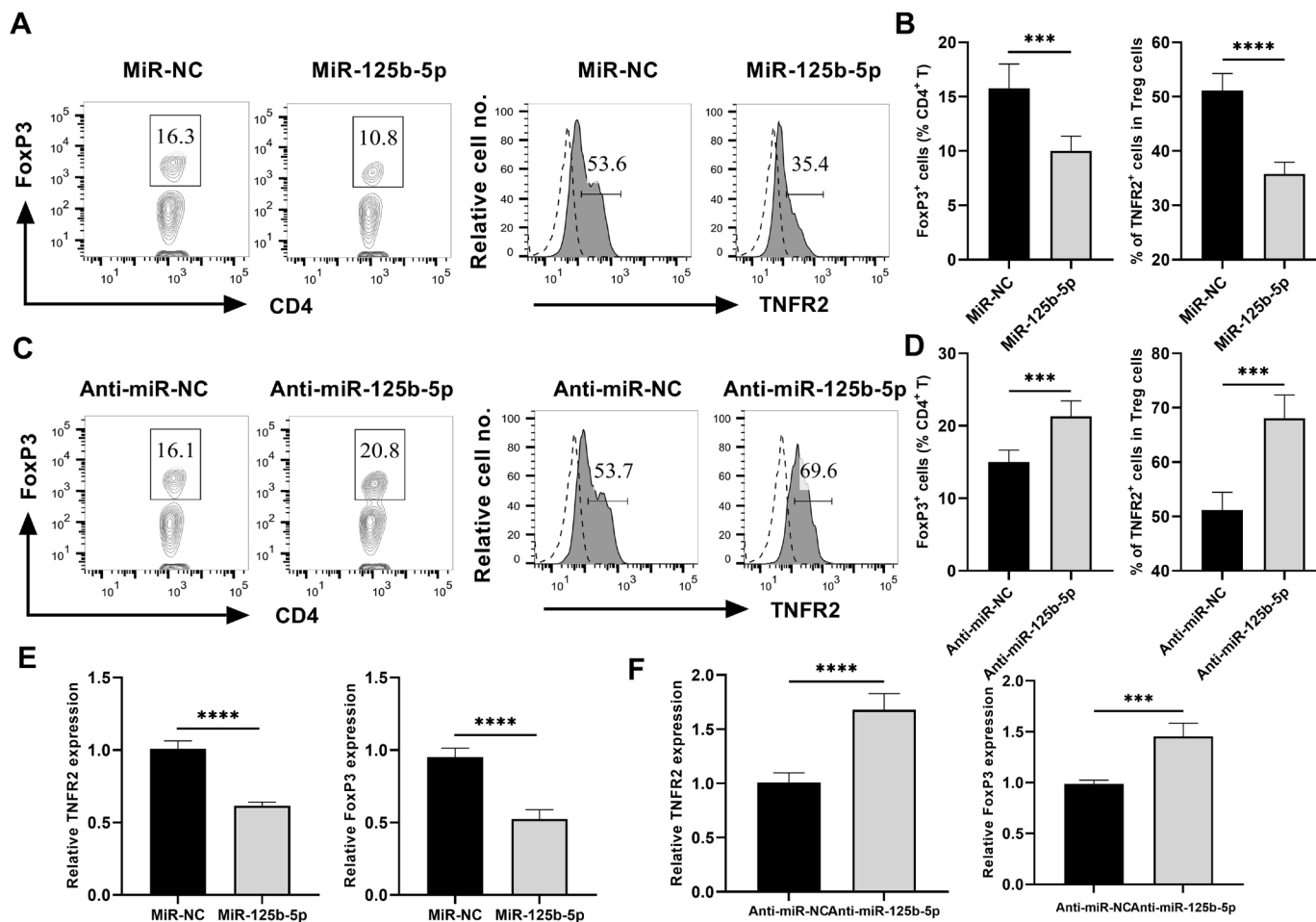


Figure 2 miR-125b-5p downregulates TNFR2 expression on Tregs. MACS-purified CD4⁺ T cells from pooled lymph nodes and spleen of wide type C57BL/6J mice were cultured in the presence of IL-2 and TNF (10 ng/mL each), then cells were transfected with 100 nM miR-125b-5p mimic/inhibitor or their relative negative control (NC). Flow cytometric plot (A) and summary (B) of the proportion of FoxP3⁺ Tregs in CD4⁺ T cells and TNFR2 expression by Tregs after transfected with miR-125b-5p. Number shows the proportion of gated cells. Flow cytometric plot (C) and summary (D) of the proportion of Tregs in CD4⁺ T cells and TNFR2 expression by Tregs after transfected with anti-miR-125b-5p. Number shows the proportion of gated cells. The mRNA expression of TNFR2 and FoxP3 by Tregs after transfected with miR-125b-5p (E), and anti-miR-125b-5p (F) were examined through qRT-PCR. Representative FACS data were from three independent experiments with similar results. The summarized data shown in figure parts B, D, E and F were presented as mean ± SEM of three independent experiments performed in triplicate (n=9). By comparison with miR-NC (or anti-miR-NC), **p<0.01, ***p<0.001, ****p<0.0001 (Student's t-test). TNFR2, tumor necrosis factor receptor type 2.

preferentially regulate the proliferation and function of Treg cells.

miR-125b-5p inhibits metabolism-related signaling pathways in Tregs

To further understand the molecular basis of regulatory effect of miR-125b-5p on Tregs, transcriptome sequence of LV-miR-125b-5p transfected Tregs showed a differentially expressed genes pattern (figure 4A), including downregulation of 112 (fold change <0.5), and upregulation of 218 genes (fold change >2) (figure 4B). As shown in figure 4C, the most upregulated genes include *Tnfrsf4*, *Bmp1*, *Hs3st1*, *Cd80*, *Gpr162*, and the downregulated genes include *Lag3*, *Ccl1*, *Pgk1*, *Cxcl9*, *Itga1*, etc. The GO and KEGG pathway analysis revealed that the differentially expressed genes mostly involved in glycolysis, carbon metabolism, and HIF-1 signaling pathways, which are known regulators of cell

metabolism (figure 4D). In addition, GSEA analysis showed that the expression of miR-125b-5p affect immune responses to tumor cell progression (figure 4E). Taken together, these findings further shed light on the important roles of miR-125b-5p in regulating the function of Tregs.

Antitumor effect of Ago-125b-5p is associated with reduction of TNFR2-expressing Tregs in murine MC38 tumor models

To further determine the effect of overexpression of miR-125b-5p on tumor growth, C57BL/6J mice bearing established subcutaneous MC38 tumors were intratumorally injected with Ago-125b-5p, the growth of tumors was monitored. The result showed that Ago-125b-5p treatment significantly reduced the tumor growth (figure 5A,B, p<0.05) and potently elongated the tumor-bearing mice survival (figure 5C, p<0.05). The treatment with Ago-125b-5p resulted in a marked increase in the infiltration

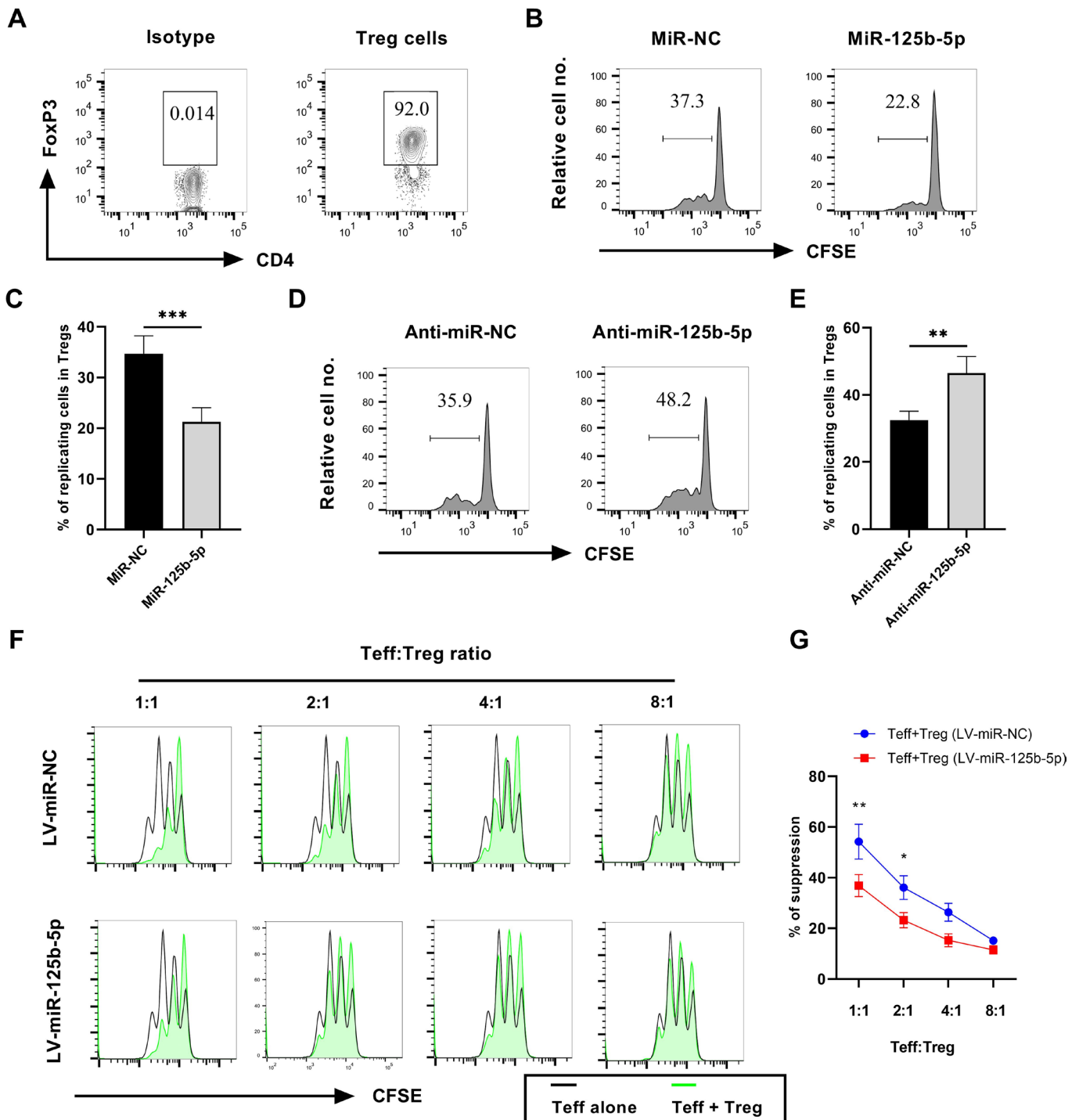


Figure 3 MiR-125b-5p inhibits the proliferation and suppressive activities of Tregs. MACS-purified $CD4^+CD25^+$ T cells from pooled lymph nodes and spleen of wide type C57BL/6J mice were cultured in the presence of IL-2 and TNF (10 ng/mL each), then transfected with 100 nM miR-125b-5p mimic/inhibitor. The proliferation of Tregs were assessed by in vitro CellTrace CFSE assay. (A) The purity of FoxP3⁺ Tregs in $CD4^+CD25^+$ T cells was examined by flow cytometry. Number shows the proportion of gated cells. Typical FACS analysis (B) and summary (C) of Tregs proliferation, as shown by dilution of CFSE expression (gated on FoxP3⁺ Tregs) by transfected with miR-125b-5p. Number in the histogram indicates the proportion of gated cells. Typical FACS analysis (D) and summary (E) of Tregs proliferation, as shown by dilution of CFSE expression (gated on FoxP3⁺ Tregs) by transfected with anti-miR-125b-5p. Number in the histogram indicates the proportion of gated cells. Furthermore, suppressive activity of miR-125b-5p overexpressed Tregs cocultured with CFSE-labeled $CD4^+CD25^-$ Teff cells at desired ratios was measured at day 2, using an irradiated (3000 rad) APCs and soluble CD3-specific antibody assay. (F) The representative CFSE profile of Teff cells at different Teff: Treg ratios (1:1, 2:1, 4:1, 8:1) as shown in the histogram. (G) The percentage of suppression at different Teff:Treg cell ratios as shown. Representative FACS data were from three independent experiments with similar results. The summarized data shown in figure parts C, E and G were presented as mean \pm SEM of three independent experiments performed in triplicate (n=9). By comparison with miR-NC (or anti-miR-NC), *p<0.05, **p<0.01 (Student's t-test).

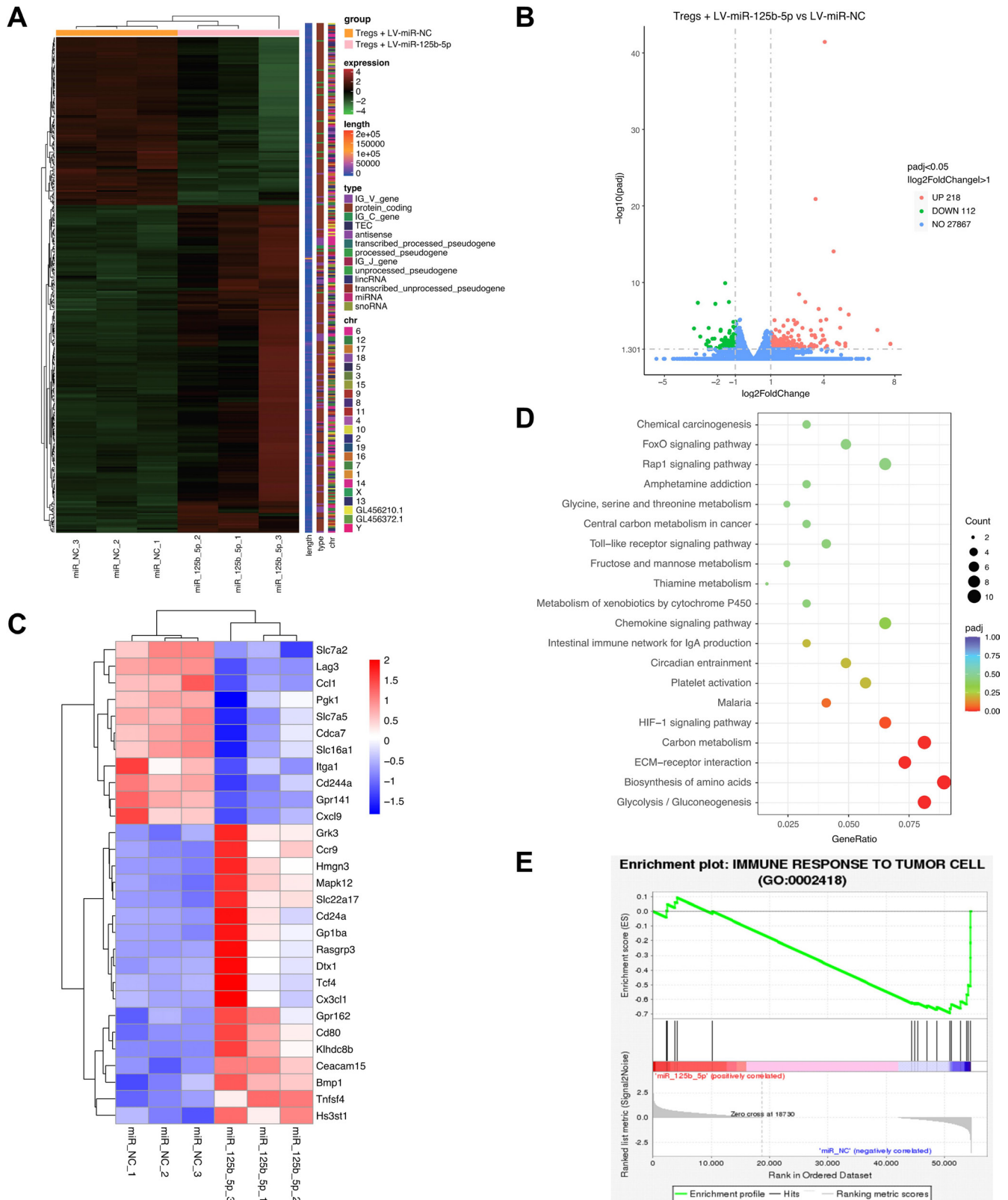


Figure 4 RNA-seq analysis of differentially expressed genes in miR-125b-5p transfected Tregs. Purified CD4⁺CD25⁺ Treg cells were transfected with 100 nM LV-miR-125b-5p or its negative control (LV-miR-NC) for 2 days, then transcriptome sequencing was performed. (A) The differentially expressed genes in miR-125b-5p overexpressed Tregs compared with control cells (n=3, each group) were shown in the heatmap. (B) The differentially expressed genes were shown in volcano plot. X-axis means log₂ foldchange; Y-axis means $-\log_{10}$ pvalue. Gray dotted line represents the screening standard threshold of different expressed genes. (C) The selected most upregulated and downregulated genes were shown in heatmap. (D) KEGG pathway analysis of enriched differentially expressed genes. (E) Gene Set Enrichment Analysis (GSEA) using Tregs RNA-seq data showed that miR-125b-5p expression levels affect regulation of immune response to tumor cell progression.

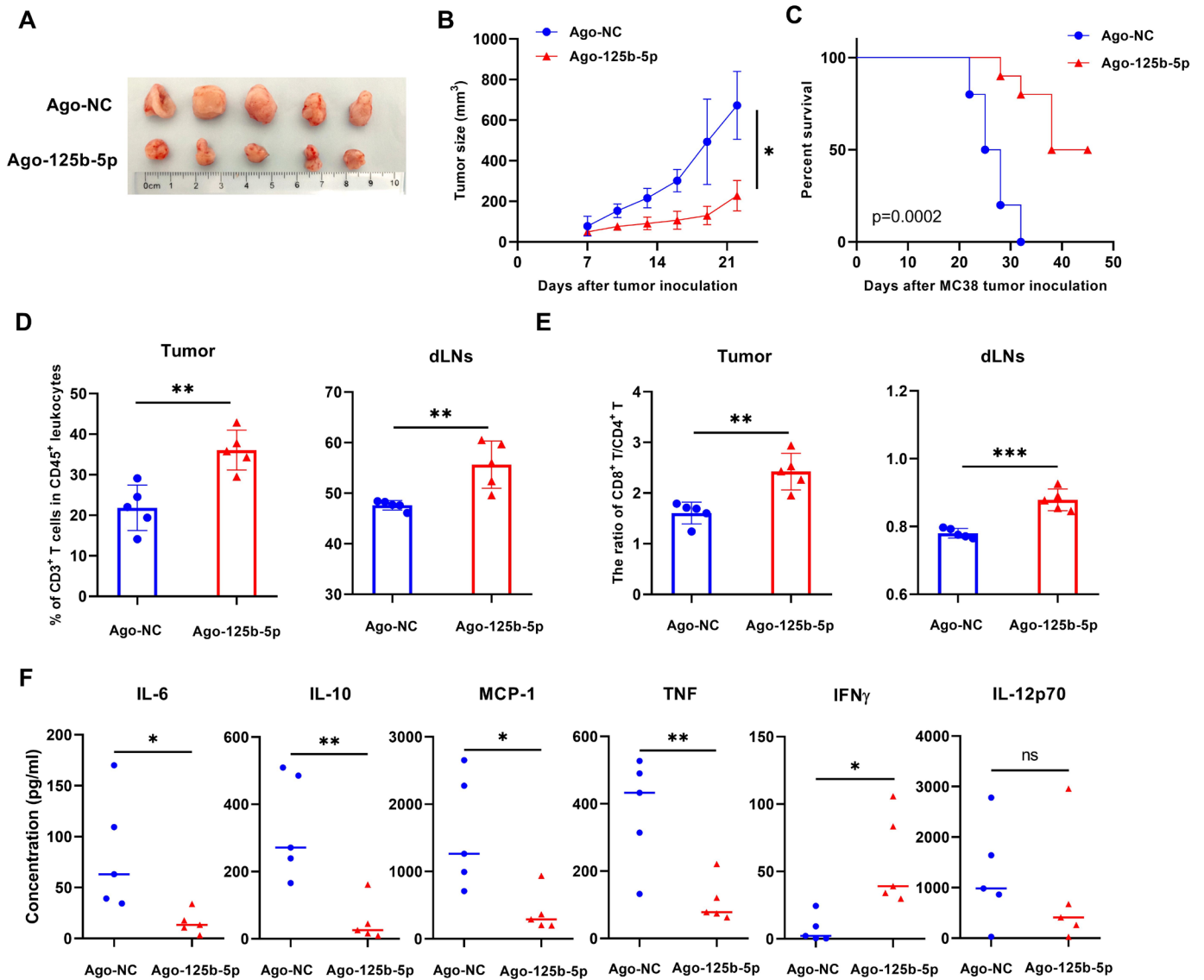


Figure 5 Therapeutic effect of Ago-125b-5p on MC38 tumor bearing mice models. C57BL/6J mice were subcutaneously injected with 5×10^5 MC38 tumor cells each mouse on the right flank on day 1. From day 7, MC38 tumor bearing mice with intratumor injections of Ago-125b-5p or Ago-NC (control) every other day at 2.5 nmol/mouse as indicated for total of five doses. (A) Representative tumor size from each mouse was shown (n=5, each group). (B) The tumor growth curve was plotted (n=5, each group). (C) The tumor bearing mice survival curve (n=10 mice, each group) from two independent experiments. After the last treatment, the tumor tissue, draining lymph nodes and periphery blood serum from each mouse were collected. The tumor tissue and draining lymph nodes were digested and grinded into single cell suspensions as indicated. (D) Summary of the percentage of CD3⁺ T cells infiltrated in tumor tissue and draining lymph nodes. (E) The ratio of CD8⁺:CD4⁺ T cells in tumor tissue and draining lymph nodes. (F) The serum levels of inflammatory cytokine IL-6, IL-10, MCP-1, TNF, IFN γ and IL-12p70 from Ago-125b-5p treated mouse. The summarized data shown in figure parts D, E, and F were displayed as mean \pm SEM, n=5 mice of each group. All data were representatives of three separate experiments. By comparison with Ago-NC, *p<0.05, **p<0.01, ***p<0.001, (Student's t-test).

of CD3⁺ T lymphocytes into the tumor and draining LNs (figure 5D, p<0.01), while the ratio of CD8 versus CD4 T cells was increased in the tumor tissue as well as draining LNs (figure 5E, p<0.01). Furthermore, the treatment with Ago-125b-5p markedly reduced the serum levels of IL-6, IL-10, TNF and MCP-1, while levels of IFN γ were increased in tumor bearing mice (figure 5F, p<0.05).

We further analyze Tregs and their TNFR2 expression in tumor environment and found that the proportion of Foxp3⁺ Treg cells in tumor-infiltrating CD4⁺ T cells was

significantly decreased in mice treated with Ago-125b-5p (figure 6A,C, p<0.001), accompanied by a marked decrease of TNFR2 expression by Treg cells (figure 6B,D, p<0.001). Intriguingly, the soluble levels of TNFR2 were reduced in serum in mice treated with Ago-125b-5p (figure 6E, p<0.01). The expression of TNFR2 was also downregulated in tumor-infiltrating CD8⁺ T cells by Ago-125b-5p treatment (figure 6F, p<0.01), while the treatment increased the expression of IFN- γ in CD8⁺ T cells in tumor tissue (figure 6G,H, p<0.01) and in dLNs (data not shown). These data suggest that the

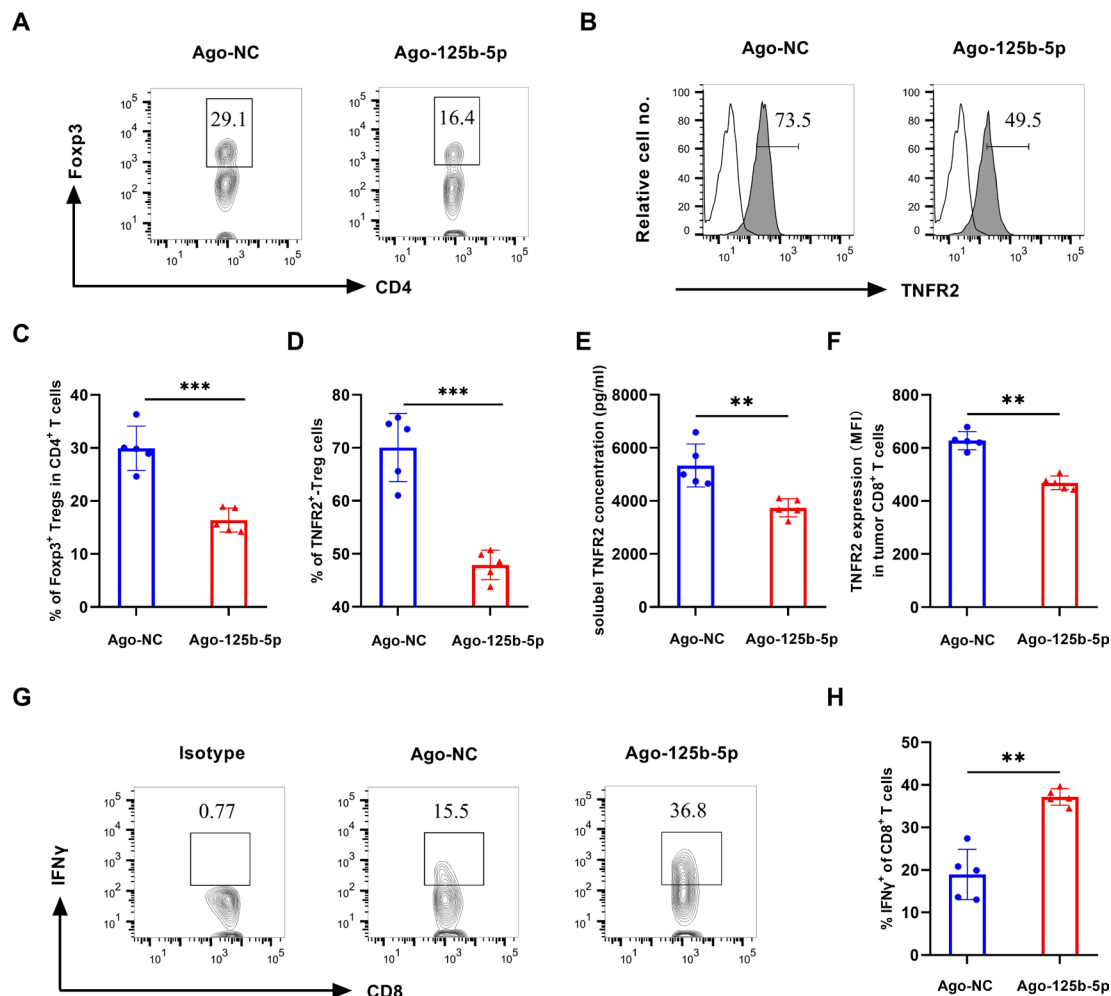


Figure 6 Effect of Ago-125b-5p on the tumor infiltration Tregs and TNFR2 expression and on cytotoxic CD8 T cells. After treatment by Ago-125b-5p for five doses on MC38 tumor-bearing mice, the immune cells in tumor tissue were detected by flow cytometry. (A) Representative flow cytometric plot of the proportion of Tregs in CD4⁺ T cells in mouse tumor tissue after treated by Ago-125b-5p. Number shows the proportion of gated cells. (B) Representative typical histograms of TNFR2 expression in Fopx3⁺ Tregs. Number in the histogram indicates the proportion of gated cells. (C) Summary of the proportion of Fopx3⁺ Tregs in CD4 populations as shown in figure part A. (D) The expression of TNFR2-expressing cells in Tregs. (E) The concentration of soluble TNFR2 levels in serum after treated by Ago-125b-5p. (F) The mean fluorescence intensity (MFI) of TNFR2 expression by tumor-infiltrating CD8⁺ T cells. (G) Representative flow cytometric plot of the proportion of IFN γ ⁺CD8⁺ T cells in CD8⁺ T cells in mouse tumor tissue after treated by Ago-125b-5p. Number shows the proportion of gated cells. (H) Summary of the proportion of IFN γ ⁺CD8⁺ T cells in CD8⁺ T cells. The summarized data shown in figure parts C, D, E, F and H were displayed as mean \pm SEM, n=5 mice of each group. All data were representatives of three separate experiments. By comparison with Ago-NC, *p<0.05, **p<0.01, ***p<0.001 (Student's t-test). TNFR2, tumor necrosis factor receptor type 2.

reduction of TNFR2-expressing Treg cells and mobilization of CD8⁺ T cells were attributable at least in part to the anti-tumor effect of miR-125b-5p.

Clinical relevance of low miR-125b-5p expression may be associated with high TNFR2 expression in human colon cancer patients

Through the bioinformatics analysis, we found that the mature sequence of miR-125b-5p is highly conserved among most vertebrate species, including human and mouse (figure 7A). To predict putative miRNAs that have binding sites in 3'UTR of human TNFR2, we analyzed the online database (TargetsCan, miRDB and miRWalk). The result indicated that human TNFR2 may also be directly targeted by miR-125b-5p (figure 7B). We also found that

human COAD tumor tissues expressed higher levels of TNFR2 and lower levels of miR-125b-5p, as compared with adjacent normal tissues according to the data from TCGA cohort (figure 7C). Furthermore, qRT-PCR analysis on 31 pairs of colon cancer tumor tissues, we could confirm that miR-125b-5p levels were significantly decreased in tumor tissues (figure 7D, p<0.05), and the relationship between TNFR2 expression and miR-125b-5p in human cancer was shown in figure 7E. χ^2 test showed that low miR-125b-5p was associated with more advanced TNM stage and lymphatic metastasis (online supplemental figure S1). Furthermore, Kaplan-Meier survival analysis indicated that poor prognosis of patients with cancer was associated with low miR-125b-5p expression (figure 7F, p=0.0008). Taken together, these

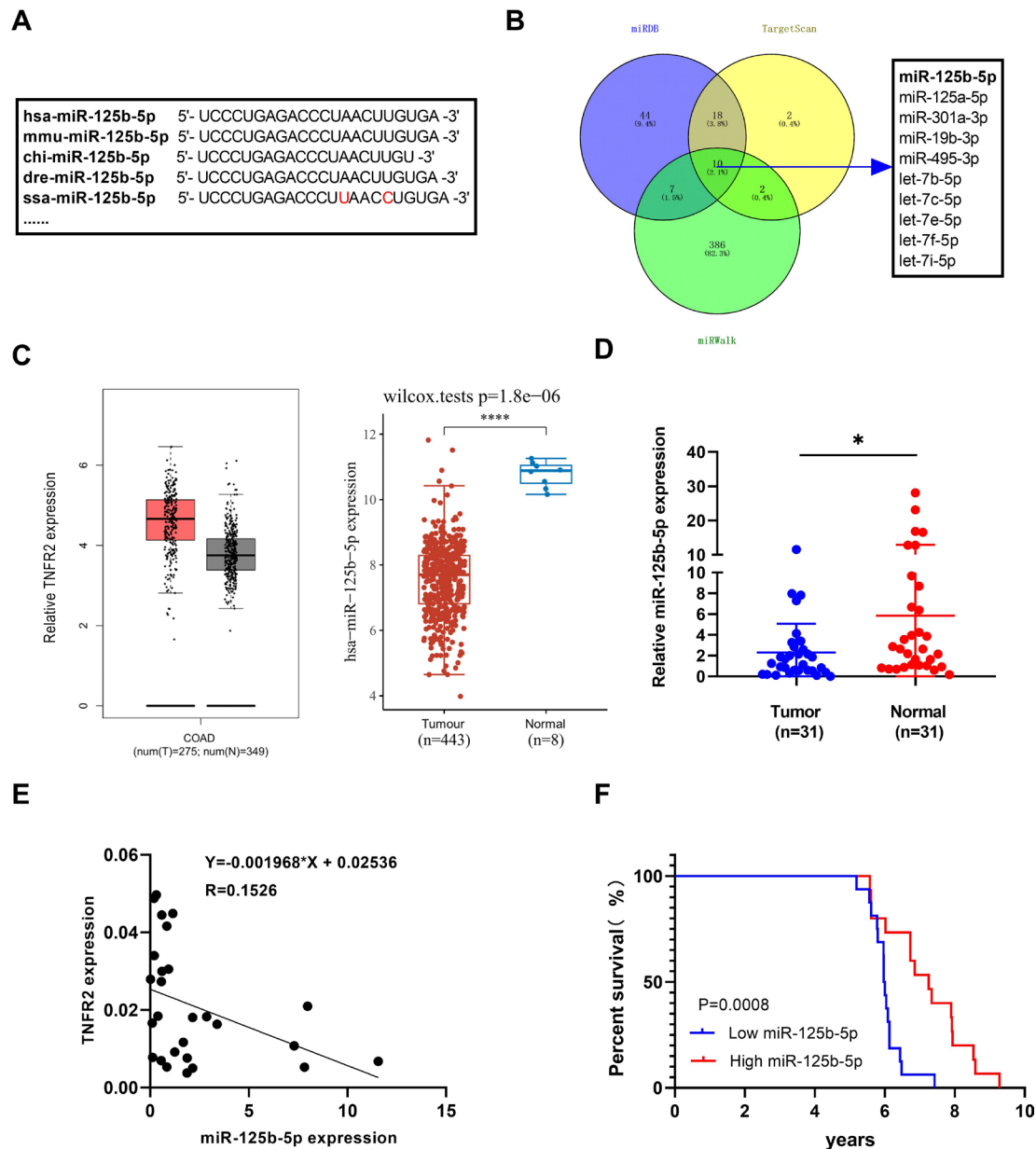


Figure 7 miR-125b-5p is negative associated with TNFR2 expression in human colon cancer patients. (A) The mature sequences of miR-125b-5p in human, mice and other species. (B) Bioinformatics search through public databases of miRWalk, TargetScan and miRDB predicted potential microRNAs that target human TNFR2 3'UTR, as shown by Venn diagram. (C) The relative expression of TNFR2 and miR-125b-5p in human colon adenocarcinoma (COAD) tissues and adjacent normal tissues from TCGA cohort. (D) qRT-PCR analysis of miR-125b-5p levels in human colon cancer patients (n=31; *p<0.05, Student's t-test). (E) The linear regression analysis of correlation between miR-125b-5p and TNFR2 expression in human COAD tissues. (F) miR-125b-5p expression is associated with overall survival (OS) of colon cancer patients (p=0.0008, Kaplan-Meier analysis). TNFR2, tumor necrosis factor receptor type 2.

results suggested that miR-125b-5p was negative correlated to TNFR2 expression in human tumor tissues, aligning well with the results of our in vitro and in vivo laboratory mouse studies.

DISCUSSION

Disruption of the synthesis of miRNA in Tregs results in the complete dysfunction of Tregs and induces the occurrence of inflammatory disease and autoimmunity,²⁸⁻³⁰ indicating that miRNAs are critical in the development

and function of Treg cells. It was proposed that miR-155 plays a role in regulating the activity of induced Treg (iTreg) and natural Treg (nTreg) by suppression of cytokine signaling 1 (a negative regulator of IL-2/STAT5 signaling pathway).³¹ Acting as an immuno-metabolic regulator, miR-142-5p was shown to be essential for controlling Treg suppressive function through the maintenance of high intracellular concentration of cAMP.³² It was also shown that miR-21 was a positive regulator, while miR-31 acting as a negative regulator of human

natural Tregs by modulating Foxp3 expression.¹⁵ Moreover, miR-17 was found to inhibit the function of Tregs by downregulating the coregulators of the Foxp3 transcriptional factor,³³ and NF- κ B-driven miR-34a could disrupt Treg/Th17 balance via targeting Foxp3.³⁴ In this study, we for the first time found that miR-125b-5p could negatively control Treg activity via, at least partially, downregulating TNFR2 expression.

The mature sequence of miR-125b-5p is evolutionarily conserved in diverse vertebrate species, making it an attractive target for translation research since the findings based on mouse study is likely to extrapolate to humans. Previously, the study on miR-125b-5p main focused on human cancer cells, such as hepatocellular carcinoma.³⁵ It was found that the expression of miR-125b-5p was markedly downregulated in tumor cells and acted as a tumor suppressor.^{36 37} Nevertheless, miR-125b-5p was also reported to play a role in the regulation of immune responses. For example, it inhibits the activation and induces the apoptosis of human $\gamma\delta$ T cells.³⁸ In this study, through bioinformatics analysis, we first predicted six miRNAs that may bind with 3'UTR of TNFR2, including miR-125b-5p and five let-7 family members. Among them, miR-125b-5p most potently inhibited TNFR2 expression by CD4 T cells. In view of the potential role of miR-125b-5p in suppressing tumor growth and regulating immune cells, we further investigated the effect of miR-125b-5p on TNFR2 expression by Tregs.

The results of our study suggest that miR-125b-5p directly acts on and negatively regulates TNFR2 expression in Tregs, consequently reduces the number of Tregs and attenuates their suppressive activity. The fact that miR-125b-5p has a similar effect on Tregs in WT and TNFR1-KO T cells, while a minor but a clear effect was observed in TNFR2-KO T cells (online supplemental figure S2A,B), suggesting that miR-125b-5p can regulate Tregs through TNFR2-dependent and TNFR2-independent mechanism. Indeed, it was reported that miR-125b could directly act on Foxp3 and promote autophagy.³⁹

TNFR2 can also be expressed by effector CD8⁺ T cells, which drives a cytotoxic ability to CD8⁺ Teff cells during the early immune response, as well as an apoptosis signal to terminate the immune response.⁴⁰ Besides, the high expression of TNFR2 on cytotoxic CD8 T cells may attenuate their antitumor effector function.¹⁹ Therefore, miR-125b-5p as a direct target on TNFR2 may mitigate the negative effect of TNFR2 expression by CD8 CTLs. This notion is supported by the fact that Ago-125b-5p treatment reduced TNFR2 expression by CD8 T cells in tumor bearing mice, while promoting the effector function of CD8 CTLs as evidenced by the upregulation of IFN γ (figure 6F,G,H). Further study is needed to clarify if the activation of CD8 CTLs in Ago-125b-5p-treated mice is resulted from the down regulation of TNFR2 on CD8 T cells, or from the elimination of Tregs. In TME, myeloid-derived suppressor cells (MDSCs) with potent immunosuppressive function can also express TNFR2 and the signal of TNFR2 can stimulate the accumulation

and activation of MDSCs.^{41 42} Whether miR-125b-5p also regulate the activity of MDSCs through decreasing TNFR2 expression should be further clarified in future study.

The soluble TNFR2 (sTNFR2) is produced by proteolytic cleavage of its membrane bound counterpart. Its level in serum is a potent immunosuppressive mediator and acts as a predictor of treatment response to inflammatory disease.^{43 44} Increased levels of sTNFR2 were reported to be associated with enhanced colorectal cancer risk and significantly accelerated their all-cause mortality.^{45 46} Therefore, serum sTNFR2 might be a powerful predictive factor for cancer treatment. This notion is also supported by the fact that, in our study, sTNFR2 were reduced remarkably by the treatment of Ago-125b-5p in tumor bearing mice, suggesting the reduction of sTNFR2 could be attributable to the mobilization of antitumor immune response.

It was reported that intracellular metabolism played crucial roles in modulating the activation and expansion of Treg cells.^{47 48} The activation of Tregs by TNF-TNFR2 signaling were attributable to stabilize human regulatory T cells switch to glycolysis, with increased intracellular lactate levels and glucose consumption.⁴⁹ To demonstrate the potential molecular mechanism of miR-125b-5p on the activity of Tregs, we performed an RNA-seq analysis in miR-125b-5p overexpressed Tregs and found that miR-125b-5p exerted its roles on Tregs by regulating the glucose and lipid metabolism related signaling pathways, including HIF-1 signaling pathway, carbon metabolism and glycolysis (figure 4). Therefore, the overexpression of miR-125b-5p partially disrupt TNF-TNFR2 signaling on suppressing Treg cells by interfering cellular metabolism related signaling.

To date, several miRNAs-based therapeutics have reached clinical trials, including a miR-34 mimic for the treatment of cancer,⁵⁰ and anti-miR-122 for the treatment of hepatitis.⁵¹ Since miRNAs are small, water soluble and can be injected subcutaneously or intravascularly, targeted delivery of miRNA with nanoparticles, plasmids, viral vectors or lipid delivery vectors into desirable site appears to be feasible and safe.⁵² Our future study will focus on the development of appropriate delivery methods for miR-125b-5p or its modified analogs in a preclinical and then clinical settings.

CONCLUSIONS

In summary, we for the first time found that miR-125b-5p could directly act on TNFR2. Our data favor the notion that downregulation of TNFR2 expression is at least partially attributable to the negative regulation of miR-125b-5p on Treg activity. Therefore, in addition to the known antitumor effect by directly acting on tumor cells, targeting of miR-125b-5p may represent a novel strategy in cancer immunotherapy by eliminating tumor infiltrating TNFR2-expressing Tregs.

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Patient consent for publication Consent obtained directly from patient(s).

Ethics approval All clinical samples were collected from consenting individuals according to the protocols approved by the Ethics Review Board at Tianjin Medical University Cancer Institute and Hospital (No. Ek2021165). The animal study protocol was approved by Animal Research Ethics Committee of University of Macau (#UMARE-AMEND-102). Participants gave informed consent to participate in the study before taking part.

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