Supplementary Materials for

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

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Figure S1. TNF increased the proportion and TNFR2 expression by Tregs.

MACS-purified $CD4^+$ T cells from pooled lymph nodes and spleen of wild type C57BL/6J mice were cultured in the presence of IL-2 with or without TNF (10 ng/ml each). (A) Flow cytometric plot and summary of the proportion of $Foxp3^+$ Tregs in $CD4^+$ T cells in the culture with IL-2 or IL-2 plus TNF. (B) Typical FACS analysis and summary of TNFR2 expression by Tregs (gated on $Foxp3^+$ Tregs) in the culture with IL-2 or IL-2 plus TNF. MACS-purified $CD4^+CD25^+$ Treg cells were transfected with miR-125b-5p mimic or inhibitor, the transfection efficiency was determined by qRT-PCR after transfected with miR-125b-5p (C) and anti-miR-125b-5p (D). Representative FACS data were from three independent experiments with similar results. Summarized data shown in A, B, and C were displayed as mean ± SEM of three independent experiments (n=9). By comparison with miR-NC (or anti-miR-NC), *** $P<0.001$ (Student's t-test).

Figure S2. MiR-125b-5p inhibited the proportion of Tregs in $TNFR1^{-/-}$ and $TNFR2^{-/-}$ mice.

MACS-purified $CD4^+$ T cells from pooled lymph nodes and spleen of $TNFR1^{-/-}$ or $TNFR2^{-/-}$ mice were cultured in the presence of 10 ng/ml IL-2 and 10 ng/ml TNF, then cells were transfected with 100 nM miR-125b-5p or miR-NC. (A) Flow cytometric plot of the proportion of Tregs in $TNFR1^{-/-}$ and $TNFR2^{-/-}$ $CD4^+$ T cells after transfected with miR-125b-5p. Number shows the proportion of gated cells. (B) Summary of the proportion of $Foxp3^+$ Tregs. (C) Representative typical histograms of TNFR2 expression in $Foxp3^+$ Tregs. Number in the histogram indicates the proportion

of gated cells. (D) Summary of the proportion of TNFR2-expressing cells in Tregs. Representative FACS data were from three independent experiments with similar results. Summarized data shown in B and D were displayed as mean ± SEM of three independent experiments (n=9). By comparison with miR-NC, * P<0.05, ** P<0.01, *** P<0.001 (Student's t-test).

Figure S3. Effect of miR-125b-5p on the cell viability of Tregs.

MACS-purified $CD4^+CD25^+$ Treg cells from pooled lymph nodes and spleen from WT C57BL/6J mice were cultured in the presence of 10 ng/ml IL-2 and 10 ng/ml TNF, then cells were transfected with 100 nM miR-125b-5p, anti-miR-125b-5p or their NC. (A) Flow cytometric plot and summary of the live $CD4^+FoxP3^+$ Treg cells after transfected with miR-125b-5p. Number shows the proportion of gated cells. (B) Flow cytometric plot and summary of the live $CD4^+FoxP3^+$ Treg cells after transfected with anti-miR-125b-5p. Number shows the proportion of gated cells. Representative FACS data were from three independent experiments with similar results. Summarized data shown in A and B were displayed as mean ± SEM of three independent experiments (n=9). By comparison with miR-NC. ns, P>0.05, indicate no significance (Student's t-test).

Figure S4. Effect of miR-125b-5p on the proportion and proliferation of Teff cells.

MACS-purified $CD4^+CD25^-$ Teff cells from pooled lymph nodes and spleen from WT C57BL/6J mice were cultured in the presence of 10 ng/ml IL-2 and 10 ng/ml TNF, then cells were transfected with 100 nM miR-125b-5p, anti-miR-125b-5p or their NC.

(A) Flow cytometric plot of the proportion of $CD4^+CD25^-$ Teff cells. Number shows the proportion of gated cells. (B) Typical FACS analysis of Tregs proliferation, as shown by dilution of CFSE expression (gated on $CD4^+CD25^-$ Teffs) by transfected with miR-125b-5p. Number in the histogram indicates the proportion of gated cells. (C) The summary of proportion of replicating Teff cells after transfected with miR-125b-5p. (D) Typical FACS analysis of Tregs proliferation, as shown by dilution of CFSE expression (gated on $CD4^+CD25^-$ Teffs) by transfected with anti-miR-125b-5p. Number in the histogram indicates the proportion of gated cells. (E) The summary of proportion of replicating Teff cells after transfected with anti-miR-125b-5p. Representative FACS data were from three independent experiments with similar results. Summarized data shown in C and E were displayed as mean ± SEM of three independent experiments (n=9). By comparison with miR-NC. ns, $P>0.05$, indicate no significance (Student's t-test).

Figure S5. Effect of miR-125b-5p on other phenotypic markers of Treg cells.

$CD4^+CD25^+$ Treg cells were transfected with miR-125b-5p or anti-miR-125b-5p for 2 days. The mean fluorescence intensity (MFI) of PD-1 and CTLA4 expression by Tregs cultured with or without TNF (A), and in Tregs after transfected with miR-125b-5p (B) or anti-miR-125b-5p (C). All experiments were performed at least three times, all values were represented as means ± SEM from 3 independent experiments. By comparison with miR-NC or anti-miR-NC, * $P<0.05$, ** $P<0.01$, *** $P<0.001$, (Student's t-test).

Table S1. Clinical pathological features of selected colon cancer patients

Pathological features		miR-125b-5p expression		P value
		low (n=15)	high (n=16)	
Age (years)	<median (54)	5	7	1
	≥median (54)	11	8	
Gender	Male	10	10	1
	Female	6	5	
Tumor size	<5cm	5	5	1
	≥5cm	11	10	
lymphatic metastasis	N0	6	12	0.0461
	N1	8	2	
	N2	2	1	
TNM stage	I	1	0	0.0253
	II	5	12	
	III	10	3	
Differentiation	Moderate/high	15	15	1
	Low	1	0	