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Supplemental Information

Sirt1-Overexpressing Mesenchymal Stem Cells Drive the Anti-tumor Effect through Their Pro-inflammatory Capacity

Fei Ye, Jinghua Jiang, Chen Zong, Xue Yang, Lu Gao, Yan Meng, Rong Li, Qiudong Zhao, Zhipeng Han, and Lixin Wei

Supplemental Figures and Legends

Figure S1. Effects of Sirt1 overexpression on the proliferation and migration of MSC

(A) MSCs were successfully transfected with recombinant adenovirus expressing mouse Sirt1 (AdSirt1). MSCs transfected with recombinant adenovirus vector expressing EGFP (AdEGFP) were used as the negative control. After 48 h, the expression of EGFP was observed under the fluorescent microscopy. Scale bars: 200 μm. (B) MSCs were successfully transfected with AdSirt1 or control AdEGFP. 8 hours after transfection, Sirt1 gene expression was detected by real-time PCR in MSC, AdEGFP-MSC and AdSirt1-MSC. 48 hours after transfection, the protein level of Sirt1 was examined by western blot analysis in MSC, AdEGFP-MSC and AdSirt1-MSC. $^{***}p < 0.001$ vs MSC. $p > 0.05$ versus MSC. NS: not significant ($p > 0.05$). (C) The effect of Sirt1 overexpression on the migration of MSC was analyzed by transwell migration assay and observed using microscopy. Representative images of the migrated cells were observed in transwell migration assay. Scale bars: 100 μm. (D) Quantitative analysis results of the migration of MSC *in vitro* migration assay. The migrated cells were counted in five random fields for each membrane under the microscopy. The three independent migration experiments were performed. Data represent mean ± s.d., $p > 0.05$ versus MSC. NS: not significant ($p > 0.05$). (E) The effect of Sirt1 overexpression on the cell proliferation of MSCs was detected by CCK8 test. The OD values of MSC, AdEGFP-MSC and AdSirt1-MSC were calculated for the cell proliferation activity. NS: not significant. CCK8, Cell Counting Kit-8; OD, optical density. $p > 0.05$ versus MSC. NS: not significant ($p > 0.05$).

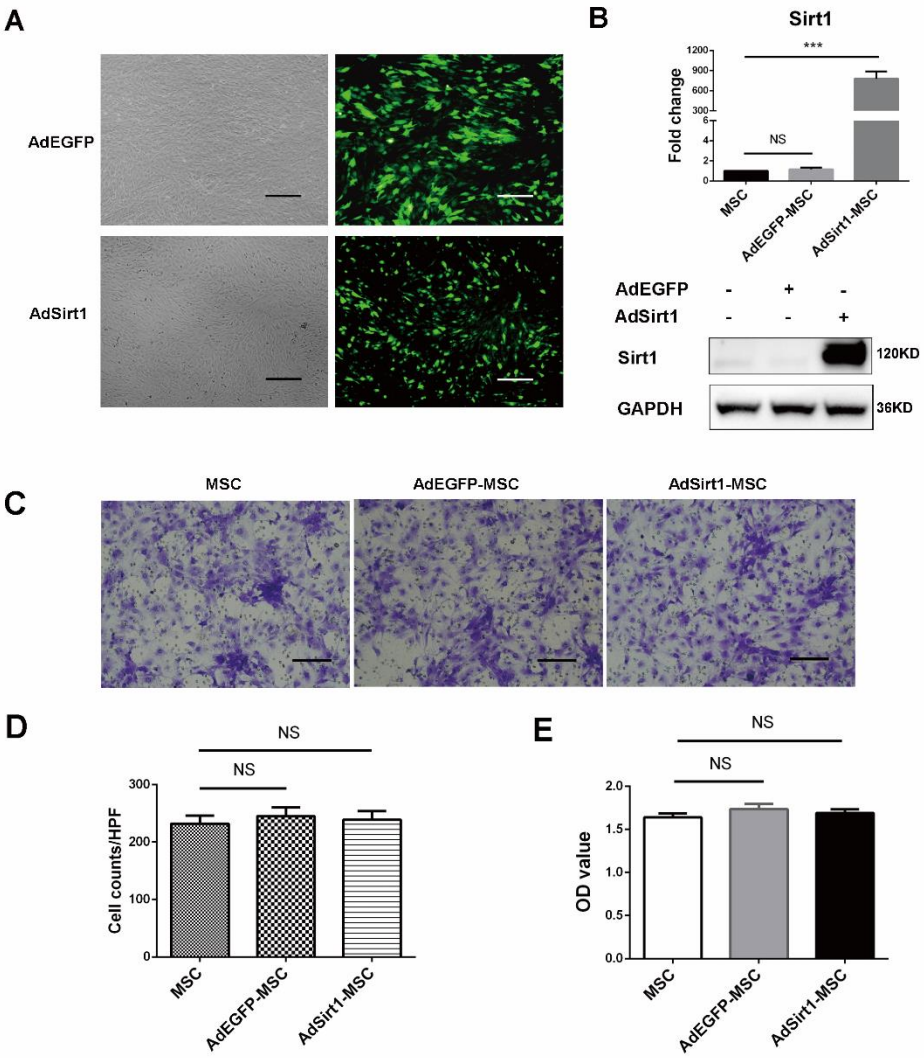


Figure S2. Administration of Sirt1-overexpressing MSC does not affect the number of hepatic CD4$^+$ T cells during hepatic metastasis of colorectal carcinoma in mice

(A) Immuno-histochemical analysis of hepatic CD4$^+$ T cells in mice (21 days post-treatment) of the CT26 group, CT26 + MSC group, CT26 + AdEGFP-MSC group and CT26 + AdSirt1-MSC group. The representative immunohistochemistry for CD4 in liver specimen from each group was shown (Scale bars, 100 μm). (B) Quantification of CD4$^+$ T cell in each group (21 days post-treatment). CD4 positive cells were counted on at least five random fields. Scale bars: 100 μm. $p > 0.05$ versus CT26. NS: not significant ($p > 0.05$).

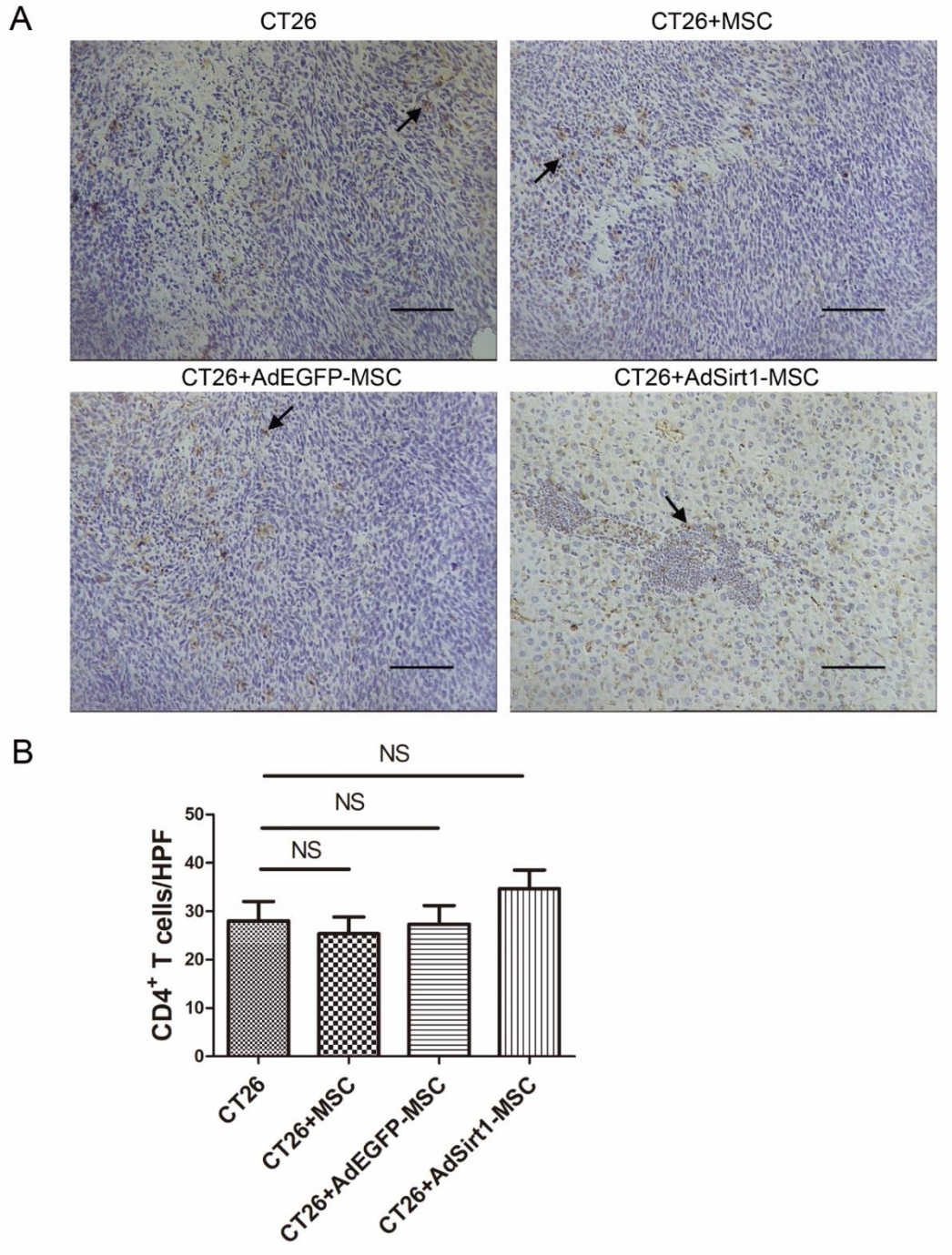


Figure S3. Administration of Sirt1-overexpressing MSC inhibits tumor progression in the established liver metastasis model of colorectal carcinoma

(A) The schematic representation depicting the experimental design to investigate the efficacy of the AdSirt1-MSC transfusion in the established liver metastasis of colorectal carcinoma in mice. Mice were randomly divided into four experimental groups as described in supplementary materials and methods. (B) Liver surface metastatic nodules were detected macroscopically. The representative photographs showed the hepatic tumor metastases from mice at day 11 post-treatment with CT26. (C) The representative photographs showed the hepatic tumor metastases at day 21 in mice treated with different groups of MSC administration from above four experimental groups. (D) H&E staining was used to evaluate the liver samples of mice treated with different groups of MSC administration at day 21 from above four experimental groups. Scale bars: 100 μm. (E) The representative immuno-histochemical staining of CD8 in liver samples of mice treated with different groups of MSC administration at day 21 from above four experimental groups was shown (Scale bars, 100 μm). The black arrows pointed to CD8-positive T cells (F) Quantitation of $CD8^+$ T cell at day 21 in mice with different groups of MSC administration from above four experimental groups. At least five fields (×200) were counted for each specimen. $^{**}p < 0.01$ vs CT26 group. $p > 0.05$ versus CT26 group. NS: not significant ($p > 0.05$).

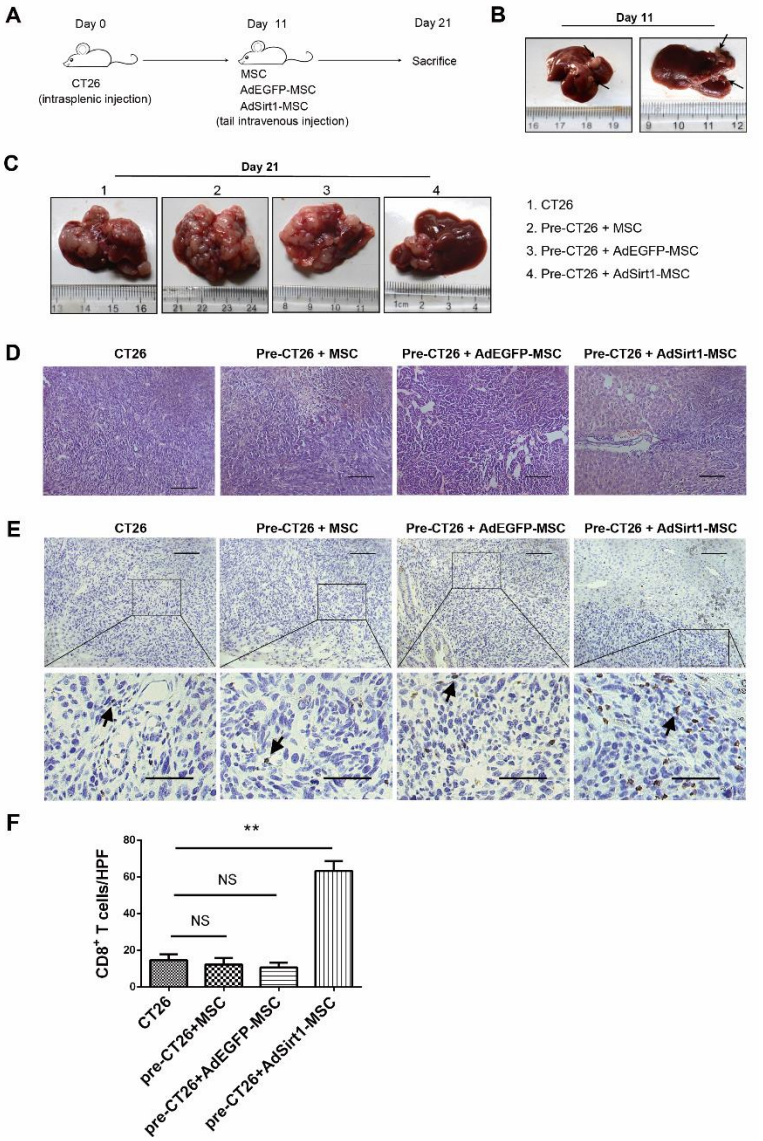


Figure S4. Administration of Sirt1-overexpressing MSC does not affect the number of hepatic $CD4^+$ T cells in CCl_4-induced acute liver injury

(A) Immuno-histochemical analysis of accumulating $CD4^+$ T cells in mice (48 hours post-treatment) of the normal control group (olive oil), CCl_4 group, CCl_4 + MSC group, CCl_4 + AdEGFP-MSC group and CCl_4 + AdSirt1-MSC group. The representative images of CD4 immunohistochemistry in liver specimen from each group were shown (Scale bars, 100 μm). (B) Quantification of accumulating $CD4^+$ T cell in each group (48 hours post-treatment). CD4 positive cells were counted on at least five random fields. Scale bars: 100 μm. $p > 0.05$ versus CCl_4 group. NS: not significant ($p > 0.05$).

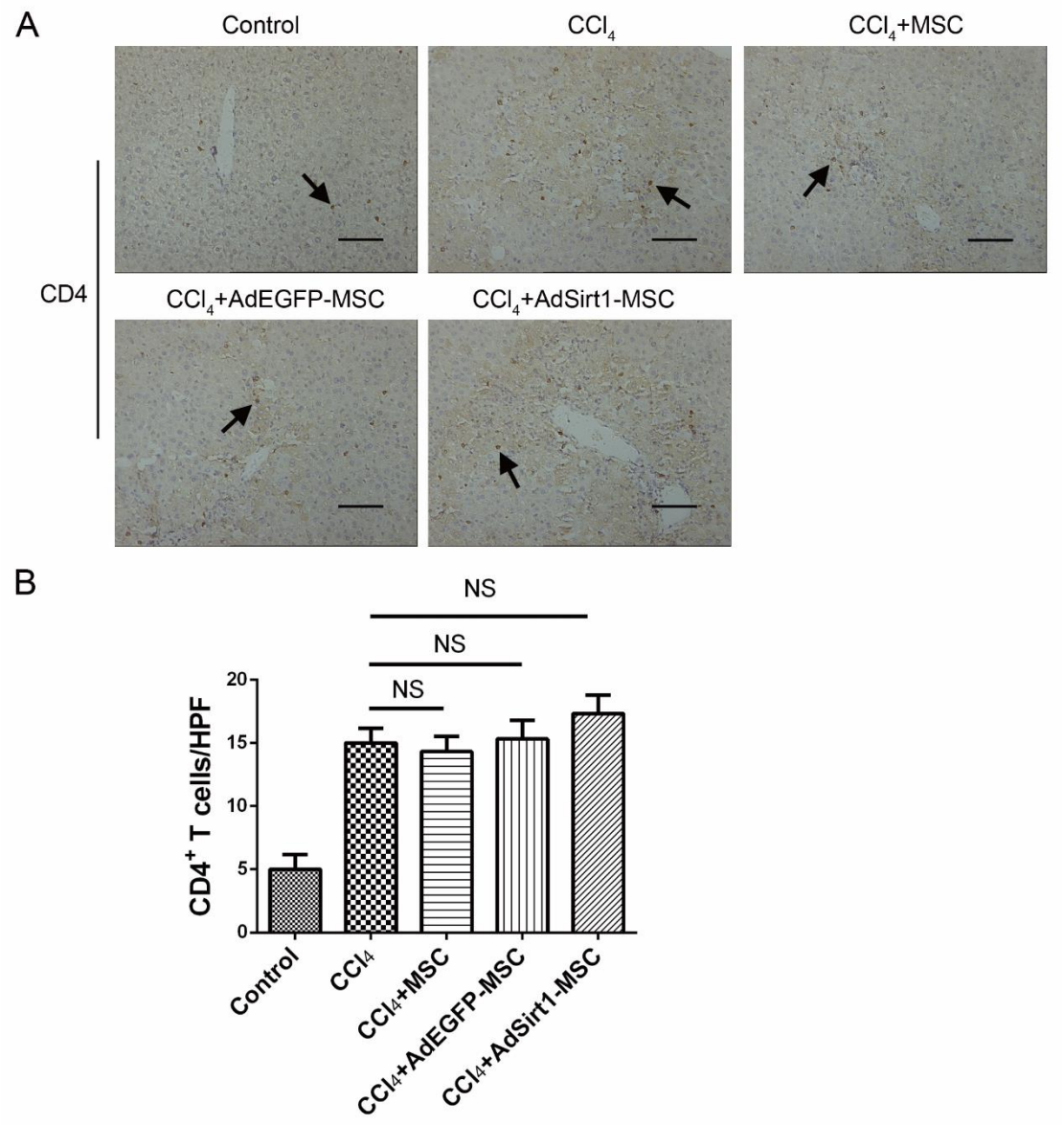


Figure S5. AdSirt1-MSCs do not inhibit the proliferation of $CD4^+$ or $CD8^+$ T cells in the coculture system of MSCs with ConA-activated splenocytes

(A) MSCs, AdEGFP-MSCs or AdSirt1-MSCs were co-cultured respectively with CFSE-labeled splenocytes at a ratio of 1: 10 in the presence of ConA (5μg/mL). The $CD4^+$ and $CD8^+$ T cells were detected by flow cytometry after 72 hours of incubation. A representative staining of three independent experiments was shown. (B) The percentage of $CD4^+$ and $CD8^+$ T cells was determined by flow cytometric quantification in each group. The values represent means ± s.d. from three independent experiments. (C) Under the same treatment conditions as in A, the total number of $CD4^+$ and $CD8^+$ T cells in each group was calculated. $^{***}p < 0.001$ vs spl+conA. $p > 0.05$ versus spl+conA. NS: not significant ($p > 0.05$).

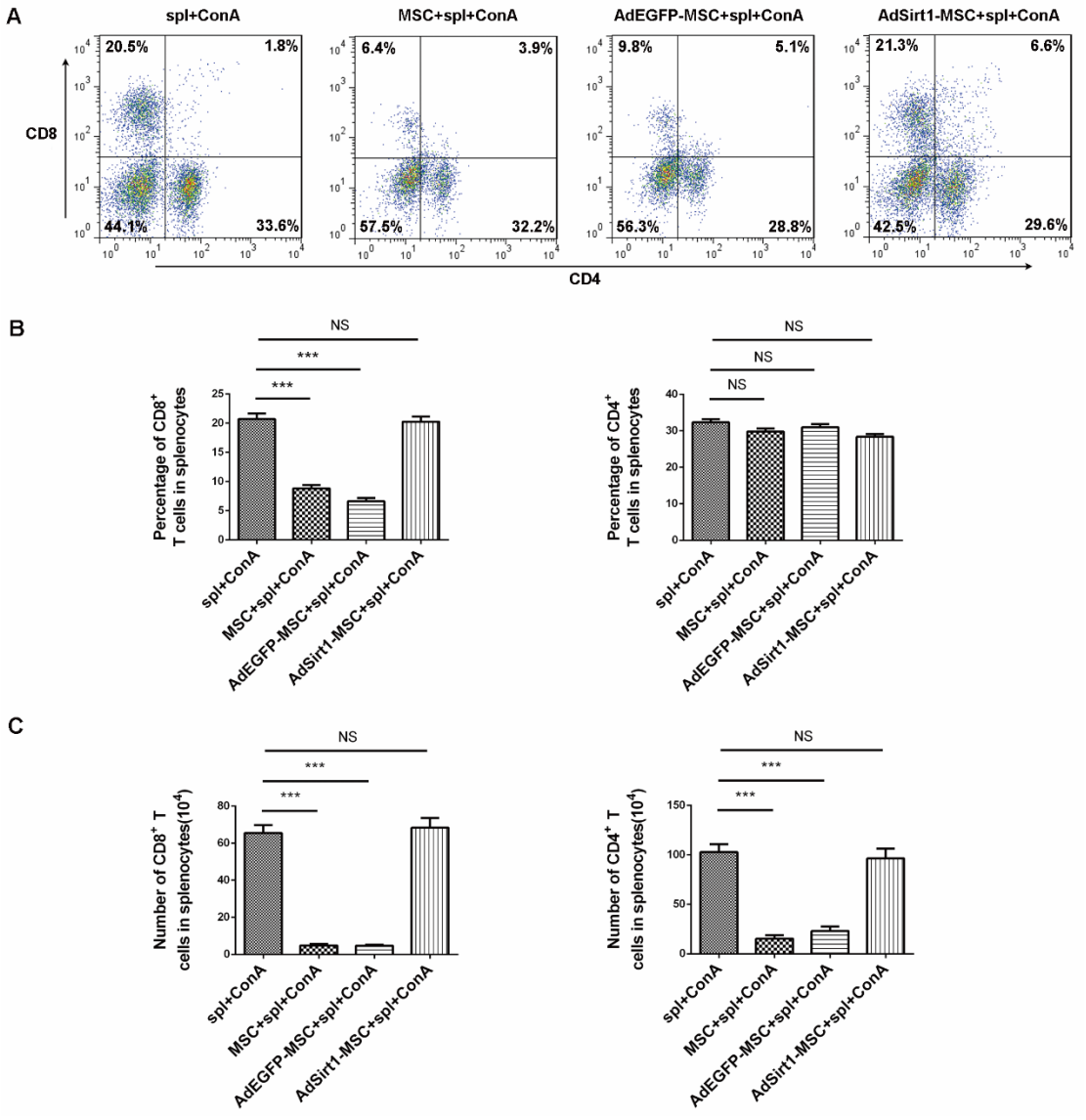


Figure S6. iNOS overexpression abolishs the anti-tumor effect of AdSirt1-MSC in the established liver metastasis model of colorectal carcinoma

(A) The schematic representation depicting the experimental design to investigate the efficacy of iNOS-overexpressing AdSirt1-MSC transfusion in the established liver metastasis of colorectal carcinoma in mice. Mice were randomly divided into four experimental groups as described in supplementary materials and methods. (B) The representative photographs showed the hepatic tumor metastases at day 21 in mice treated with different groups of MSC administration from above four experimental groups. (C) H&E staining was used to evaluate the liver sample of mice treated with different groups of MSC administration at day 21 from above four experimental groups. Scale bars: 100 μm. (D) The representative immunohistochemical staining of CD8 in liver samples of mice with different groups of MSC administration at day 21 from above four experimental groups was shown (Scale bars, 100 μm). The black arrows pointed to CD8-positive T cells. (E) Quantitation of $CD8^+$ T cell in mice with different groups of MSC administration at day 21 from above four experimental groups. At least five fields (×200) were counted for each specimen. $^{**}p < 0.01$ vs CT26 group. $p > 0.05$ versus CT26 group. NS: not significant ($p > 0.05$).

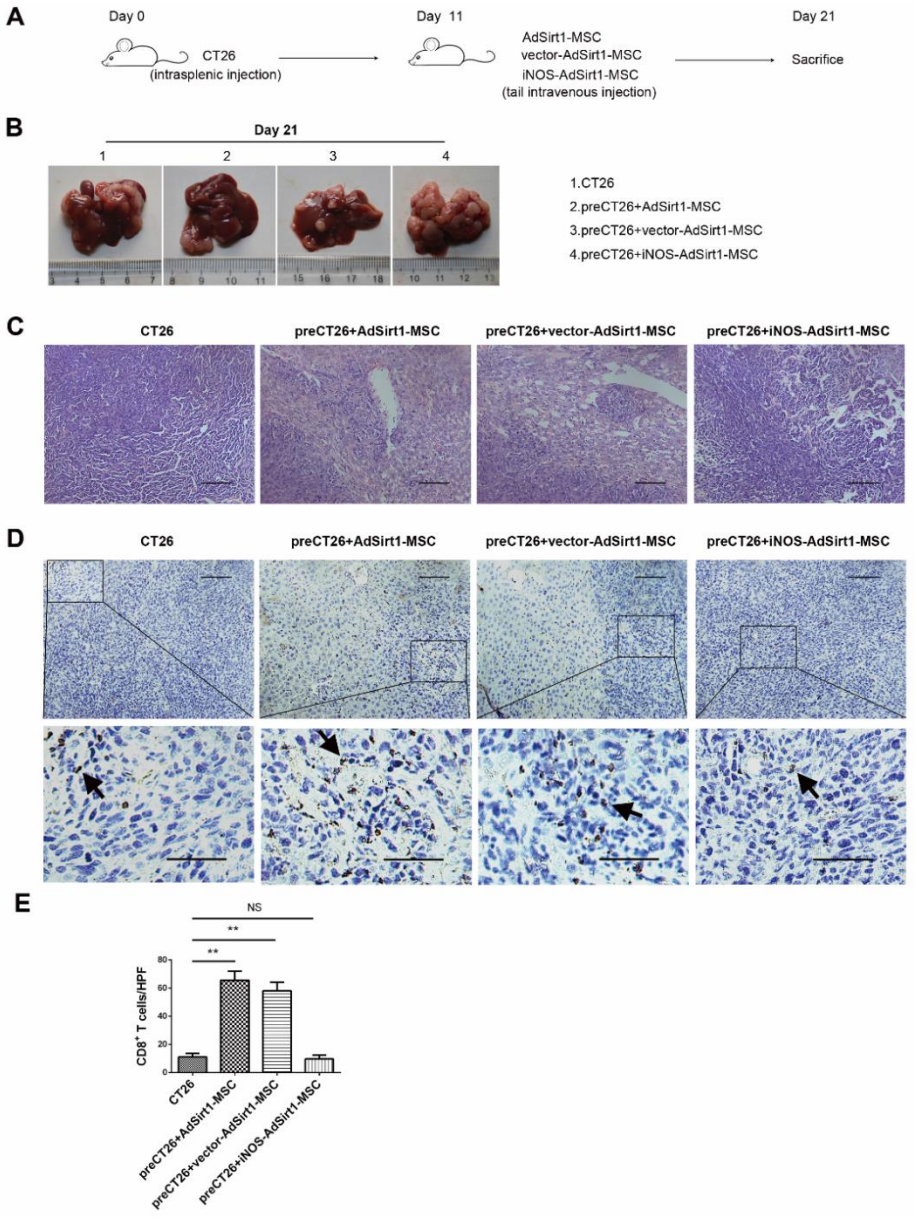
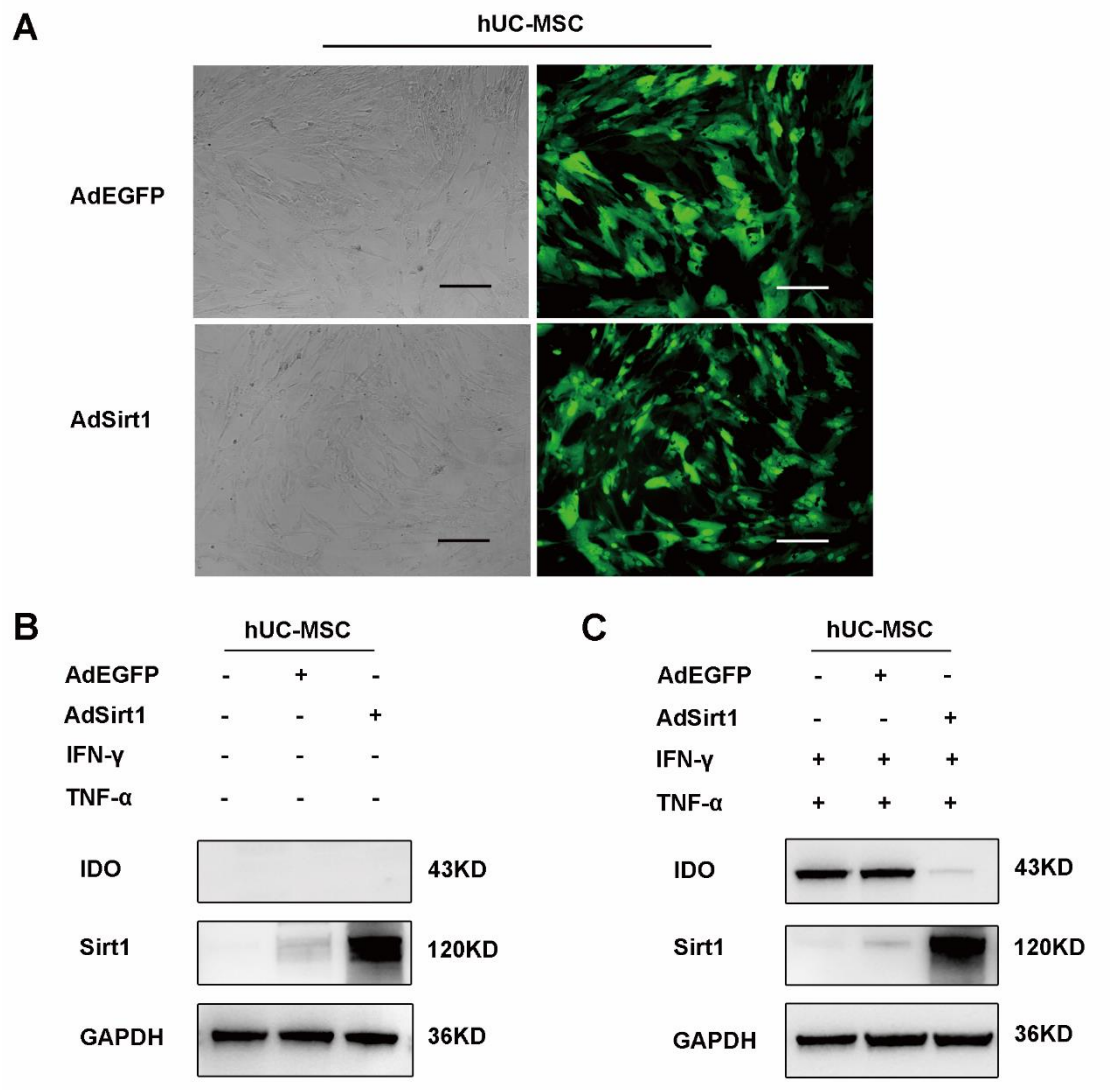


Figure S7. Sirt1 overexpression inhibits IDO production in inflammatory cytokines-induced human umbilical cord derived MSC

(A) Human umbilical cord derived MSC (hUC-MSC) was successfully transfected with recombinant adenovirus expressing human Sirt1 (AdSirt1). After 48 h, the expression of EGFP was observed under the fluorescent microscopy. The hUC-MSC transfected with recombinant adenovirus vector expressing EGFP (AdEGFP) was used as a negative control. Scale bars: 200 μm. (B-C) The protein level of Sirt1, IDO and GAPDH was examined by western blot analysis in hUC-MSC stimulated with or without TNF-α and IFN-γ (IT, 10ng/mL, each) for 24h.



Supplemental Methods

Generation of Sirt1-overexpressing MSCs

The stable overexpression of Sirt1 in mouse MSC was generated by transduction with recombinant adenovirus vectors expressing mouse Sirt1 which was provided by Heyuan Bio-technology Company (Shanghai, China). The mouse MSC transfected with combinant adenovirus vectors encoding EGFP was used as the control. To transfect mouse MSC, cells were incubated with adenoviral vectors co-expressing Sirt1 at a multiplicity of infection (MOI) of 60 or adenoviral vectors expressing EGFP at a MOI of 30, as well as 1 μg/mL of polybrene in the medium for 8 hours, then cells were washed and used as described in each experiment. To transfect human umbilical cord-derived MSC, cells were incubated with adenoviral vectors co-expressing human Sirt1 at a multiplicity of infection (MOI) of 40 or adenoviral vectors expressing EGFP at a MOI of 20, as well as 1 μg/mL of polybrene in the medium for 8 hours, then cells were washed and used as described in each experiment. The level of transduction was determined by assessing EGFP positive cells under fluorescent microscopy observation after 48 h infection.

Transwell Migration Assay

The assay for cell migration was performed in transwell chambers. Briefly, 200 μL of FBS-free medium (low-glucose DMEM) containing MSC, AdEGFP-MSC or AdSirt1-MSC at a density of 1×10^5 cells/mL was seeded on the upper chamber of the transwell assembly (6.5-mm diameter inserts, 8.0-μm pore size; Corning Costar, Corning, NY, USA). 500 μL of medium (low-glucose DMEM + 5% FBS) was then added to the lower chamber. The chambers were then incubated at 37°C in 5% CO_2 for 72h. At the end of the incubation, the cells on the upper side of the membrane were mechanically removed. Cells that had migrated to the lower side of the membrane were fixed for 15 min in 4% paraformaldehyde and stained with crystal violet.

The co-culture assay of MSCs with ConA-activated splenocytes

Freshly isolated splenocytes from the BALB/c mice were co-cultured respectively with MSCs, AdEGFP-MSCs or Adirt1-MSCs at the ratio of 10:1 for 72 hours in the presence of 5μg/mL ConA (eBioscience), and then were collected for flow cytometric measurement of the percentage and number of $CD4^+$ and $CD8^+$ T cells.

Animal model

The established hepatic metastasis model of colorectal carcinoma was performed to investigate the effects of AdSirt1-MSCs. 6-8 weeks old male BALB/c mice were randomly divided into four experimental groups (n = 5 per group) including Group I (CT26), Group II (preCT26 + MSC group), Group III (preCT26 + AdEGFP-MSC group), Group IV (preCT26 + AdSirt1-MSC group). At day 0, mice were inoculated intrasplenically with CT26 cells (2×10^5) to induce the hepatic metastasis model of colorectal carcinoma in Group I- IV. 11 days later. In Group II- IV, mice were administrated with MSCs, AdEGFP-MSCs or AdSirt1-MSCs (2×10^5) respectively via tail intraveneously injection. Animals were sacrificed on day 21 after administration with different groups of MSCs, murine livers were removed and processed for histology assessment.

The established hepatic metastasis model of colorectal carcinoma was also performed to investigate the effects of iNOS overexpressing AdSirt1-MSCs. 6-8 weeks old male BALB/c mice were randomly divided into four experimental groups (n = 5 per group) including Group I (CT26), Group II (preCT26 + AdSirt1-MSC group), Group III (preCT26 + vector-AdSirt1-MSC group), Group IV (preCT26 + iNOS-

AdSirt1-MSC group). At day 0, mice were inoculated intrasplenically with CT26 cells (2×10^5) to induce the hepatic metastasis model of colorectal carcinoma in Group I- IV. 11 days later. In Group II- IV, mice were administrated with AdSirt1-MSCs, vector-AdSirt1-MSCs or iNOS-AdSirt1-MSCs (2×10^5) respectively via tail intraveneously injection. Animals were sacrificed on day 21 after administration with different groups of MSCs, murine livers were removed and processed for histology assessment.