

Expanded View Figures

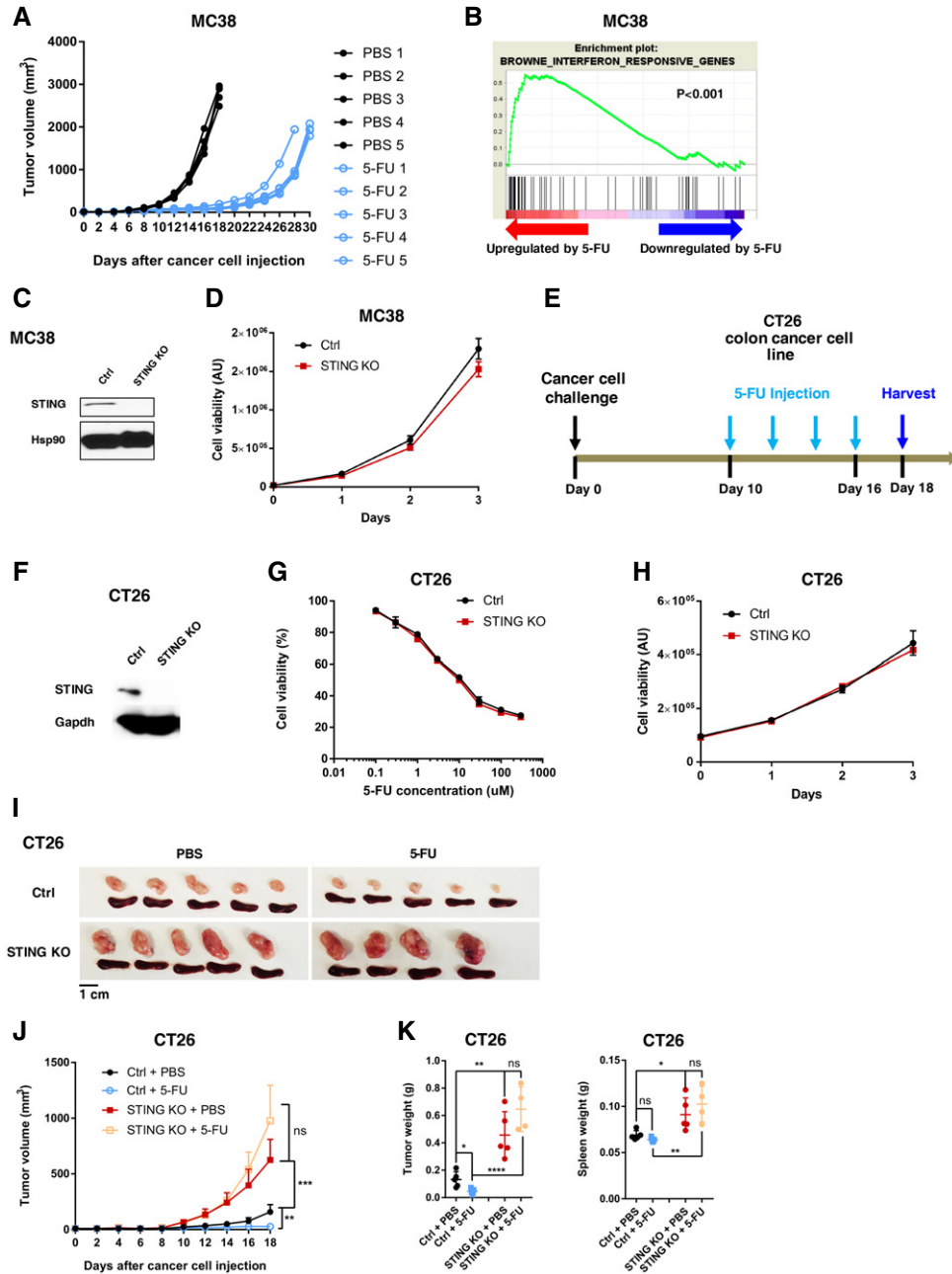


Figure EV1.

Figure EV1. 5-FU responses of MC38 and CT26 colon cancer cells.

- A Mice were injected with WT MC38 cells, and treated with PBS or 5-FU every other day from the sixth day after the cancer cell injection, and continued until the tumor surpassed size limit. Tumor volumes were quantified every 2 days. $N = 5$. Each line represents one tumor.
- B MC38 cells were treated with 5-FU or PBS for 24 h *in vitro* and subjected to RNA-Seq analyses. Gene expression profiles were queried on interferon-induced gene sets in MSigDB using Gene Set Enrichment Analysis. Result from a typical gene set is shown. The indicated P value was obtained by gene permutation during the GSEA procedure.
- C Western blot for STING levels in control (Ctrl) sgRNA and STING-KO MC38 cells. Hsp90 was used as a loading control.
- D MC38 cells with Ctrl sgRNA and STING-KO MC38 cells were subjected to proliferation assay. Cell viability was determined on the indicated days after cell plating using the CellTiter-Glo assay. Luminescence data in arbitrary units (AU) are plotted. $N = 3$. Data are representative of two independent experiments.
- E Schematic illustration of the syngeneic tumor model in which CT26 colon cancer cells were injected subcutaneously into WT BALB/c mice. 5-FU (25 mg/kg) were administered intraperitoneally from day 10 after cancer cell injection, with one dose every 2 days. Tumor and spleen were harvested on day 18.
- F Western blot of Ctrl and STING-KO CT26 cells on STING and GAPDH levels.
- G STING-KO or sgRNA control (Ctrl) CT26 cells were treated *in vitro* with the indicated concentrations of 5-FU for 2 days. Cell viability was determined using the CellTiter-Glo assay and normalized based on the level of PBS treatment. $N = 3$.
- H STING-KO or Ctrl CT26 cells were subjected to proliferation assays, with cell viability determined on indicated days after plating. $N = 3$.
- I-K Ctrl or STING-KO CT26 cells were injected into mice. Tumor growth and 5-FU response was followed based on the schematics in (E). (I) Representative images of tumors and spleens. Image panels were cropped from the same picture. (J) Mean tumor volumes of Ctrl or STING-KO CT26 with or without 5-FU treatment were followed during the experiment. $N = 5$ for all except for 5-FU-treated STING-KO group ($N = 4$). (K) Tumor (left panel) and spleen (right panel) weights were determined at the endpoint. Each dot represents one mouse.

Data information: For all panels, error bars represent SD, and center values represent mean. Two-tailed unpaired Student's t -test was used. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; ns: not significant. Data are representative of at least two independent experiments.

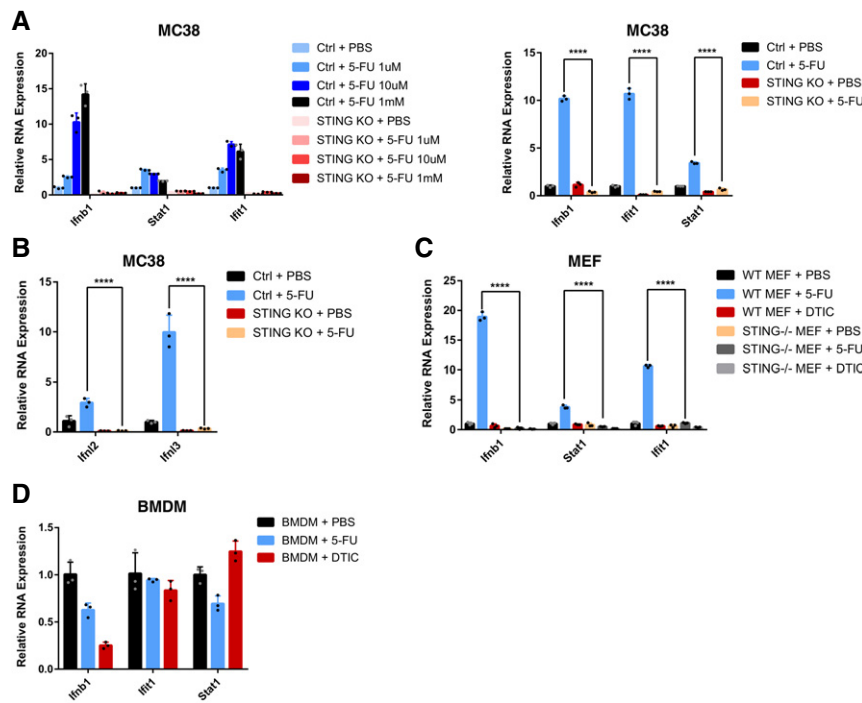


Figure EV2. Induction of IFN response by 5-FU in cancer and normal cells.

- A Ctrl or STING-KO MC38 cells were treated with the indicated concentrations (left panel) and IC50 (right panel) of 5-FU for 24 h. Cells were analyzed for *Ifnb1* and ISG RNA levels using qRT-PCR. $N = 3$.
- B Ctrl or STING-KO MC38 cells were treated with 5-FU or PBS for 24 h. Cells were analyzed for *Ifnl2* and *Ifnl3* RNA levels using qRT-PCR. $N = 3$.
- C WT or STING-KO MEF cells were treated with 5-FU, DTIC or PBS for 24 h. Cells were analyzed for *Ifnb1* and ISG RNA levels using qRT-PCR. $N = 3$.
- D WT BMDM cells were treated with 5-FU, DTIC, or PBS for 24 h. Cells were analyzed for *Ifnb1* and ISG RNA levels using qRT-PCR. $N = 3$.

Data information: For all panels, error bars represent SD, and center values represent mean. Two-tailed unpaired Student's t -test was used. **** $P < 0.0001$. Data are representative of two independent experiments.

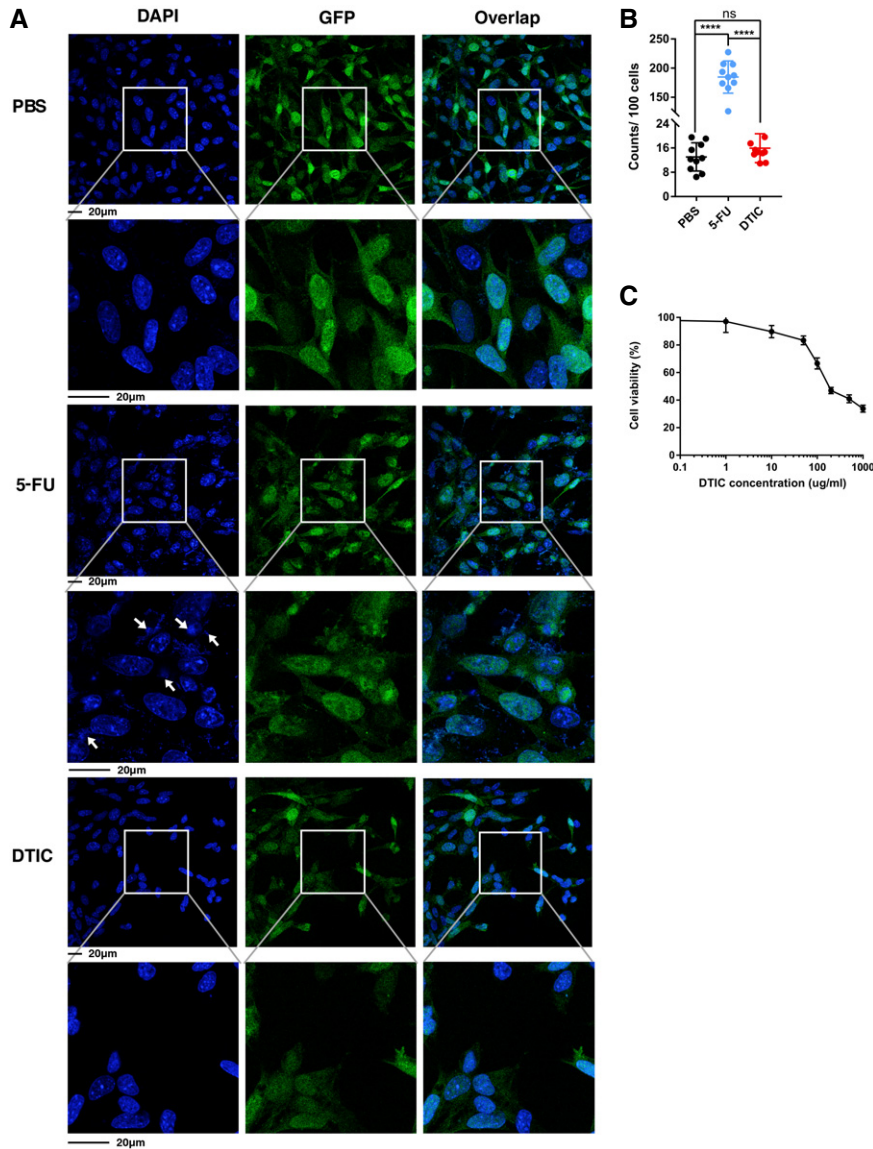


Figure EV3. 5-FU treatment leads to the appearance of micronuclei-like DNA structures.

- A Ctrl MC38 cells stably expressing GFP were treated with 0.3 μ M 5-FU, 300 μ g/ml DTIC or PBS for 48 h. The cells were stained with DAPI (blue). Representative pictures are shown, with enlarged areas indicated by white boxes. Arrows point to examples of micronuclei-like DNA structures.
- B Quantification of micronuclei-like DNA structures per 100 nuclei. $N = 10$ fields.
- C Ctrl MC38 cells were treated *in vitro* with the indicated concentrations of DTIC for 2 days. Cell viability was determined using the CellTiter-Glo assay and normalized based on the level of PBS treatment. $N = 3$.

Data information: For all panels, error bars stand for SD, and center values represent mean. Two-tailed unpaired Student's *t*-test was used. **** $P < 0.0001$; ns: not significant.

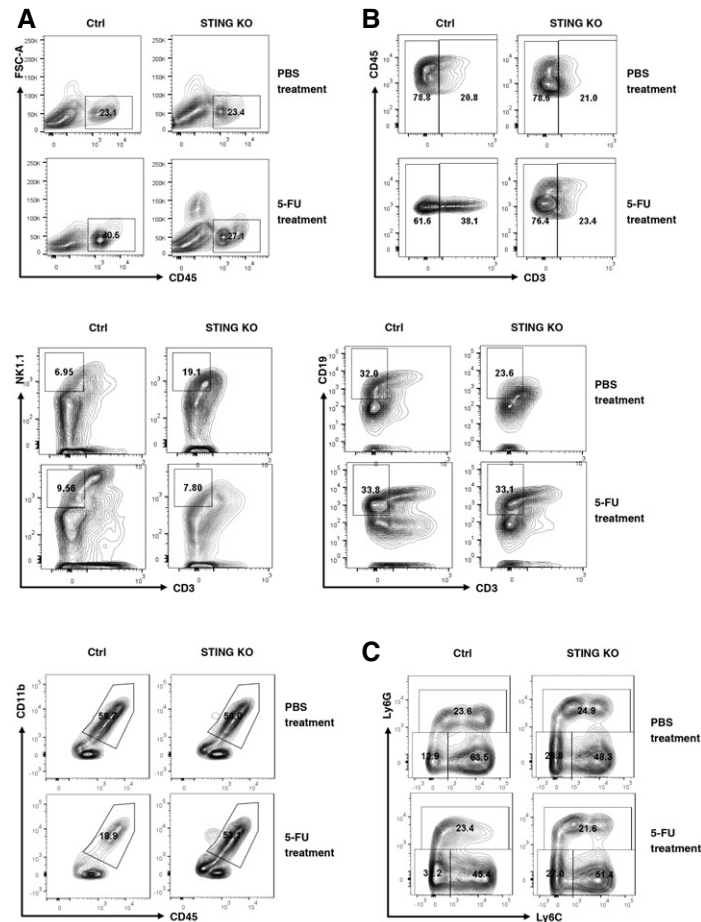


Figure EV4. Flow cytometry analyses of intratumoral immune cell populations.

Mice were injected with control (Ctrl) or STING-KO MC38 cells and treated with PBS or 5-FU. Tumors were harvested 2 weeks after cancer cell injection and intratumoral immune cells were examined by flow cytometry.

- A Representative flow cytometry plots showing the percentages of CD45⁺ cells within FSC and SSC gated live cell population from the tumor mass.
- B Representative flow cytometry plots showing the percentages of CD3⁺ T cells, CD3⁻NK1.1⁺ NK cells, CD3⁻CD19⁺ B cells, and CD11b⁺ myeloid cells within intratumoral CD45⁺ cells.
- C Representative flow cytometry plots analyzing the CD11b⁺ population with Ly6G and Ly6C.
- D The counts of intratumoral CD11b⁺F4/80⁺ macrophages per gram of tumor were quantified. *N* = 5. Each dot represents one mouse.
- E The counts of intratumoral CD11b⁺Ly6G⁺, CD11b⁺Ly6C⁺ Ly6G⁻, and CD11b⁺Ly6C⁻ Ly6G⁻ cells per gram of tumor were quantified. *N* = 5. Each dot represents one mouse.
- F The percentage of F4/80⁺ cells within the CD11b⁺ population is shown. *N* = 5. Each dot represents one mouse.
- G The percentage of Ly6G⁺, Ly6G⁻Ly6C⁺, and Ly6G⁻Ly6C⁻ cells within the CD11b⁺ population is shown. *N* = 5. Each dot represents one mouse.

Data information: For all panels, error bars represent SD, and center values represent mean. Two-tailed unpaired Student's *t*-test was used. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001; ns: not significant. Data in all figures are representative of two or more independent experiments.

