

5-Fluorouracil Efficacy Requires Anti-tumor Immunity Triggered by Cancer-cell-intrinsic STING

Jingru Tian, Dingyao Zhang, Vadim Kurbatov, Qinrong Wang, Yadong Wang, Dorothy Fang, Lizhen Wu, Marcus Bosenberg, Mandar Muzumdar, Sajid Khan, Qianjin Lu, Qin Yan, and Jun Lu

DOI: [10.15252/embj.2020106065](https://doi.org/10.15252/embj.2020106065)

Corresponding author: Jun Lu (jun.lu@yale.edu)

Review Timeline:

Submission Date:	25th Jun 20
Editorial Decision:	31st Aug 20
Revision Received:	17th Nov 20
Editorial Decision:	23rd Dec 20
Revision Received:	2nd Jan 21
Accepted:	8th Jan 21

Editor: Daniel Klimmeck

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dear Dr Lu,

Thank you for the submission of your manuscript (EMBOJ-2020-106065) to The EMBO Journal. Please accept my apologies for the delay with the peer-review of your work due to protracted referee input and detailed discussions in the team. Your manuscript has been sent to three reviewers and we have received reports from all of them, which I enclose below.

As you will see, the referees acknowledge the potential interest and novelty of your results, although they also express a number of issues that will have to be conclusively addressed before they can be supportive of publication of your manuscript in The EMBO Journal.

Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

In light of the extensive experimentation requested by the reviewers, I would appreciate if you could contact me during the next weeks via e.g. a video call to discuss your perspective on the comments and potential plan for revisions.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

In this context I also want to point to our adjusted GTA. We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic and have therefore extended our 'scooping protection policy' to cover the period required for a full revision to address the experimental issues highlighted in the editorial decision letter. Please contact us at any time to discuss an adapted revision plan for your manuscript should you need additional time, and also if you see a paper with related content published elsewhere.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Kind regards,

Daniel Klimmeck

Daniel Klimmeck, PhD
Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:

<http://bit.ly/EMBOPressFigurePreparationGuideline>

IMPORTANT: When you send the revision we will require

- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (<https://www.embopress.org/page/journal/14602075/authorguide>).
- Expanded View files (replacing Supplementary Information)

Please see out instructions to authors

<https://www.embopress.org/page/journal/14602075/authorguide#expandedview>

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

Further information is available in our Guide For Authors:

<https://www.embopress.org/page/journal/14602075/authorguide>

The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 29th Nov 2020.

Referee #1:

Tian et al investigated the role of STING in the antitumor effects of 5-FU, a commonly used anticancer drug. Surprisingly, they found that STING in cancer cells, but not in the host (mice), is required for the anticancer effect of 5-FU. This was shown in several tumor cell lines including MC38, CT26 and YUMM1.7 (a melanoma cell line). The authors further showed that 5-FU induces type-I interferons and ISGs in tumor cells in a STING dependent manner, that the loss of IFN α and IFN β in cancer cells or the loss of IFN α in bone marrow cells led to defective antitumor effect of 5-

FU. Evidence was also presented to show that T cells accumulate in the tumors after 5-FU treatment, and that depletion of CD4 and CD8 T cells abrogated the therapeutic effect of 5-FU. Analysis of the TCGA database suggests that STING expression correlates with a better survival of human colon cancer patients.

Overall, the data showing an essential role of cGAS and STING in tumor cells in the therapeutic effects of 5-FU are quite convincing. This is interesting and unexpected, as several other studies showed a more important role of STING in the stromal cells in other mouse tumor models. This paper will contribute to a better understanding of the role of the cGAS-STING pathway in antitumor immunity. Considering that 5-FU has been a backbone of standard cancer therapy for many decades, the new mechanistic understanding of the action of 5-FU is an important advance in the field of cancer therapy.

The paper can be improved by addressing the following questions:

- 1) How does 5-FU activate the cGAS-STING pathway in cancer cells? Other cytotoxic drugs such as Decarbazine apparently don't have such an effect. What makes 5-FU uniquely capable of activating cGAS? Does 5-FU activate cGAS or induce IFNs in non-cancer cells?
- 2) Figure 7J: although the authors stated that DMXAA did not induce IFNs strongly in the pancreatic cancer cell line, STING agonists may still have an antitumor effect in vivo through acting on stromal cells. The authors should compare the effect of DMXAA or cGAMP with polyI:C in the same experiment.

Referee #2:

General Summary

The study by Tian et al investigated the mechanisms of the chemotherapeutic drug 5-Fluorouracil (5-FU) in immunocompetent mouse models of colon carcinoma and melanoma. They found that tumor reduction was dependent on activation of cancer cell-intrinsic cGAS, STING and type I interferon production and IFN sensing by bone marrow derived cells which were predominantly T cells. Other reagents applied locally that induce IFN such plpC were also found to induce anti-tumor effects supporting an important role for IFN signalling. In addition the authors found that higher STING expression in patient tumors is associated with better survival and responses to chemotherapy. The results of this study may have important implications for better utilizing chemotherapy drugs such as 5-FU for more effective treatment of cancer.

Major points

The following concerns require attention particularly with regard to mechanism to substantiate conclusions.

In previous studies, 5-FU has been shown to enhance the immunogenicity of tumor cells by upregulation of immune markers such as MHCI, Fas and CD80. Was expression of any of these markers increased on the tumor lines in vivo after 5-FU chemotherapy administration?

In all of the in vivo experiments only short term tumor growth experiments are shown. What were the effects of 5-FU on survival of mice? Did 5-FU lead to complete eradication of tumor?

In Fig 4, the authors show a significant increase in percentage of CD3+ cells after 5-FU treatment of MC38 tumors yet interestingly the increase in CD4 or CD8 T cells was not significant. Were other CD3+ cells modulated by 5-FU therapy such as NKT cells or T cells to account for this difference? Although the effect on percentage of immune cells is shown in Fig. 4, the quantitation on the numbers of these immune subsets following 5-FU treatment should also be shown.

Does 5-FU therapy through activation of the cGAS-STING pathway impact on the functional responses of intratumoral T cells (cytokine, proliferation). There is no data shown in the paper on this. Does 5-FU treatment induce a memory response in mice?

Did 5-FU treatment impact on numbers of dendritic cells within the tumor and draining lymphnodes particularly with regard to CD11c+ CD103+ DC cells?

Can intratumoral injection of DMXAA or plpC induce abscopal effects against distant tumors in mice?

Have the authors examined whether low dose 5-FU can synergise with checkpoint blockade to enhance therapeutic effects?

Referee #3:

The manuscript by Tian et al., provide in vivo data suggesting that efficient 5-FU treatment of mice carrying cell line tumors is mediated by STING/IFNa/b activity within the cancer cells triggering anti-tumor immunity (next to the cytotoxic effect).

The authors used two CRC (most analysis performed with MC38) and one melanoma line to show similar growth behavior +/- 5-FU, which is in part distinct from the effects in culture.

Elimination of STING, IFNa/b in the cancer cells or IFNAR1 in the mouse microenvironment led to (1) increased tumor growth and (2) insensitivity to 5-FU. A partial effect was achieved by antibody mediated elimination of CD4/CD8 T cells. The authors also show a correlation with human TCGA samples, although the effect is moderate and in these "bulk" samples no distinction between the expression in cancer or microenvironment can be made (acknowledged by the authors).

This is an interesting study linking 5-FU sensitivity of cancer cells in vivo to intrinsic STING/IFNa/b and the tumor microenvironment (TME).

While the models have been well analysed and presented, the analysis of the corresponding TME cell compartments should be strengthened. Altogether it is a very interesting and timely study with high relevance to a better understanding of responders and non-responders to chemotherapy and the data raise a particular interesting link to the immune microenvironment. The suggestions below should guide the authors to solidify their conclusions.

Major points:

(1) The FACS analysis of the tumors analysed in Fig. 4A-C and Fig. S6 are critical. Since tumors of very different sizes are analyzed the number of the different immune cells in relation to the number of tumor cells should be determined to make strong conclusions.

(2) More extensive FACS panels including rationale pre-gatings to exclude certain populations should be used to better characterize the various myeloid and lymphoid cell populations within the TME. For example in S6B (left): is the change in the CD45 pattern after 5-FU due to the loss of

myeloid cells compared to the STING KO samples? If yes, why? 5-FU is highly cytotoxic to myeloid cells, does STING KO protect from this?

(3) In Fig. 4A-C about 20-40% of cells are CD45+; what are the rest - tumor cells? Some show 100% CD45+ cells (Fig. 4A), can this be explained?

(4) The decline of myeloid cells in controls is very interesting. Next to CD11b also other markers such as Gr1 and F4/80 and other should be included in a co-staining to better characterize the changing myeloid cell types.

(5) This reviewer is not sure how much Figure 7 contributes to the storyline. The high production of interferons in response to plpC may simply lead to a blockade of cancer cell proliferation (testable in vitro). These data should be considered to either be taken out or supplemented with an analysis directly showing that at least some of the effects are mediated by immune cells (along the storyline) rather than directly inhibiting tumor cell growth.

Minor points:

(6) Page 5 : should say "spleen growth" is different in 5-FU treated mice?

(7) Page 5 : The lack of STING in the microenvironment does have a significant effect (which is interesting and could be discussed) and should not be played down so much, it does not jeopardize the storyline.

(8) Fig. 2A: order of panels should be exchanged to match text

(9) Page 6 bottom: reference to S1D and 5-FU concentrations seems wrong/ or not shown?

(10) Fig. 3D: did T cells already readily reconstitute at 45 days ?

(11) Page 9 last paragraph: should it no say one day prior "5-FU injection" (rather than "WT MC38")

(12) S7C: poor quality H&E staining

(13) Are type III interferons differentially expressed?

(14) A model describing the cancer and immune cell effects would be helpful to guide the reader along.

Overview:

We thank all reviewers for thoughtful and constructive suggestions that helped to improve this manuscript. We have performed new experiments and included new data in the revised manuscript.

In addition to the suggestions by the reviewers, we have rearranged figures according to the journal requirements. Some of the previous supplementary figures are now in the Main Figures, Expanded View (EV) Figures, or Appendix Figures. Revised text is shown in red font.

Reviewer #1:

Comment: Overall, the data showing an essential role of cGAS and STING in tumor cells in the therapeutic effects of 5-FU are quite convincing. This is interesting and unexpected, as several other studies showed a more important role of STING in the stromal cells in other mouse tumor models. This paper will contribute to a better understanding of the role of the cGAS-STING pathway in antitumor immunity. Considering that 5-FU has been a backbone of standard cancer therapy for many decades, the new mechanistic understanding of the action of 5-FU is an important advance in the field of cancer therapy.

Response: We thank the reviewer for considering our work interesting and of importance for the field.

Comment 1: How does 5-FU activate the cGAS-STING pathway in cancer cells? Other cytotoxic drugs such as Decarbazine apparently don't have such an effect. What makes 5-FU uniquely capable of activating cGAS? Does 5-FU activate cGAS or induce IFNs in non-cancer cells?

Response: We thank the reviewer for raising the interesting question on 5-FU's effect on normal cells. We performed experiments by treating two types of normal cells with 5-FU, early passage MEF cells that are actively proliferating, and bone marrow derived macrophages (BMDMs) that are mostly post-mitotic. In MEF cells, 5-FU, but not DTIC, induces *Ifnb1* and ISGs (*Ifit1* and *Stat1*), in a STING-dependent manner. In contrast, neither 5-FU nor DTIC induces IFN response in BMDM cells. These data support that in some normal cells, 5-FU can also induce IFN, and suggest that this capacity may be influenced by cellular context or proliferation status. These data are included as **revised Figure EV1F** and **Figure EV1G**, as well as shown below.

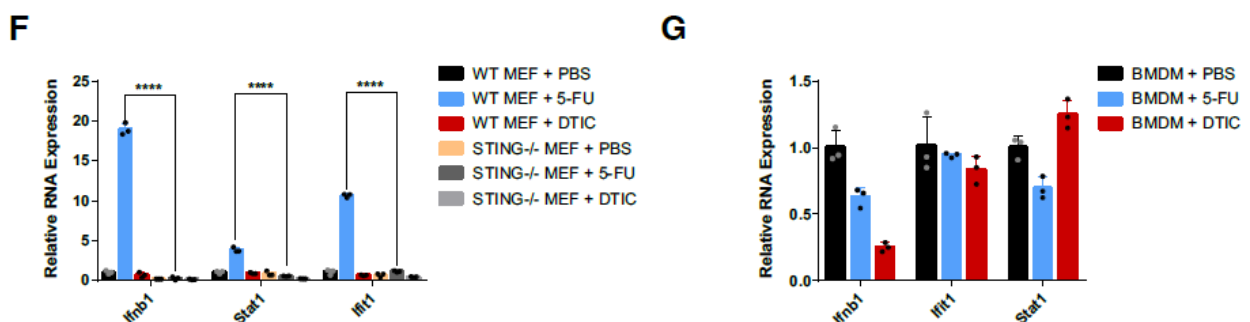


Figure EV1. (F) WT or STING KO MEF cells were treated with 5-FU or DTIC or vehicle control for 24 hours. Cells were analyzed for *Ifnb1*, *Stat1* and *Ifit1* RNA levels using qRT-PCR. N=3. **(G)** WT BMDM cells were treated with 5-FU or DTIC or vehicle control for 24 hours. Cells were analyzed for *Ifnb1*, *Stat1* and *Ifit1* RNA levels using qRT-PCR. N=3. For all panels, error bars stand for standard deviation. ****: $p < 0.0001$.

The reviewer also asked the interesting question why 5-FU can activate the cGAS-STING pathway whereas Decarbazine (DTIC) cannot. To address this question, we performed new experiments to visualize the integrity of nuclei upon 5-FU and DTIC treatment. We focused on MC38 cells, and treated cells with the IC₅₀ concentration of 5-FU (0.3 μ M) which caused robust STING-dependent induction of type I IFN and ISG expression (**revised Figure EV1E**). This treatment of 5-FU led to the disruption of normal nuclear morphology, with a substantial increase of DAPI-positive micronuclei-like DNA structures (**revised Figure EV3A and EV3B, and shown below**). Of note, we used MC38 cells stably expressing GFP, so that cell boundary can be better demarcated. To examine the effects of DTIC, we also determined the IC₅₀ of DTIC in MC38 cells (**revised Figure EV1I and shown below**), and used a concentration (300 μ g/ml) slightly higher than IC₅₀. In contrast to 5-FU, DTIC treatment did not result in an increase of micronuclei-like DNA structures (**revised Figure EV3A and EV3B, and shown below**). Because it has been well documented that micronuclei can trigger cGAS-STING activation (**Reference 28-31** in the revised text), these data suggest the involvement of 5-FU-induced micronuclei-like DNA structures in cGAS activation.

We have also attempted to visualize cGAS localization to these micronuclei-like DNA structures in MC38 cells by using the standard approach of transducing cGAS-GFP into MC38 cells. However, the signal intensity of cGAS-GFP was too low to be imaged, despite multiple attempts. Of note, in HEK293T cells, we did observe the co-localization cGAS-GFP with micronuclei-like DNA structures (**Figure R1** below). We did not include these HEK293T cell data in the revised manuscript because HEK293T cells are defective in the cGAS-STING pathway and are thus not strongly relevant to our findings.

The deeper question why 5-FU, but DTIC, can trigger micronuclei-like DNA structure formation will require a large number of experiments. We feel that this interesting and extended question will be best addressed in a separate future study.

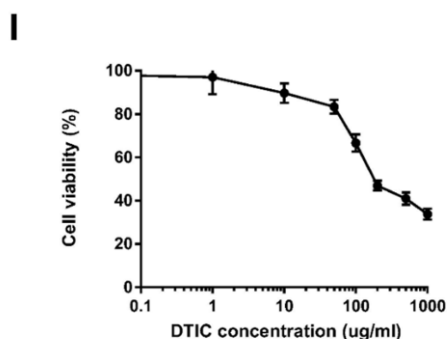


Figure EV1. (I) Ctrl MC38 cells were treated *in vitro* with the indicated concentrations of DTIC. Cell viability were determined using the CellTiter-Glo assay after two days, with relative luminescence shown in arbitrary units (AU). N=3.

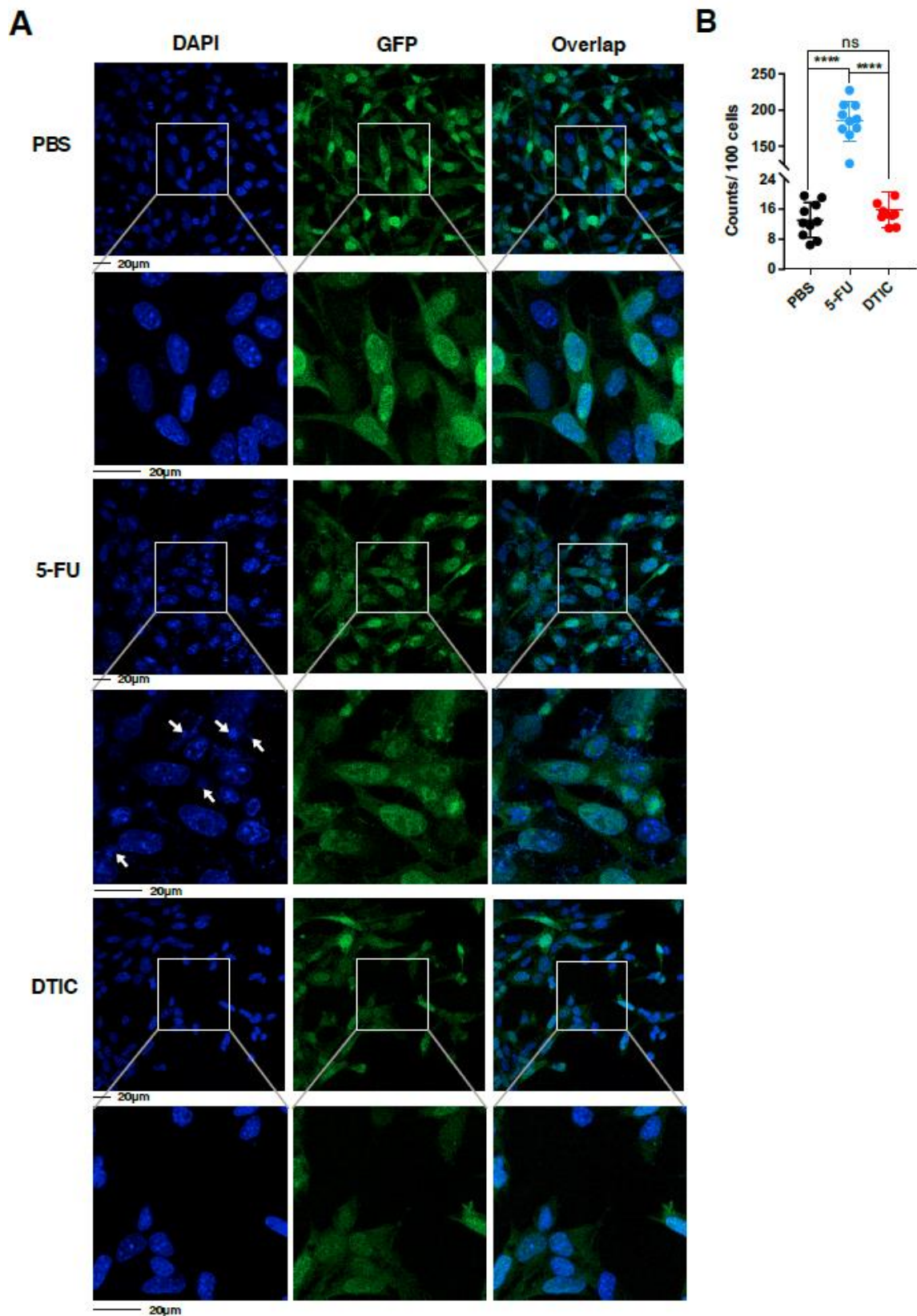


Figure EV3. (A) Ctrl MC38 cells stably expressing GFP were treated with 0.3 μ M 5-FU, 300 μ g/ml DTIC or vehicle control for 48 hours. The cells were stained with DAPI (blue). Representative pictures are shown, with enlarged area indicated by white boxes. Arrows point

to examples of micronuclei-like DNA structures. **(B)** Quantification of micronuclei-like DNA structures per 100 cells. N=10 fields from two slides. For all panels, error bars stand for standard deviation. ****: $p < 0.0001$; ns: not significant.

Figure for referees removed

Comment 2: Figure 7J: although the authors stated that DMXAA did not induce IFNs strongly in the pancreatic cancer cell line, STING agonists may still have an antitumor effect *in vivo* through acting on stromal cells. The authors should compare the effect of DMXAA or cGAMP with polyI:C in the same experiment.

Response: We thank the reviewer for pointing out potential issues in the old Figure 7J. Following the reviewer's suggestion, we performed experiments with the pancreatic cancer model and compared intratumoral injection of DMXAA vs polyI:C. As shown in **Figure R2** below, while polyI:C was more effective in reducing tumor size, intratumoral injection of DMXAA also led to statistically significant reduction of tumor size, although not as strong as polyI:C. Additionally, to address whether the effect of intratumoral injection of polyI:C was due to IFN-sensing by host cells, we performed the following experiment. Using *Ifnar1* knockout mice as host, we found that tumors grow larger than those in WT hosts, and tumors from *Ifnar1* KO mice were not as sensitive to polyI:C treatment (**Figure R2A-D**). Thus, polyI:C-induced tumor size reduction is at least partially dependent on host cells' type I IFN receptor.

Despite the above findings, as the reviewer pointed out, these results could either be interpreted as an effect of DMXAA and/or polyI:C on cancer cells, on cells in the tumor microenvironment, or a combination of effects on cancer cells and on stromal cells, and thus cannot be conclusive in terms of which cells responded to polyI:C and DMXAA. Related to this comment, reviewer 3 has suggested us to remove the old Figure 7 completely, due to this Figure not being tightly linked to our overall finding that the efficacy of 5-FU *in vivo* requires cancer-intrinsic STING activation. Considering all of the above, especially the difficulty of data

interpretation and the fact that data in this figure are not essential for our findings, we have followed the suggestion from reviewer 3 and removed this figure from the revised manuscript.

Figure for referees removed

Reviewer #2:

Comment 1: The results of this study may have important implications for better utilizing chemotherapy drugs such as 5-FU for more effective treatment of cancer.

Response: We thank the reviewer for appreciating the significance of our study.

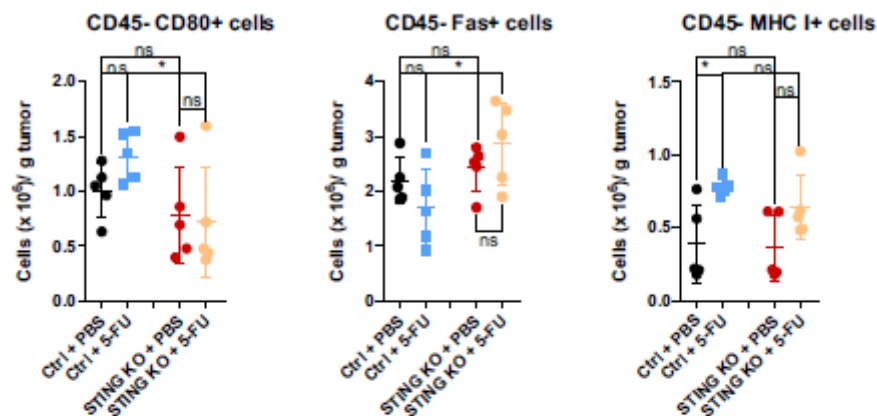
Comment 2: In previous studies, 5-FU has been shown to enhance the immunogenicity of tumor cells by upregulation of immune markers such as MHC-I, Fas and CD80. Was expression of any of these markers increased on the tumor lines in vivo after 5-FU chemotherapy administration?

Response: We followed the reviewer's suggestion and examined the levels of MHC-I, Fas and CD80 using flow cytometry. Due to the lack of a cancer-cell-specific cell surface marker, we used CD45-negative cells for quantification, which are composed primarily of cancer cells. The number of MHC-I⁺CD45⁻ cells was mildly increased after 5-FU treatment on WT MC38 tumors, whereas the number of cells positive for CD80 and Fas within the CD45-negative gate did not significantly change after 5-FU treatment (**revised Appendix Figure 2A, and also shown below**). Of note, due to concerns on FACS quantification in the previous version using percent

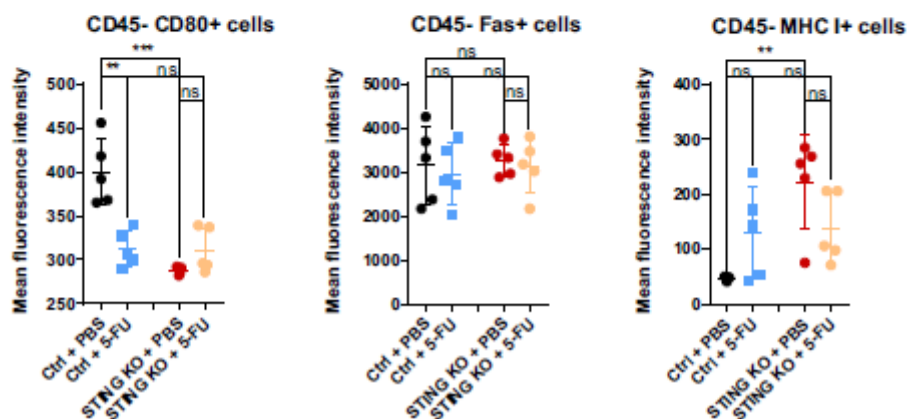
of live cells, we have modified the quantification to show data as the number of cells within a gram of tumor (see response to comment 4 below for details).

In addition, we also quantified the expression levels of MHC-I, Fas and CD80, using mean fluorescence intensity, in CD45-negative cells that are positive for these markers, respectively. (revised Appendix Figure 2B, and also shown below)

A



B



Appendix Figure 2. (A-B) 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. Intratumoral cells were examined by flow cytometry. **(A)** The number of CD45⁺CD80⁺, CD45⁺Fas⁺, and CD45⁺MHC-I⁺ cells per gram of tumor was quantified. **(B)** The mean fluorescence intensities of CD80, Fas and MHC-I in CD45⁺CD80⁺, CD45⁺Fas⁺, and CD45⁺MHC-I⁺ cells, respectively, were quantified. Each dot represents one mouse. N=5. For all panels, error bars stand for standard deviation. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ns: not significant. Data are representative of two independent experiments.

Comment 3: In all of the in vivo experiments only short term tumor growth experiments are shown. What were the effects of 5-FU on survival of mice? Did 5-FU lead to complete eradication of tumor?

Response: We thank the reviewer for this intriguing question, and performed experiments as suggested. We treated tumor-bearing mice with PBS or 5-FU. For the 5-FU treatment group, we gave a dose of 5-FU (25 mg/kg per dose) every other day from the sixth day after the cancer cell injection, and continued until the tumor became too large. Due to IACUC regulation that tumor-bearing mice need to be euthanized after tumors reach a certain size, we included new data on the growth of tumor size for each individual mouse (**revised Figure EV1A, also shown below**). We observed that while 5-FU can effectively slow down the growth of tumors in the early stage of treatment, it cannot completely eradicate the tumor mass. After ~18 to 20 days, the tumors increased rapidly in size. Because of this finding, we have modified our wording in the text, so that we refer to 5-FU's effect as "reduction of tumor burden", rather than "5-FU-induced tumor regression". We also specified in the revised text that this study is focusing on the 5-FU response in the relatively early phase.

There are several possibilities that may explain the eventual emergence of 5-FU resistance, including, but not limited to, cancer-cell-intrinsic resistance to 5-FU due to selection of genetically mutated subclones, cancer-cell-intrinsic resistance to 5-FU due to epigenetic reprogramming, remodeling of anti-tumor immunity, remodeling of non-immune tumor microenvironment, or combinations of the above. We discussed these possibilities in the **revised Discussion**. We feel that the elucidation of the exact mechanism of resistance should be the topic of a new study, and we plan to follow this up in the future.

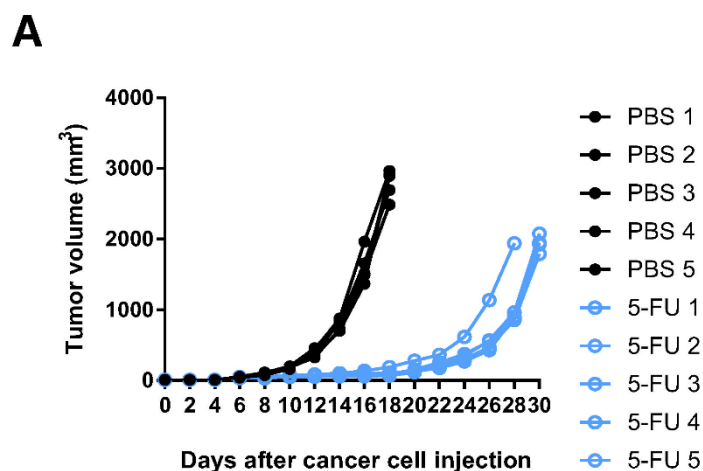


Figure EV1. (A) Mice were injected with Ctrl MC38 cells, and treated with PBS or 5-FU every other day from the sixth day after the cancer cell injection, and continued until the tumor reaches size limit. Tumor volumes were quantified every two days post cancer cell injection. Each line represents one mouse. N=5.

Comment 4: In Fig 4, the authors show a significant increase in percentage of CD3+ cells after 5-FU treatment of MC38 tumors yet interestingly the increase in CD4 or CD8 T cells was not significant. Were other CD3+ cells modulated by 5-FU therapy such as NKT cells or T cells to account for this difference? Although the effect on percentage of immune cells is shown in Fig. 4, the quantitation on the numbers of these immune subsets following 5-FU treatment should also be shown.

Response: We apologize for the confusing nature of the **old Figure 4**, which shows the

percentage of CD4+ and CD8+ cells within the CD3+ gate, and such percentages did not significantly change after 5-FU treatment, while total CD3+ cells within the CD45+ gate increased. So our interpretation was that both CD4+ cells and CD8+ cells increased after 5-FU treatment.

To avoid this confusion, and also to avoid concerns of preferential cancer cell death during single cell preparation from tumor tissue (raised by reviewer #3), we performed new experiments to quantify the absolute number of immune cells normalized by the weight of tumor tissue. This was achieved by carefully weighing the resected tumor mass and carefully processing samples to avoid cell loss during the whole procedure. After the tumor tissue was digested, we added defined amounts of synthetic counting beads. Both cells and beads were then quantified through flow cytometry. We then used the bead counts from the FACS to normalize the cell number of a given cell population to reflect the number of cells per gram of tumor. The new data of absolute cell numbers for CD3+, CD4+ and CD8+ cells are shown in **revised Figure 6**, as well as below, and the percentage data are shown in **revised Figure EV4**. These new data confirm that in WT MC38 tumors, the number of intratumoral CD4+ and CD8+ cells increased after 5-FU treatment.

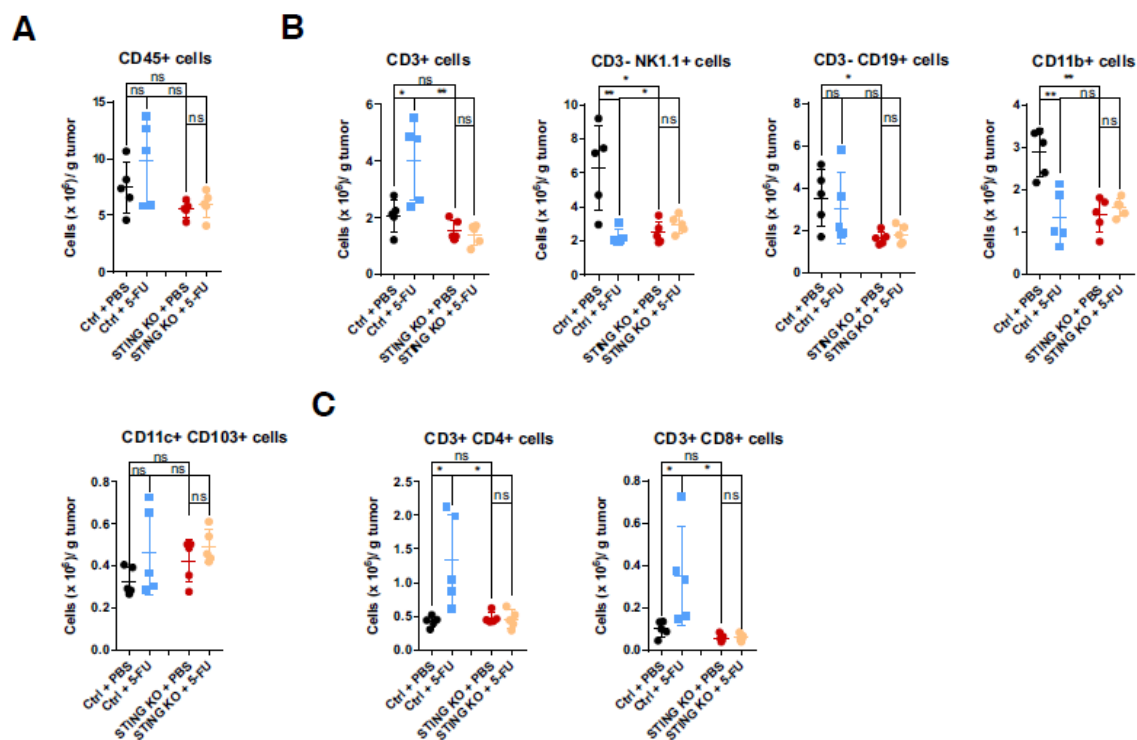


Figure 6. (A-C) Mice were injected with control (Ctrl) or STING-KO MC38 cells and treated with PBS or 5-FU. Tumors were harvested 2 weeks after cancer cell injection and intratumoral immune cells were examined by flow cytometry, and quantified as millions of cells per gram of tumor. Data shown are for **(A)** CD45+ cells, **(B)** CD3+, CD3-NK1.1+, CD3-CD19+, CD11b+ and CD11c+CD103+ cells, and **(C)** CD4+ and CD8+ T cells. For all panels, N=5. Error bars stand for standard deviation. *: $p < 0.05$; **: $p < 0.01$; ns: not significant. Data are representative of two independent experiments.

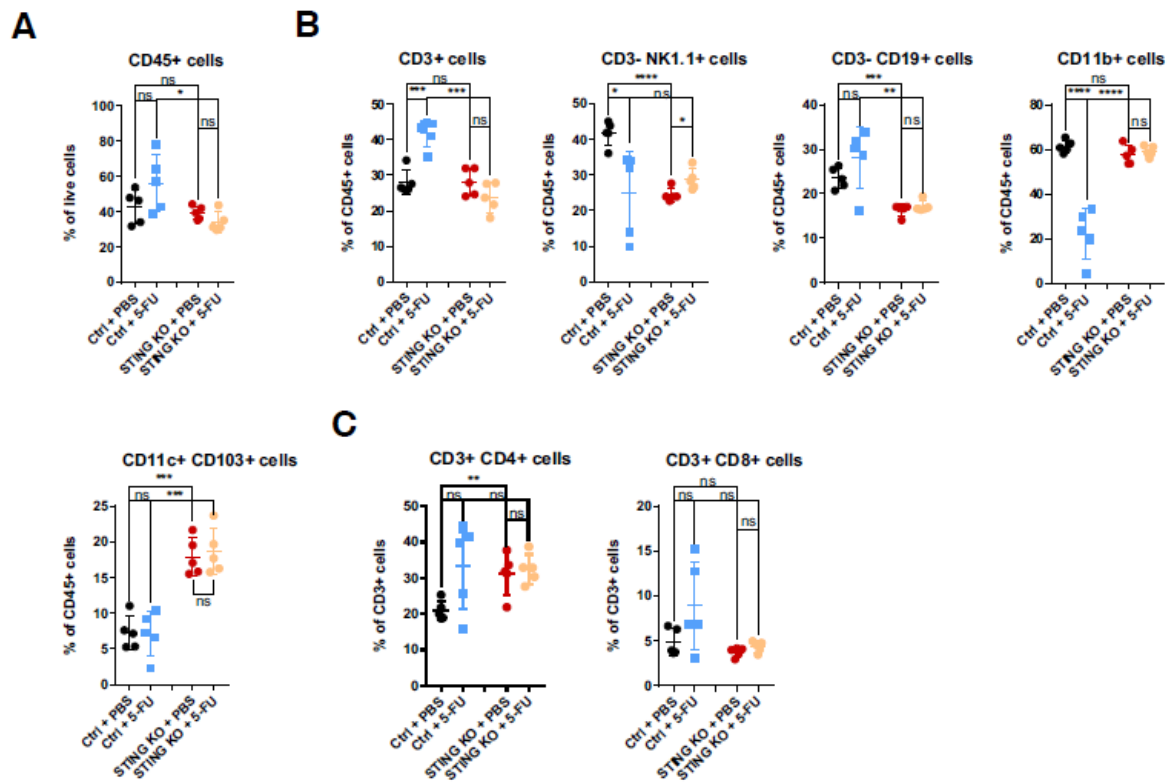
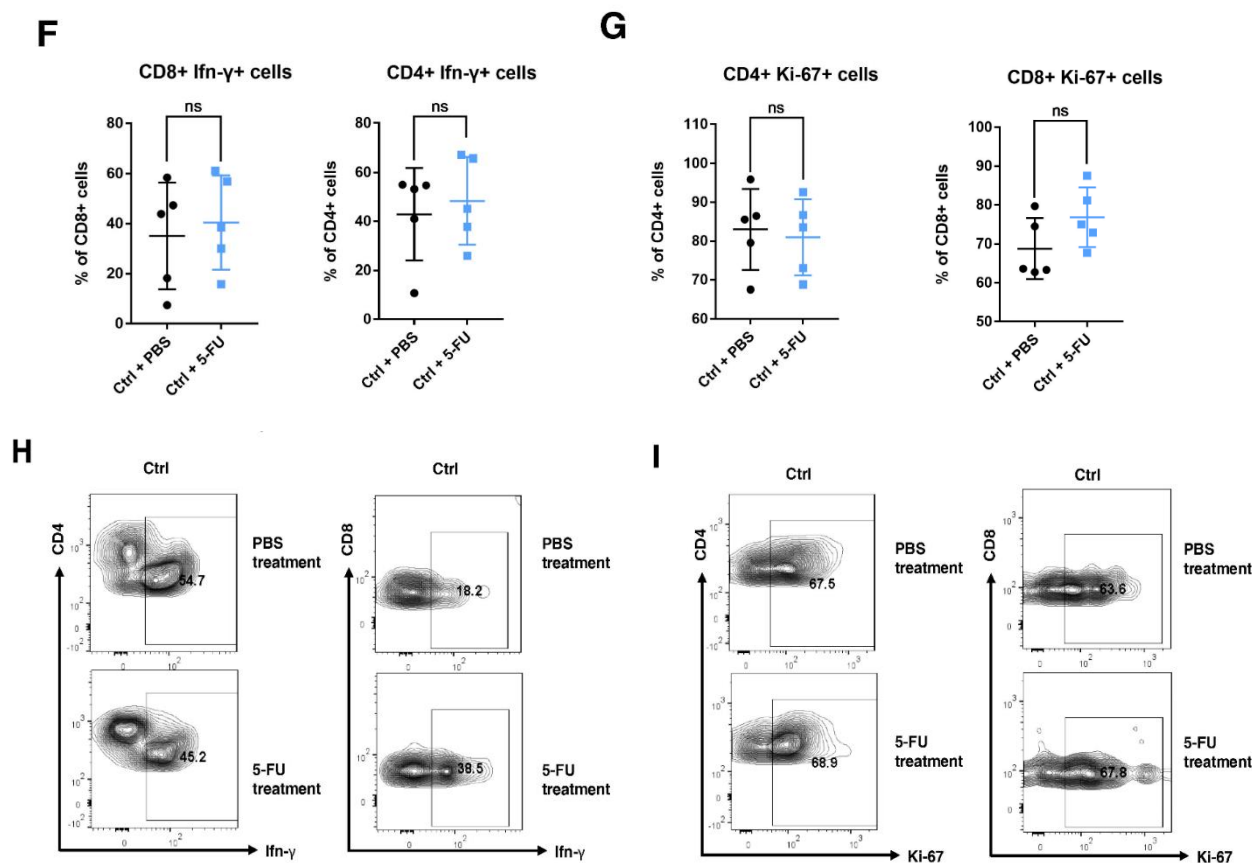


Figure EV4. (A-C) Mice were injected with control (Ctrl) or STING-KO MC38 cells and treated with PBS or 5-FU. Tumors were harvested 2 weeks after cancer cell injection and intratumoral immune cells were examined by flow cytometry. **(A)** The percentages of CD45+ cells among FSC and SSC-gated live cell population, **(B)** the percentages of CD3+, CD3⁻NK1.1⁺, CD3⁻CD19⁺, CD11b⁺ and CD11c⁺CD103⁺ cells among CD45+ cells, and **(C)** the percentages of CD4+ and CD8+ cells among CD3+ T cells are shown. For all panels, N=5. Error bars stand for standard deviation. *: p < 0.05; **: p < 0.01; ***: p < 0.001; ****: p < 0.0001; ns: not significant. Data for all panels are representative of two or more independent experiments.

Comment 5: Does 5-FU therapy through activation of the cGAS-STING pathway impact on the functional responses of intratumoral T cells (cytokine, proliferation). There is no data shown in the paper on this. Does 5-FU treatment induce a memory response in mice?

Response: To address the question on cytokine and proliferation responses of intratumoral T cells, we used intracellular flow cytometry to examine Ifn- γ ⁺ T cells and Ki-67⁺ T cells. The percentage of Ifn- γ ⁺ cells within CD4⁺ or CD8⁺ T cells did not significantly change, nor did the percentage of Ki-67⁺ cells within CD4⁺ or CD8⁺ gates (**revised Appendix Figure 1F, 1G, also shown below**). Representative flow cytometry plots are shown in **revised Appendix Figure 1H-I**, and also shown below.



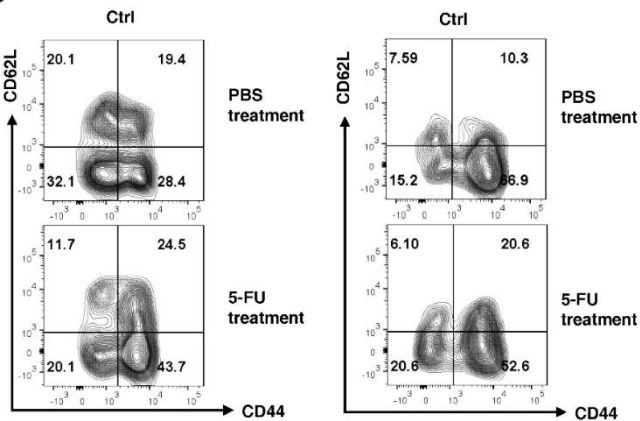
Appendix Figure 1. Control MC38 cells were injected into mice and mice were treated with 5-FU or PBS. Tumors were harvested at 2 weeks post cancer cell injection. **(F)** Percentages of intratumoral Ifn- γ + cells within CD8+ (left panel) or CD4+ (right panel) cells were quantified within CD8+ and CD4+ cells, respectively. **(G)** Percentages of intratumoral Ki67+ cells within CD4+ (left panel) or CD8+ (right panel) cells were quantified. Each dot represents one mouse. **(H)** Representative flow cytometry plots showing the percentages of Ifn- γ + cells within intratumoral CD4+ and CD8+ cells. **(I)** Representative flow cytometry plots showing the percentages of Ki-67+ cells within intratumoral CD4+ and CD8+ cells. For all panels, error bars represent standard deviation. ns: not significant.

To address the question on memory T cells, we performed two sets of experiments. First, we used CD44 and CD62L to further analyze intratumoral T cells within CD4+ and CD8+ gates, respectively. Other than a decrease of CD44⁺CD62L⁻ % within CD8 population and an increase of absolute numbers of CD62L⁺CD44⁺ CD8 cells after 5-FU treatment, we did not observe any significant changes in other populations (**revised Appendix Figure 1C-E, also shown below**).

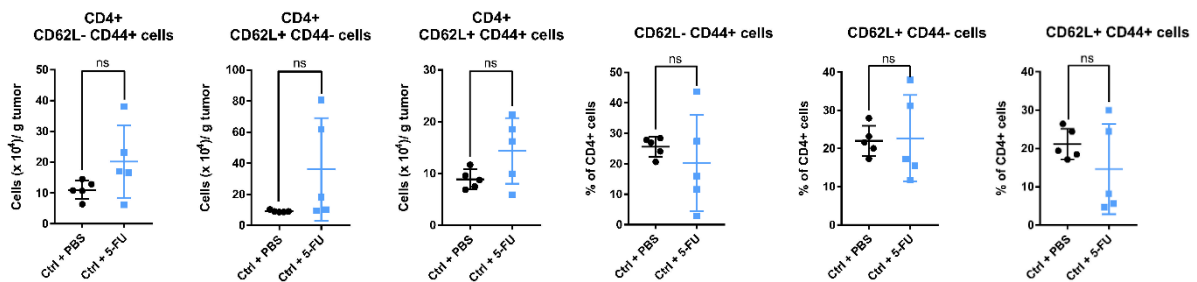
To address whether there is a functional anti-tumor memory induced by 5-FU, we performed the following experiment. First, the mice were injected with cancer cells subcutaneously on one of the two flanks. From day 4, PBS or 5-FU were given 3 times, once every other day. On day 10, we injected cancer cells on the other flank of the same mice. After another 10 days, the tumor size and weight were quantified from both flanks. **Figure R3** below shows that while 5-FU effectively reduced the first tumor (note that the reduction was less

compared to our other experiments, because only three doses of 5-FU was administered), the second tumor did not show difference between PBS and 5-FU groups, suggesting there is not a strong memory effect that can affect a distal site. We recognize that this experiment is limited by how long we can keep the mice due to the size of the first tumor becoming too big, and thus the conclusion is only suggestive rather than definitive. As such, we decided not to include these data in the revised manuscript.

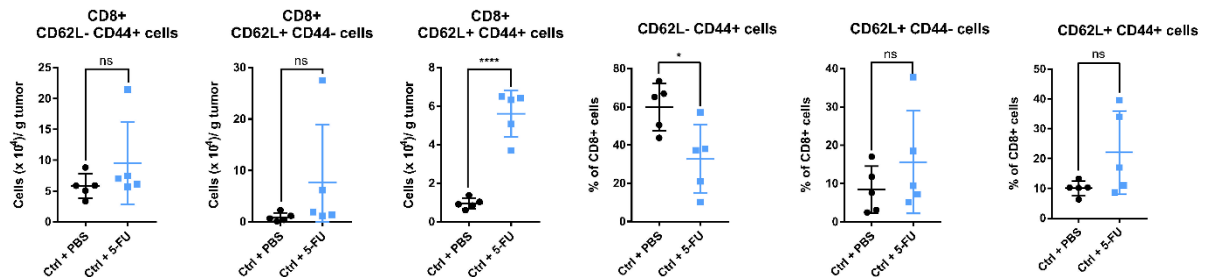
C



D



E



Appendix Figure 1. Control MC38 cells were injected into mice and mice were treated with 5-FU or PBS. Tumors were harvested at 2 weeks post cancer cell injection. **(C)** Representative flow cytometry plots showing CD62L and CD44 staining among intratumoral CD4⁺ (left panel) or CD8⁺ (right panel) cells. **(D-E)** The number of CD62L-CD44⁺, CD62L⁺CD44⁻, and CD62L⁺CD44⁺ cells per gram of tumor, as well as the percentages of these populations were quantified within **(D)** CD4⁺ or **(E)** CD8⁺ gates. Each dot represents one mouse. For all panels, error bars represent standard deviation. **: p < 0.01; ****: p < 0.0001; ns: not significant.

Figure for referees removed

Comment 6: Did 5-FU treatment impact on numbers of dendritic cells within the tumor and draining lymphnodes particularly with regard to CD11c+ CD103+ DC cells?

Response: We analyzed intratumoral CD11c+ and CD11c+CD103+ dendritic cells using flow cytometry. For WT MC38 tumors, 5-FU significantly reduced CD11c+ cells, but did not significantly change the CD11c+CD103+ cells, due to a relative enrichment of CD103+ cells within the CD11c+ gate. These data are included as **revised Figure 6B** (please refer to data in response to reviewer's comment #4 above), **Figure EV4B, and Appendix Figure 3**.

We did not perform experiments on draining lymphnodes, because it is unclear how to precisely define which lymphnodes are draining lymphnodes in our tumor models.

Comment 7: Can intratumoral injection of DMXAA or plpC induce abscopal effects against distant tumors in mice?

Response: The reviewer suggested an intriguing experiment, and we tried it out. We injected MC38 cancer cells on both flanks at the same time to induce two tumors in the same mouse. After 6 days, we performed intratumoral injection of DMXAA or plpC into one of the tumors and followed the changes of tumor mass on both flanks. As expected, the tumors directly treated by DMXAA or plpC were reduced in size. Interestingly, the untreated tumors in the

same mice were also significantly reduced, but the reduction was not as strong (**Figure R4 below**). The problem is with data interpretation. Although we were careful when performing the intratumoral injection of DMXAA and plpC, we cannot exclude the possibility that some of the chemicals leaked out of the treated tumor and thus may affect a distal tumor. So we decided not to include these data in the revised manuscript due to this concern.

Figure for referees removed

Comment 8: Have the authors examined whether low dose 5-FU can synergise with checkpoint blockade to enhance therapeutic effects?

Response: Experiments similar to those suggested by the reviewer have been reported by Dosset et al. *Oncoimmunology*, 2018 (**Reference 35** in our revised manuscript), in which a synergy between a 5-FU-based chemotherapy regimen and anti-PD-1 was observed, although the mechanism why there is synergy was not fully understood. Our proposed model thus may provide a mechanistic explanation to this reported observation. We discussed on this in the **revised Discussion**.

Reviewer #3:

Comment: This is an interesting study linking 5-FU sensitivity of cancer cells in vivo to intrinsic STING/IFN α /b and the tumor microenvironment (TME).

While the models have been well analysed and presented, the analysis of the corresponding TME cell compartments should be strengthened. Altogether it is a very interesting and timely study with high relevance to a better understanding of responders and non-responders to chemotherapy and the data raise a particular interesting link to the immune microenvironment. The suggestions below should guide the authors to solidify their conclusions.

Response: We thank the reviewer for considering our work interesting and timely, and for suggesting important revisions. We detail our responses below.

Major Comment 1: The FACS analysis of the tumors analysed in Fig. 4A-C and Fig. S6 are critical. Since tumors of very different sizes are analyzed the number of the different immune cells in relation to the number of tumor cells should be determined to make strong conclusions.

Response: We thank the reviewer for pointing this issue out. Additionally, we have considered the issue raised by this reviewer's major comment 3. What happened is that during the digestion of tumor tissue to prepare for single cell suspension for flow cytometry, some of the cancer cells die (this is a frequently encountered phenomenon seen by many laboratories). The preferential death of cancer cells during sample preparation in our model is the reason why the CD45+% can get close to 100% in some WT MC38 tumors after 5-FU treatment.

To overcome the issue raised in this comment and also that in comment 3, we performed new experiments to quantify the absolute number of immune cells within a gram of tumor tissue. This was achieved by carefully weighing the resected tumor mass and carefully processing samples to avoid cell loss during the whole procedure. After the tumor tissue was digested, we added defined amounts of synthetic counting beads. Both cells and beads were then quantified through flow cytometry. We then used the bead counts from the FACS to normalize the cell number of a given cell population to reflect the number of cells per gram of tumor. New data for old Fig 4A-C are shown as numbers of cells per gram of tumor (**revised Figure 6**, also shown below). The percentage of the populations are shown in **revised Figure EV4**. Representative flow cytometry plots are shown in **revised Figure EV5A-B**.

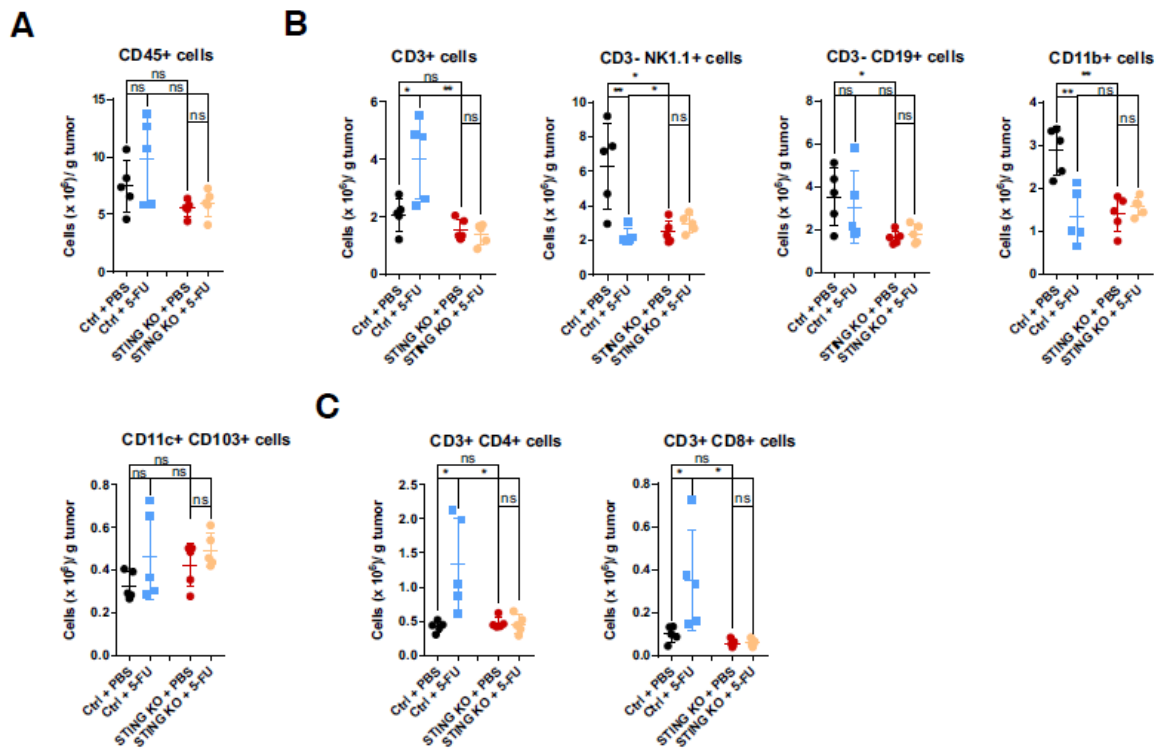


Figure 6. (A-C) Mice were injected with control (Ctrl) or STING-KO MC38 cells and treated with PBS or 5-FU. Tumors were harvested 2 weeks after cancer cell injection and intratumoral immune cells were examined by flow cytometry, and quantified as millions of cells per gram of tumor. Data shown are for **(A)** CD45+ cells, **(B)** CD3+, CD3-NK1.1+, CD3-CD19+, CD11b+ and CD11c+CD103+ cells, and **(C)** CD4+ and CD8+ T cells. For all panels, N=5. Error bars stand for standard deviation. *: $p < 0.05$; **: $p < 0.01$; ns: not significant. Data are representative of two independent experiments.

Major Comment 2: More extensive FACS panels including rationale pre-gatings to exclude certain populations should be used to better characterize the various myeloid and lymphoid cell populations within the TME. For example in S6B (left): is the change in the CD45 pattern after 5-FU due to the loss of myeloid cells compared to the STING KO samples? If yes, why? 5-FU is highly cytotoxic to myeloid cells, does STING KO protect from this?

Response: We have followed reviewer's suggestion, and performed new experiments to quantify the absolute number of immune cells normalized by the weight of tumor. We have also analyzed a number of new populations, including subsets of myeloid, dendritic, and T cells. These new data are included as **revised Figure 6, revised Figure EV4, EV5, and Appendix Figures 1-3.**

In terms of the reviewer's question on 5-FU's toxicity on myeloid cells, indeed 5-FU significantly reduced CD11b+ myeloid cells in WT MC38 tumors, and this toxicity is dependent on cancer-cell-intrinsic STING. These findings are revealed in the **revised Figure EV4B** (also shown below) that quantifies the percentage of CD11b+ cells within CD45+ cells. These data are accompanied by those on absolute normalized cell counts (**revised Figure 6B**, see above).

The reviewer is also correct that the reduction of the CD45-high population after 5-FU treatment is due to a reduction of CD11b+ myeloid cells, as in **revised Figure EV5B** (also shown below). We further used F4/80, Ly6C and Ly6G to characterize the specific myeloid population affected (please see response to reviewer's major comment #4). Although we do not know the exact mechanism why the cancer cells' STING status can determine myeloid cell toxicity, we speculate that type I IFNs produced from cancer cells trigger myeloid cell death. We discussed this point in the revised manuscript.

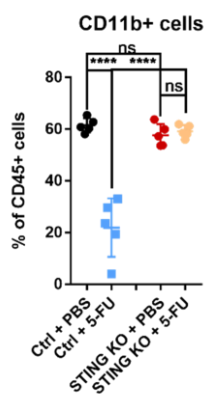


Figure EV4. (B) The percentages of CD11b+ cells among CD45+ cells were quantified. N=5 from a representative experiment. For all panels, error bars stand for standard deviation. ****: $p < 0.0001$; ns: not significant. Data are representative of two independent experiments.

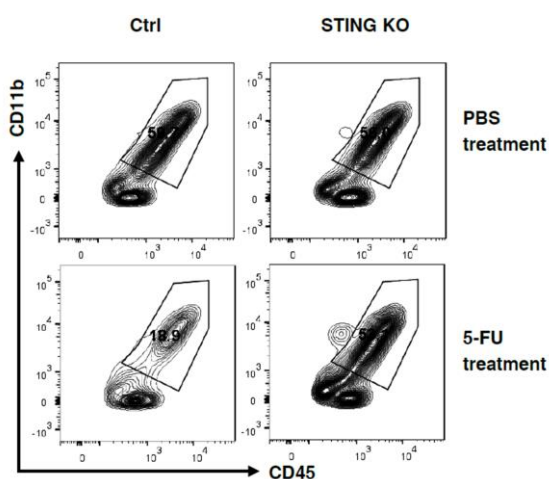


Figure EV5. (B) Representative flow cytometry plots of CD11b and CD45, in WT or STING-KO tumors treated with PBS or 5-FU.

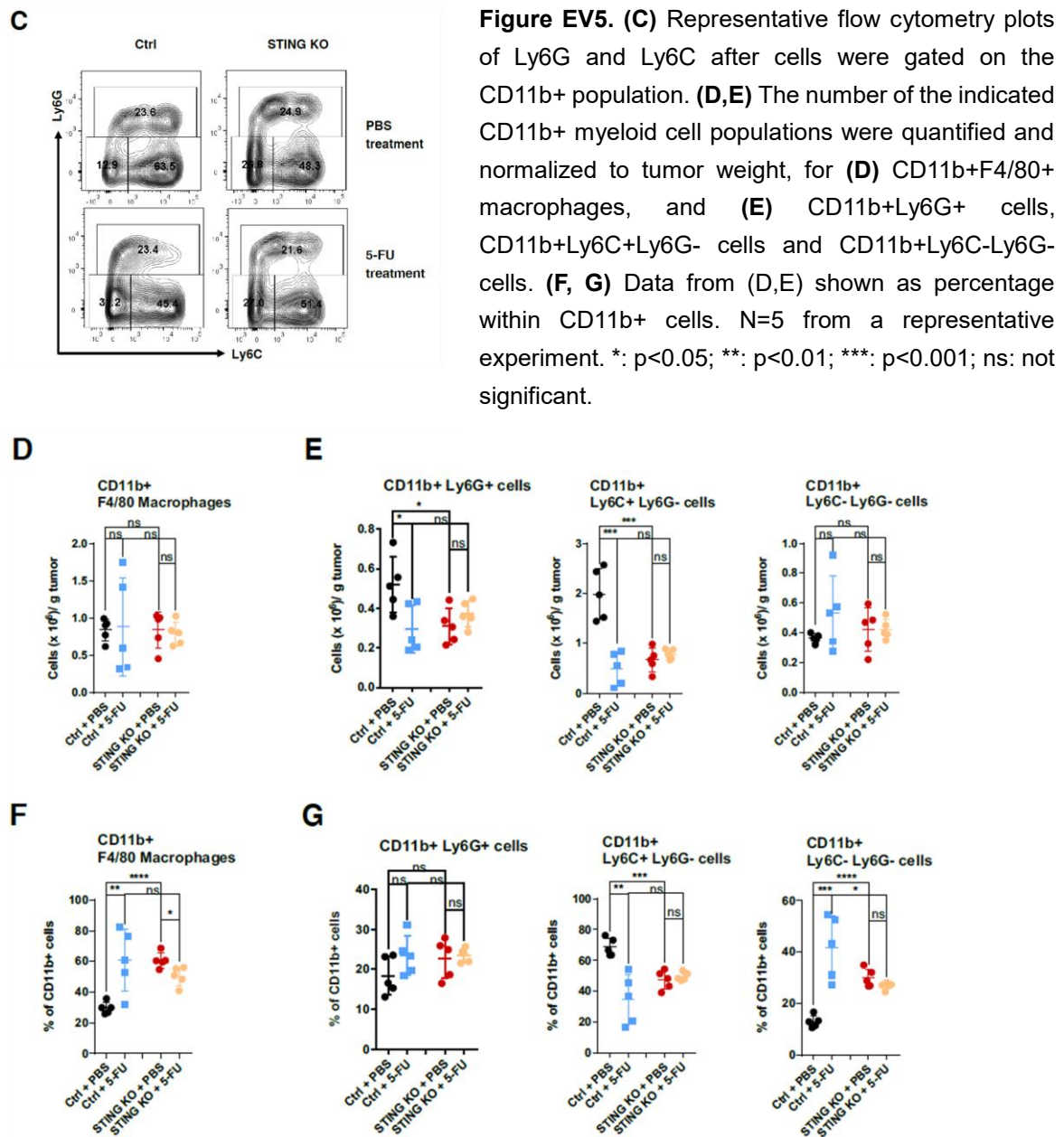
Major Comment 3: In Fig. 4A-C about 20-40% of cells are CD45+; what are the rest - tumor cells? Some show 100% CD45+ cells (Fig. 4A), can this be explained?

Response: We addressed the near 100% CD45+ cell issue in response to the reviewer's major comment 1. As for the nature of CD45- cells, we believe the vast majority are cancer cells.

Major Comment 4: The decline of myeloid cells in controls is very interesting. Next to CD11b also other markers such as Gr1 and F4/80 and other should be included in a co-staining to better characterize the changing myeloid cell types.

Response: Following the reviewer's suggestion, we have further characterized the changes

in myeloid cell populations after 5-FU treatment. We define macrophages as CD11b+F4/80+ cells. Due to Gr1 antibody recognizing both Ly6C and Ly6G antigens, we have used CD11b together with Ly6C and Ly6G to characterize intratumoral myeloid cells. Quantification based on the number of cells per gram of tumor shows that 5-FU induced a significant reduction of CD11b+ cells in WT MC38 tumors. Furthermore, Ly6C+Ly6G- cells (both absolute number and percentage within CD11b+ cells) were reduced, which seems to be the main contributor to this reduction (**revised Figure 6B and EV5C-G**). **Figure EV5C-G** is also shown below.



Major Comment 5: This reviewer is not sure how much Figure 7 contributes to the storyline. The high production of interferons in response to plpC may simply lead to a blockade of cancer cell proliferation (testable in vitro). These data should be considered to either be taken out or supplemented with an analysis directly showing that at least some of the effects are mediated

by immune cells (along the storyline) rather than directly inhibiting tumor cell growth.

Response: We agree with the reviewer that the old Figure 7 does not contribute significantly to the storyline. We have also performed additional experiments on this pancreatic cancer model in response to reviewer 1's comment 2. Overall, we feel that it is difficult to reach firm conclusions on which cells responded to intratumoral plpC in vivo (cancer cell, immune cells and other stromal cells). So we followed the reviewer's suggestion and removed the data in old Figure 7 (and the accompanying old Figure S7).

Minor Point 1: Page 5: should say "spleen growth" is different in 5-FU treated mice?

Response: We thank the reviewer for pointing this out. We feel that a better word to describe the enlargement of spleen in the presence of tumor is "splenomegaly", a word that has been used frequently to describe "spleen growth" or "spleen enlargement". So we replaced "spleen enlargement" with splenomegaly.

Minor Point 2: Page 5: The lack of STING in the microenvironment does have a significant effect (which is interesting and could be discussed) and should not be played down so much, it does not jeopardize the storyline.

Response: We agree with the reviewer. We have reworded sentences in the text to reflect this point.

Minor Point 3: Page 6 bottom: reference to S1D and 5-FU concentrations seems wrong/ or not shown?

Response: We thank the reviewer for catching this omission. We have now referenced twice the **revised Figure EV1E** (old Fig S1D) in the revised text and mentioned about 5-FU concentrations.

Minor Point 4: Fig. 3D: did T cells already readily reconstitute at 45 days?

Response: We routinely perform bone marrow transplantation in our laboratory. A typical experiment is shown below in **Figure R7**, which indicates that CD3+ T cells have been largely reconstituted one month after transplantation, accounting for ~33% of all CD45+ mononuclear cells within peripheral blood.

Figure for referees removed

Minor Point 5: Page 9 last paragraph: should it no say one day prior "5-FU injection" (rather than "WT MC38")

Response: We thank the reviewer for pointing out this error. It has been corrected.

Minor Point 6: S7C: poor quality H&E staining

Response: We have removed the old Figure 7 and old Figure S7 in response to the reviewer's major comment 5.

Minor Point 7: Are type III interferons differentially expressed?

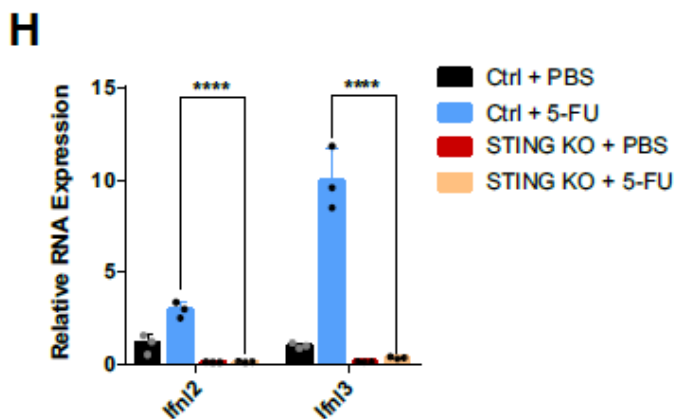


Figure EV1. (H) WT (Ctrl) or STING-KO MC38 cells were treated with 5-FU or vehicle control for 24 hours. Cells were analyzed for Ifnl2 and Ifnl3 RNA levels using qRT-PCR. N=3. For all panels, error bars represent standard deviation. ****: $p < 0.0001$.

Response: Following the reviewer's suggestion, we measured type III interferon expression. Due to murine Ifnl1 being a pseudogene, we measured Ifnl2 and Ifnl3 RNA levels after 5-FU treatment in both control and STING KO MC38 cells. The result showed that type III interferons can be induced by 5-FU treatment, and the induction is also dependent on STING (**revised Figure EV1H, and also shown above**). This is consistent with literature (e.g. Kim et al, Journal of Investigative Dermatology, 137(10):2101-2109, 2017) that type III IFN (IFN- λ) induction is partly through a STING-dependent pathway.

Minor Point 8: A model describing the cancer and immune cell effects would be helpful to guide the reader along.

Response: Following the reviewer's suggestion, we now include a diagram of our model as the **revised Figure 9E**.

Dear Dr Lu,

Thank you for submitting your revised manuscript (EMBOJ-2020-106065R) to The EMBO Journal. Please accept my sincere apologies for getting back to you with unusual delay due to protracted reviewer input. Your amended study was sent back to all referees for re-evaluation, and we have received comments from referee #3, which I enclose below. Please note that while referees #1 and #2 were at this time not able to reassess the work, we have editorially evaluated your response to their concerns and found them to be convincingly addressed. As you will see, the other referee stated that his/her issues have been comprehensively resolved and s/he is now broadly in favour of publication.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal, pending a number of minor points related to formatting and data representation as detailed below, which should be addressed at re-submission.

Further, I will share additional changes and comments from our production team during the next days to be considered.

Please contact me at any time if you have additional questions related to below points.

As you might have noticed, every paper at the EMBO Journal now includes a 'Synopsis', displayed on the html and freely accessible to all readers. The synopsis includes a 'model' figure as well as 2-5 one-short-sentence bullet points that summarize the article. I would appreciate if you could provide this figure and the bullet points.

Thank you for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to your final revision.

Again, please contact me at any time if you need any help or have further questions.

Kind regards,

Daniel Klimmeck

Daniel Klimmeck PhD
Senior Editor
The EMBO Journal

Formatting changes required for the revised version of the manuscript:

>> Please provide a separate 'Conflict of Interest' statement, and add up to five keywords to your manuscript.

>> Release the privacy from your GEO data set.

- >> Please specify distinct author contributions for L.W. .
- >> Adjust the reference format to EMBO Journal style, limiting to 10 authors et al. .
- >> Add a ToC on the first page of the Appendix file.
- >> Provide figures and EV figures as individual, high-resolution .tiff files. The figure legends should stay in the manuscript.
- >> Recheck figure callouts and their correct order in the main text: Fig 2E is called out after Fig 3A, Fig EV 1E-G are called out after Fig EV2 A-G; Fig EV 4C is called out after Fig EV5A-G.
- >>Mention reuse of 5-FU control tumor data display 4B in the legend of Fig 5C.
- >> EV figure legends should be added to the MS, after main figure legends.

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

Further information is available in our Guide For Authors:
<https://www.embopress.org/page/journal/14602075/authorguide>

The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 23rd Mar 2021.

Link Not Available

Referee #3:

The authors extensively revised and improved the study, which strengthened their conclusions. This reviewer has no other comments or concerns.

The authors performed the requested editorial changes.

Dear Dr Lu,

Thank you for submitting the revised version of your manuscript. I have now evaluated your amended manuscript and concluded that the remaining minor concerns have been sufficiently addressed.

Thus, I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. I would thus like to ask for your consent on keeping the additional rebuttal figures included in this file.

Also in case you might NOT want the transparent process file published at all, you will also need to inform us via email immediately. More information is available here:
http://emboj.embopress.org/about#Transparent_Process

Please note that in order to be able to start the production process, our publisher will need and contact you regarding the following forms:

- PAGE CHARGE AUTHORISATION (For Articles and Resources)

[http://onlinelibrary.wiley.com/journal/10.1002/\(ISSN\)1460-2075/homepage/tej_apc.pdf](http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1460-2075/homepage/tej_apc.pdf)

- LICENCE TO PUBLISH (for non-Open Access)

Your article cannot be published until the publisher has received the appropriate signed license agreement. Once your article has been received by Wiley for production you will receive an email from Wiley's Author Services system, which will ask you to log in and will present them with the appropriate license for completion.

- LICENCE TO PUBLISH for OPEN ACCESS papers

Authors of accepted peer-reviewed original research articles may choose to pay a fee in order for their published article to be made freely accessible to all online immediately upon publication. The EMBO Open fee is fixed at \$5,200 (+ VAT where applicable).

We offer two licenses for Open Access papers, CC-BY and CC-BY-NC-ND.

For more information on these licenses, please visit: <http://creativecommons.org/licenses/by/3.0/> and http://creativecommons.org/licenses/by-nc-nd/3.0/deed.en_US

- PAYMENT FOR OPEN ACCESS papers

You also need to complete our payment system for Open Access articles. Please follow this link and select EMBO Journal from the drop down list and then complete the payment process:

https://authorservices.wiley.com/bauthor/onlineopen_order.asp

Should you be planning a Press Release on your article, please get in contact with embojournal@wiley.com as early as possible, in order to coordinate publication and release dates.

On a different note, I would like to alert you that EMBO Press is currently developing a new format for a video-synopsis of work published with us, which essentially is a short, author-generated film explaining the core findings in hand drawings, and, as we believe, can be very useful to increase visibility of the work.

Please see the following link for a representative example:

https://www.embopress.org/video_synopses

The videos are embedded in the respective article html page, see e.g.

<https://www.embopress.org/doi/abs/10.15252/embojournal.2019103009>

Please let me know, should you be interested to engage in commissioning a similar video synopsis for your work. According operation instructions are available and intuitive.

If you have any questions, please do not hesitate to call or email the Editorial Office.

Thank you again for this contribution to The EMBO Journal and congratulations on a successful publication! Please consider us again in the future for your most exciting work.

Kind regards,

Daniel Klimmeck

Daniel Klimmeck, PhD
Senior Editor
The EMBO Journal
EMBO
Postfach 1022-40
Meyerhofstrasse 1
D-69117 Heidelberg
contact@embojournal.org
Submit at: <http://emboj.msubmit.net>

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Jun Lu
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2020-106065

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if $n < 5$, the individual data points from each experiment should be plotted and any statistical test employed should be justified.
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
 - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
 - are tests one-sided or two-sided?
 - are there adjustments for multiple comparisons?
 - exact statistical test results, e.g., P values = x but not P values < x;
 - definition of 'center values' as median or average;
 - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Animal experiments generally used 5-10 mice. These numbers were estimated based on initial experiments to detect potential changes. Sample numbers of other experiments were similarly determined.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	We have included such a sentence in the methods, under "Mice".
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	We did not exclude data from analyses for successfully measured samples or animals. Collections of samples for which measurements were not successful (e.g. without a band in western blot even for positive control) were not considered in analysis.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	In animal experiments, mice were of similar age, and were randomly divided into groups. The weight and health status of mice in each group were similar.
For animal studies, include a statement about randomization even if no randomization was used.	We have included a sentence on randomization in the methods, under "Mice".
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	The experiments were primarily performed by one of the investigators. No blinding was designed.
4.b. For animal studies, include a statement about blinding even if no blinding was done	We have included a sentence on the absence of blinding in the methods, under "Mice".
5. For every figure, are statistical tests justified as appropriate?	Yes, we have evaluated the statistical tests and deem them as appropriate.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We used two-tailed unpaired student's t-test for comparisons between the means of two variables. We also used Welch's t-test, which resulted in similar conclusions. For the types of experiments requiring statistical tests, it is well recognized that they are under the influence of multiple stochastic factors, thus making the assumption of normal distribution appropriate. No explicit method was used to verify the distribution of our measurements.
Is there an estimate of variation within each group of data?	We also used Welch's t-test, which assumes unequal variance, and reached similar conclusions.

USEFUL LINKS FOR COMPLETING THIS FORM

<http://www.antibodypedia.com>
<http://1degreebio.org>
<http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor>

<http://grants.nih.gov/grants/olaw/olaw.htm>
<http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm>
<http://ClinicalTrials.gov>
<http://www.consort-statement.org>
<http://www.consort-statement.org/checklists/view/32-consort/66-title>

<http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tum>

<http://datadryad.org>

<http://figshare.com>

<http://www.ncbi.nlm.nih.gov/gap>

<http://www.ebi.ac.uk/ega>

<http://biomodels.net/>

<http://biomodels.net/miriam/>
<http://jij.biochem.sun.ac.za>
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
<http://www.selectagents.gov/>

Is the variance similar between the groups that are being statistically compared?	We also used Welch's t-test, which assumes unequal variance, and reached similar conclusions
---	--

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	We purchased antibodies from well-trusted commercial sources that carry out their quality control tests routinely. We have included the vendor, catalog number, clone number when available, as well as the dilutions that we have used in our experiments.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	We have indicated the sources of the cell lines used in this study. We have not tested them for mycoplasma contamination.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	We have detailed all of these in the methods, under "Mice".
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	We have indicated that studies were under an approved protocol by Yale University's Institutional Animal Care and Use Committee (IACUC).
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	All animal experiments were in compliance with guidelines.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	We have included such a section and indicated that RNA-Seq data from this study has been submitted to GEO (GSE160985).
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	RNA-seq data have been deposited.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biocompare (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	No open-source or custom code was used to collect data for this paper.

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA
---	----