nature research

Peer Review Information

Journal: Nature Immnuology
Manuscript Title: The ubiquitin ligase MDM2 sustains STAT5 stability to control T cell-mediated anti-tumor immunity
Corresponding author name(s): Weiping Zou

Editorial Notes:

Transferred manuscripts This manuscript has been previously reviewed at another journal that is not operating a transparent peer review scheme. This document only contains reviewer comments, rebuttal and decision letters for versions considered at Nature Immunology.

Reviewer Comments & Decisions:

Decision Letter, initial version:

Subject: Decision on Nature Immunology submission NI-A29928-T
Message: 12th Jun 2020

Dear Weiping,
Thank you for your response letter detailing how you will address the issues raised by the Referees. I've now discussed this with my colleagues and we are willing to consider a revision that addresses all the concerns of the Referees as outlined in your response letter.

Please note, the Referees are not especially supportive of this manuscript so I suspect a thorough revision would be required and we'd be looking for a clear endorsement following re-review. The Referees raise a substantial number of issues

We hope you will find the referees' comments useful as you decide how to proceed. If you wish to submit a substantially revised manuscript, please bear in mind that we will be reluctant to approach the referees again in the absence of major revisions. For instance the rescue experiment suggested by Ref. 2 (activation of STAT5 in the MDM2 deficient CTL) will likely be essential to convince them.

Please do not hesitate to get in touch if you would like to discuss these issues further or if there are lockdown-related problems. Please also let us If you choose to revise your manuscript taking into account all reviewer and editor comments, please highlight all changes in the manuscript text file.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

If revising your manuscript:

* Include a “Response to referees” document detailing, point-by-point, how you addressed each referee comment. If no action was taken to address a point, you must provide a compelling argument. This response will be sent back to the referees along with the revised manuscript.

* If you have not done so already please begin to revise your manuscript so that it conforms to our Article format instructions at http://www.nature.com/ni/authors/index.html. Refer also to any guidelines provided in this letter.

* Include a revised version of any required reporting checklist. It will be available to referees (and, potentially, statisticians) to aid in their evaluation if the manuscript goes back for peer review. A revised checklist is essential for re-review of the paper.

The Reporting Summary can be found here:
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If you wish to submit a suitably revised manuscript we would hope to receive it within 6 months. If you cannot send it within this time, please let us know. We will be happy to consider your revision so long as nothing similar has been accepted for publication at Nature Immunology or published elsewhere. Should your manuscript be substantially delayed without notifying us in advance and your article is eventually published, the received date would be that of the revised, not the original, version.

Nature Immunology is committed to improving transparency in authorship. As part of our efforts in this direction, we are now requesting that all authors identified as 'corresponding author' on published papers create and link their Open Researcher and Contributor Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from the home page of the MTS by clicking on 'Modify my Springer Nature account'. For more information please visit please visit www.springernature.com/orcid.

Please do not hesitate to contact me if you have any questions or would like to discuss the required revisions further.

Thank you for the opportunity to review your work.

Sincerely,

Zoltan Fehervari, Ph.D.
Senior Editor
Nature Immunology

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Referee expertise:

Referee #1: p53, cancer immunology

Referee #2: cancer immunology

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
Reference Number: NI-A29928-T

Title: MDM2 sustains STAT5 stability to control T cell-mediated anti-tumor immunity

The manuscript by Zhou et al. show that loss of MDM2 in T cells affects cancer progression of transplanted cell lines into mice. The authors find that MDM2 controls STAT5 protein expression via inhibition of Cbl-mediated degradation of STAT5. They show that APG115 stabilizes MDM2 and p53 in T cells and delays tumor growth of various cell lines in mice. This effect is independent of p53 status of the cell lines, but dependent on p53 expression in T cells. Depletion of CD8 T cells reverses tumor growth of APG115-treated mice indicating that CD8 T cells are required for the efficacy of APG115. The authors also show an association between high expression of MDM2 in human T cells and an anti-tumor phenotype.

In recent years, there have been a few studies addressing the role of p53 in immune cells during inflammation and cancer. Scott Lowe's lab has done this in macrophages (Lujambio et al., Cell 2013). One study has shown that down-regulation of p53 is required for CD4 T cell response (Watanabe et al. Immunity 2014). Inhibiting MDM2 sustains p53 expression and prevents T cell proliferation – these data seem to contradict the data under review here. Another study reported that APG115 monotherapy (at the same dosage used here 10mg/kg) has no affect on tumor growth using MH-22A or MC38 cell lines (Fang et al., J for Immunothearpy of Cancer 2019). These data are also different to the data presented here.

Overall, the study presents new mechanistic data on the role of MDM2 in anti-tumor T cells. The evidence includes both gain-of-function and loss-of-function experiments, as well as genetic and pharmacological approaches to test the hypothesis. Most experiments are well controlled. It is difficult to get a sense of the robustness of the data, especially the western blots, since the variation between replicates is not shown. Some conclusions are over-stated and not support by evidence. Some methodological details are missing. Below are some comments to help strengthen the manuscript:

1. Figures 1-3: The link between MDM2 and p53 is not clear from the data presented. How does loss or silencing of Mdm2 in T cells affect p53 expression levels? Is the increase in T cell death and decrease in Bcl2 family members seen in Mdm2-deficient T cells dependent on p53? How do these data relate to the Watanabe study on CD4 T cells?
2. The figures include many western blots without quantification. Therefore it is difficult to determine the extent of variation between replicate experiments. The authors should include graphical representation of their western blots throughout the manuscript.
3. Point out to reader that both CD4 and CD8 T cells are affected in Cd4-Cre mice, as most people will not know this trick and please provide a reference. Referring to Cd4-Cre;Mdm2F/F mice as Mdm2–/– mice and Cd4-Cre;Trp53F/F mice as p53–/– mice suggests that knockout mice have been used, which is confusing. I suggest that the authors use Cd4-Cre;Mdm2F/F mice and Cd4-Cre;Trp53F/F mice throughout the text for clarity.

4. In Figure 1, it is unclear whether the reduction in T cell survival from Cd4-Cre;Mdm2F/F mice is dependent on the tumor. What are numbers of CD4s and CD8s like in the thymus, spleen, lymph nodes etc compared to controls? Are T cells less abundant in Mdm2-deficient mice? Are there any differences, such as lymphoid organ pathology or body weight, between Cd4-Cre;Mdm2F/F mice and controls? How does antigen independent proliferation compare between CD8 T cells from Cd4-Cre;Mdm2F/F mice and controls? These mice need further characterization. Results of this comparison will affect the interpretation of the tumor growth curves.
5. I have never seen such nice staining for Granzyme B with clone GB11 or magnitudes of expression reaching 50%. My lab cannot do this. How were the cells stimulated ex vivo? What concentration of antibody was used? This information was not included in the Methods section.
6. In Figure 3, the biochemical interaction between MDM2, STAT5 and Cbl is shown. How is proliferation of T cells affected by loss of Mdm2 or Cbl? Can silencing of Cbl rescue decreased proliferation of Mdm2-deficient cells?
7. Cd4-Cre;Trp53F/F mice are prone to inflammatory disease and smaller when compared to controls (Kawashima et al. JI 2013, 191:000), and these differences should be pointed out to the reader. Were there any signs of leukemia or lymphoma in Cd4-Cre;Trp53F/F mice? Were the mice in Figure 4j age matched or weight matched for experiments?
8. The data in Figure 4 are not convincing for some tumor models, such as B16 in 4C and CT26 in 4E – even if these differences managed to reach statistical significance, I disagree with the interpretation that APG115 slows tumor growth. It seems the B16 tumors were grown to nearly double the size of other tumor models, surpassing 2000 mm3 at the end of the experiment. I don't believe this is ethical. The experiment seems forced to generate a mediocre difference. The way in which tumor volume was calculated is missing from the Methods. It does not appear that tumor-bearing mice were randomized in any way before treatment.
9. Controls are missing from Figure 5a. Please show tumor growth between wild-type and NSG mice. Presumably, MC38 cells grow faster in NSG mice than wild-type if CD8 T cells are involved.
10. There is insufficient evidence for the statement made at the top of page 12, "Thus, APG115 regulates CD8 T cell survival and function through MDM2-dependent STAT5 stabilization." This statement can only be supported by blocking STAT5 in APG115-treated cells/tumors.
11. Statistical analysis is incorrect throughout the manuscript: one-way ANOVA should be used for 3 groups or more, and Mann-Whitney should be used for 2 groups, not a two-tailed t test. Error bars and statistical analysis are missing from Extended Data Figure 6a-c.
12. Please include more details in the Methodology for every experiment. How much protein was loaded on SDS-PAGE gels? How was IP performed? How much pull down antibody was used?

Reviewer #2:
Remarks to the Author:

In this paper, Zhou et al. analyzed the role of MDM2-P53 pathway in CD8+ T cells antitumor responses. The results highlight the expression of MDM2 on governing T cell-mediated tumor control and the loss of MDM2 expression in CD8+ T cells led to increased apoptosis and failure to produce cytotoxic cytokines. The authors have found that MDM2, by interacting with c-Cbl, prevents the interaction between c-Cbl and STAT5, which in turn prevents STAT5 degradation. The MDM2-mediated stabilization of STAT5 might be responsible for improved survival and effector function of CD8+ T cells infiltrating the tumor. The authors showed that the drug APG115, which is known to impede the interaction between MDM2 and p53, could enhance MDM2 expression and therefore be used as an immune modulator. Tumor bearing mice treated with APG-115 showed stronger anti-tumor responses in a CD8+ T cell-dependent manner and the therapeutic benefits of APG-115 relies on the presence of p53 and MDM2 in T cells. Moreover, Zhou et al. showed that APG-115 synergizes with PD-1 blockade to enhance antitumor immune responses and that MDM2 expression correlates with functional T-cell responses in cancer patients.
In fact, Watanabe et al. in 2014 have shown that downmodulation of p53 mediated by TCR is critical for antigen specific T cell proliferation. Moreover, a work from Banerjee et al. in 2016 has demonstrated that the absence of p53 in T cells leads to increased effector function and tumor control. On this line, the lack of MDM2 would prevent p53 downmodulation and therefore alters cytotoxic T-cell response. These two publications are not cited and discussed and the Banerjee's work presented contradictory results. In addition, Fang et al. in 2019 have shown that the drug APG-115 in combination with PD-1 blockade is able to reduce wt, mutated or p53 KO tumor (MH-22Awt, MC38 and MH-22Amut), but this study is not cited and discussed as well. Even though the finding of the mechanism through which MDM2 regulates CD8 antitumor response via STAT5 is interesting, the novelty of this work is compromised by several previous publications and the results presented here do not demonstrate MDM2-mediated enhancement of antitumor responses is controlled by increased STAT5 expression.

Specific points could be addressed to improve the significance of the work:

• Given the importance of STAT5 in CD8+ T cell activation, could be possible to exclude that CD8+ T cell lacking MDM2 are sub-optimally activated, which results in impaired effector functionality? The analysis of activation markers such as CD44 and inhibitory receptors such as PD-1 could help in defining the dysfunctional status of MDM2 KO CD8+ T-cells.
• To confirm that the effect of MDM2 expression is mediated by the presence of STAT5, would be important to prove that overexpression of STAT5 could rescue the generation of antitumor CD8+ T cell response in the absence of MDM2 or silencing STAT5 in T cells. This analysis is critical to support their claim that MDM2 modulate T cell anti-tumor responses via stabilization of STAT5.
• To further validate the interaction of MDM2 with c-Cbl, would be important to show in Figure 3h the level of myc-MDM2 in IP:HA.
• The fact that APG-115 treatment requires wt p53 gene in T cells points out that the effect reached with the treatment is not exclusively mediated by MDM2-c-Cbl-STAT5 axis. Would be important to

further dissect this point to reveal the mechanism of action of APG-115 beside p53. Would be interesting to show that the expression of STAT5 can rescue the functionality of CD8+ T-cell lacking p53 and MDM2 under APG-115 treatment or that lack of c-Cbl would also improve T-cell function in absence of MDM2 under APG-115 treatment. Moreover, the discrepancy between this study and Banerjee's study published in 2016 should be discussed.

• The depletion of MDM2 in CD4 expressing cells doesn't exclude a possible role in CD4 T-cells. Would be interesting to explore this point? On this line, the work of Fang et al. in 2019 has shown that APG-115 treatment enhances CD4+ T- cell activation and, in combination with PD-1 blockade, increases the percentage of CD4+ T-cells which produce IFNγ.

Author Rebuttal to Initial comments

Reviewer #1

(Remarks to the Author)
Reference Number: NI-A29928-T
Title: MDM2 sustains STAT5 stability to control T cell-mediated anti-tumor immunity

The manuscript by Zhou et al. show that loss of MDM2 in T cells affects cancer progression of transplanted cell lines into mice. The authors find that MDM2 controls STAT5 protein expression via inhibition of Cbl-mediated degradation of STAT5. They show that APG115 stabilizes MDM2 and p53 in T cells and delays tumor growth of various cell lines in mice. This effect is independent of p53 status of the cell lines, but dependent on p53 expression in T cells. Depletion of CD8 T cells reverses tumor growth of APG115-treated mice indicating that CD8 T cells are required for the efficacy of APG115. The authors also show an association between high expression of MDM2 in human T cells and an anti-tumor phenotype.

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Overall, the study presents new mechanistic data on the role of MDM2 in anti-tumor T cells. The evidence includes both gain-of-function and loss-of-function experiments, as well as genetic and pharmacological approaches to test the hypothesis. Most experiments are well controlled. It is difficult to get a sense of the robustness of the data, especially the western blots, since the variation between replicates is not shown. Some conclusions are over-stated and not support by evidence. Some methodological details are missing. Below are some comments to help strengthen the manuscript:

Response: We appreciate the reviewer's encouraging comments. The reviewer indicated that our study presents new mechanistic data on the role of MDM2 in anti-tumor T cells, and most experiments are well controlled. We have experimentally addressed their critiques and concerns (see point-by-point responses).

We thank the reviewer for making reference to the work from the Lowe group. Dr. Lowe has shown that p53-expressing senescent stellate cells stimulate immune surveillance by polarizing M1 macrophages and activating natural killer (NK) cells. They did not explore the cross-talk between MDM2 and STAT5 in T cells and T cell-mediated anti-tumor immunity. We have discussed this and other reviewer-referred articles in the revision. Please also see our general responses and specific responses to Reviewer #2.

1. Figures 1-3: The link between MDM2 and p53 is not clear from the data presented. How does loss or silencing of Mdm2 in T cells affect p53 expression levels? Is the increase in T cell death and decrease in Bcl2 family members seen in Mdm2-deficient T cells dependent on p53? How do these data relate to the Watanabe study on CD4 T cells?

Response: In response to the reviewer, we demonstrate that MDM2 contributes to $CD8^+$ T cell survival in the absence of p53 (page 6, new Fig.1r-w). We included new data showing the effects of loss or silencing of MDM2 on expression of p53 (page 6, new Fig.1r), and Bcl2 and caspase family members (page 6, new Extended Data Fig. 1m-q), and the impact of p53 on MDM2-regulated T cell survival (page 6, new Fig.1r-w). Results in the Watanabe work do not contradict to our observation. They demonstrated an effect of p53 on wild type ($MDM2^{+/+}$) $CD4^+$ T cell survival (please also see our response to general comments from Reviewer 2).

2. The figures include many western blots without quantification. Therefore it is difficult to determine the extent of variation between replicate experiments. The authors should include graphical representation of their western blots throughout the manuscript.

Response: Western blot bands are quantified and included in the revision. This is included in the new extended data figures related to each individual main figure.

3. Point out to reader that both CD4 and CD8 T cells are affected in Cd4-Cre mice, as most people will not know this trick and please provide a reference. Referring to Cd4-Cre;Mdm2F/F mice as Mdm2–/– mice and Cd4-Cre;Trp53F/F mice as p53–/– mice suggests that knockout mice have been used, which is confusing. I suggest that the authors use Cd4-Cre;Mdm2F/F mice and Cd4-Cre;Trp53F/F mice throughout the text for clarity.

Response: This is clarified in the revision. As the reviewer suggested, T cell specific $MDM2^{-/-}$ mice are referred to as $CD4^{Cre}MDM2^{f/f}$, T cell specific $p53^{-/-}$ mice are referred to as $CD4^{Cre}p53^{f/f}$, and T cells from these mice are referred to as $MDM2^{-/-}$ and $p53^{-/-}$ T cells, respectively.

4. In Figure 1, it is unclear whether the reduction in T cell survival from Cd4-Cre;Mdm2F/F mice is dependent on the tumor. What are numbers of CD4s and CD8s like in the thymus, spleen, lymph nodes etc compared to controls? Are T cells less abundant in Mdm2-deficient mice? Are there any differences, such as lymphoid organ pathology or body weight, between Cd4-Cre;Mdm2F/F mice and controls? How does antigen independent proliferation compare between CD8 T cells from Cd4-Cre;Mdm2F/F mice and controls? These mice need further characterization. Results of this comparison will affect the interpretation of the tumor growth curves.

Response: We have addressed these questions and included the characteristics of $CD4^{Cre}Mdm2^{f/f}$ mice in the revision (page 5, new Extended Data Fig.1e-l). We added new data on T cell subset numbers and percentages in different lymphoid organs (page 5, new Extended Data Fig.1g-h, j), antigen-independent proliferation (page 5, new Extended Data Fig.1k-l), and survival (apoptosis) protein expression in $MDM2^{-/-}$ T cells (page 6, new Extended Data Fig. 1m-q). In order to provide complementary and confirmatory data on a role of MDM2 in T cells, we have extended several lines of experiments in addition to $CD4^{Cre}Mdm2^{f/f}$ mice, including in vitro and in vivo studies with genetically and pharmacologically manipulated T cells and adoptive T cell transfusion. As the reviewer suggested, based on a role of MDM2 in T cell immunity in homeostasis and tumor stress, we have accordingly revised our interpretation and added detailed nuance in our discussion. We appreciate that the reviewer raised these comments. Addressing their comments additionally reinforces the importance of MDM2 in T cell biology.

5. I have never seen such nice staining for Granzyme B with clone GB11 or magnitudes of expression reaching 50%. My lab cannot do this. How were the cells stimulated ex vivo? What concentration of antibody was used? This information was not included in the Methods section.

Response: The detailed method and antibody information are provided in the revision (see page 27). We are confident with this staining.

6. In Figure 3, the biochemical interaction between MDM2, STAT5 and Cbl is shown. How is proliferation of T cells affected by loss of Mdm2 or Cbl? Can silencing of Cbl rescue decreased proliferation of Mdm2-deficient cells?

Response: We examined a role of MDM2 and c-Cbl in T cell proliferation and survival, and have included new data in the revision (page 9, Fig. 3f, Extended Data Fig. 3j-k).

7. Cd4-Cre;Trp53F/F mice are prone to inflammatory disease and smaller when compared to controls (Kawashima et al. JI 2013, 191:000), and these differences should be pointed out to the reader. Were there any signs of leukemia or lymphoma in Cd4-Cre;Trp53F/F mice? Were the mice in Figure 4j age matched or weight matched for experiments?

Response: According to Drs. Kawashima et al (Kawashima et al. JI 2013, 191:000), body weight loss in $CD4^{Cre}p53^{f/f}$ mice is noticeable at 4 months of age, and inflammatory symptoms manifest at 6-9 months of age. We used 6-8 weeks old mice in our immunological experiments. We found neither physical abnormalities nor lymphoproliferative symptoms. Nonetheless, we have included the new data in the revision (page 7, Extended Data Fig. 1v-x).

8. The data in Figure 4 are not convincing for some tumor models, such as B16 in 4C and CT26 in 4E – even if these differences managed to reach statistical significance, I disagree with the interpretation that APG115 slows tumor growth. It seems the B16 tumors were grown to nearly double the size of other tumor models, surpassing 2000 mm3 at the end of the experiment. I don't believe this is ethical. The experiment seems forced to generate a mediocre difference. The way in which tumor volume was calculated is missing from the Methods. It does not appear that tumor-bearing mice were randomized in any way before treatment.

Response: It is well known that B16 is not a strong immunogenic tumor and weakly responds to anti-PD-L1 and PD-1 therapy. In line with this, we observed a moderate anti-tumor role of APG115 in B16 bearing mice. We have taken several steps to address and ease the important concerns raised: (a) We have tested a role of APG115 in mice bearing several types of tumor. Similar to PD-L1 and PD-1 blockade, the anti-tumor efficacy of APG115 varies in different types of tumor. We feel the inclusion of different types of tumor in murine models is useful and informative to the field. (b) In addition to tumor volume, we have included tumor weight as an additional tumor read-out. (c) We included the tumor volume calculation formula in the revised Methods. (d) Our tumor volume read-out has been approved in our animal protocol. Nonetheless, the reviewer's point is well received. We must minimize unnecessary mouse suffering, including controlling tumor volume.

9. Controls are missing from Figure 5a. Please show tumor growth between wild-type and NSG mice. Presumably, MC38 cells grow faster in NSG mice than wild-type if CD8 T cells are involved.

Response: The controls are included (revised Fig. 5a).

10. There is insufficient evidence for the statement made at the top of page 12, "Thus, APG115 regulates CD8 T cell survival and function through MDM2-dependent STAT5 stabilization." This statement can only be supported by blocking STAT5 in APG115-treated cells/tumors.

Response: We agree that we failed to provide direct evidence supporting a role of APG115 in T cell survival and function via MDM2-dependent STAT5 stabilization in the first version of the manuscript. We have made significant efforts to address this important point. Using a specific STAT5 inhibitor, a constitutive active STAT5 mutant, and specific STAT5 siRNAs, our new in vitro and in vivo studies demonstrate a direct role of MDM2-dependent STAT5 in T cell survival and function (page 13, new Fig.6f-m, new Extended Data Fig. 6i-j).

11. Statistical analysis is incorrect throughout the manuscript: one-way ANOVA should be used for 3 groups or more, and Mann-Whitney should be used for 2 groups, not a two-tailed t test. Error bars and statistical analysis are missing from Extended Data Figure 6a-c.

Response: The statistical analysis is now corrected. Error bars and statistical analysis are included in the revised Extended Data Figure 6f-h.

12. Please include more details in the Methodology for every experiment. How much protein was loaded on SDS-PAGE gels? How was IP performed? How much pull down antibody was used?

Response: The information is included in the revision (page 24).

Reviewer #2

(Remarks to the Author)
In this paper, Zhou et al. analyzed the role of MDM2-P53 pathway in CD8+ T cells antitumor responses. The results highlight the expression of MDM2 on governing T cell-mediated tumor control and the loss of MDM2 expression in CD8+ T cells led to increased apoptosis and failure to produce cytotoxic cytokines. The authors have found that MDM2, by interacting with c-Cbl, prevents the interaction between c-Cbl and STAT5, which in turn prevents STAT5 degradation. The MDM2-mediated stabilization of STAT5 might be responsible for improved survival and effector function of CD8+ T cells infiltrating the tumor. The authors showed that the drug APG115, which is known to impede the interaction between MDM2 and p53, could enhance MDM2 expression and therefore be used as an immune modulator. Tumor bearing mice treated with APG-115 showed stronger anti-tumor responses in a CD8+ T cell-dependent manner and the therapeutic benefits of APG-115 relies on the presence of p53 and MDM2 in T cells. Moreover, Zhou et al. showed that APG-115 synergizes with PD-1 blockade to enhance antitumor immune responses and that MDM2 expression correlates with functional T-cell responses in cancer patients.

In fact, Watanabe et al. in 2014 have shown that downmodulation of p53 mediated by TCR is critical for antigen specific T cell proliferation. Moreover, a work from Banerjee et al. in 2016 has demonstrated that the absence of p53 in T cells leads to increased effector function and tumor control. On this line, the lack of MDM2 would prevent p53 downmodulation and therefore alters cytotoxic T-cell response. These two publications are not cited and discussed and the Banerjee's work presented contradictory results. In addition, Fang et al. in 2019 have shown that the drug APG-115 in combination with PD-1 blockade is able to reduce wt, mutated or p53 KO tumor (MH-22Awt, MC38 and MH-22Amut), but this study is not cited and discussed as well. Even though the finding of the mechanism through which MDM2 regulates CD8 antitumor response via STAT5 is interesting, the novelty of this work is

compromised by several previous publications and the results presented here do not demonstrate MDM2-mediated enhancement of antitumor responses is controlled by increased STAT5 expression.

Response: We appreciate that the reviewer indicated that our finding of the mechanism through which MDM2 regulates CD8 antitumor response via STAT5 is interesting. We agree that we need to provide direct and compelling evidence that MDM2-mediated enhancement of antitumor responses is controlled by increased STAT5 expression (page 13, Fig. 6a-m, new Extended Data Fig. 6i-j)

We provided the detailed experiments to address the reviewer's specific comments. In response to their general comments, we would like to clarify the novelty of our work and the differences between our work and the cited papers. We focus on the mechanistic connection between MDM2 and STAT5 in controlling T cell-mediated anti-tumor immunity. We have used specific MDM2 deficient- and p53-deficient T cells to avoid well-known developmental issues in general p53 knock out mice. In addition, we have generated genetic, biochemical, and functional evidence demonstrating that the MDM2-c-Cbl-STAT5 pathway controls T cell immunity. Our experimental approach and central scientific questions are different than the published works. The detailed comparison and analyses:

a. The Watanabe study: They used Trp53$^{-/-}$ KLH-specific CD4^{+} T cells and examined a role of p53 in TCR-induced CD4^{+} T cell proliferation in their work. We focused on MDM2 and the MDM2-c-Cbl-STAT5 pathway in T cell survival, proliferation, and function in vitro and in vivo. As we have generated p53 specific knock out T cells, we have included studies on p53 in both CD4^{+} and CD8^{+} T cells in the revision. Notably, Dr. Watanabe and colleagues did not examine MDM2, STAT5, and their interaction in controlling CD8^{+} T cell-mediated anti-tumor immunity. We agree that one could assume that the lack of MDM2 would prevent p53 down-regulation, therefore altering cytotoxic T-cell response. However, given the unknown role of MDM2 in CD8^{+} T cell-mediated tumor immunity and the well-known p53-independent and -dependent biological activities of MDM2 in tumor cells, our work remains novel in T cell biology in general and in cancer immunity specifically. In support of this, to our surprise, we found MDM2 can regulate T cell survival and function in the absence of p53 in T cells (page 6, new Fig.1r-w). Please note that this is different from the p53-dependent role of APG115 in T cells (page 11, new Extended Data Fig.4k; page 15, new Extended Data Fig.6k). APG115 targets the interaction between MDM2 and p53 to enhance MDM2 expression in T cells (page 10, Fig.4 a-b, new Extended Data 4 b, d). Thus, APG115 is not pharmacologically active in the absence of p53. Finally, we highly appreciate the reviewer's comment. In addition to experimentally addressing their comments, we understand that scientific novelty does not exist in a vacuum. Therefore, we have now placed our work in the context of p53 literature in the revision.

b. The Banerjee study: They used p53 knockout h3T TCR transgenic mice and p53 inhibitor PFT-μ in their work. PFT-μ prevents p53 binding to Bcl-xL and Bcl-2. PFT-μ does not alter p53 expression and transactivation activities. Thus, it seems PFT-μ may not mimic p53 genetic deficiency. They did not examine MDM2, STAT5, and their interaction in controlling CD8^{+} T cell-mediated anti-tumor immunity.

c. The Fang study: We regret that we missed this sole study connecting APG115 to immune responses. Consistent with our results (Fig 4h, j), they found that treatment with APG115 inhibits MC38 tumor growth. They observed APG115 stimulated IFNγ expression in CD4^{+} T cells. We found that the anti-tumor effect of APG115 depends on CD8^{+} T cells, not CD4^{+} T cells. Furthermore, Dr. Fang and colleagues neither dissect a direct role of APG115 on T cells, nor the novel molecular mechanisms (e.g. the connection of MDM2-c-Cbl-STAT5 in T cells), and nor the relevance of P53 in T cells. Additionally, we tested multiple tumor types, different doses of APG115, and multiple

genetic models in vitro and in vivo (e.g. MDM2 and p53 knockdown and knockout T cells in vitro, and specific MDM2 and p53 deficient mouse models).

Specific points could be addressed to improve the significance of the work:

• Given the importance of STAT5 in CD8+ T cell activation, could be possible to exclude that CD8+ T cell lacking MDM2 are sub-optimally activated, which results in impaired effector functionality? The analysis of activation markers such as CD44 and inhibitory receptors such as PD-1 could help in defining the dysfunctional status of MDM2 KO CD8+ T-cells.

Response: We have included the characteristics of $CD4^{Cre}Mdm2^{f/f}$ mice in the revised version (page 5, new Extended Data Fig.1e-l). Furthermore, we have included results in optimally activated $MDM2^{+/+}$ and $MDM2^{-/-}$ T cells, and additional markers (CD25 and PD-1) (page 5, new Extended Data Fig.1i).

• To confirm that the effect of MDM2 expression is mediated by the presence of STAT5, would be important to prove that overexpression of STAT5 could rescue the generation of antitumor CD8+ T cell response in the absence of MDM2 or silencing STAT5 in T cells. This analysis is critical to support their claim that MDM2 modulate T cell anti-tumor responses via stabilization of STAT5.

Response: This is a valid and important point. Using a specific STAT5 inhibitor, a constitutive active STAT5 mutant, and specific STAT5 siRNAs, our new in vitro and in vivo studies demonstrate a direct role of MDM2-dependent STAT5 in T cell survival and function (page 13, new Fig. 6f-m, new Extended Data Fig. 6i-j).

• To further validate the interaction of MDM2 with c-Cbl, would be important to show in Figure 3h the level of myc-MDM2 in IP:HA.

Response: The myc-MDM2 levels are included (revised Fig. 3i).

• The fact that APG-115 treatment requires wt p53 gene in T cells points out that the effect reached with the treatment is not exclusively mediated by MDM2-c-Cbl-STAT5 axis. Would be important to further dissect this point to reveal the mechanism of action of APG-115 beside p53. Would be interesting to show that the expression of STAT5 can rescue the functionality of CD8+ T-cell lacking p53 and MDM2 under APG-115 treatment or that lack of c-Cbl would also improve T-cell function in absence of MDM2 under APG-115 treatment. Moreover, the discrepancy between this study and Banerjee's study published in 2016 should be discussed.

Response: These points are well received and experimentally addressed. To explore a role of MDM2 in T cells and T cell-mediated anti-tumor immunity, we need to biochemically and genetically manipulate MDM2 in T cells. We generated MDM2-specific knock out T cells. As APG115 enhances MDM2 expression, we used APG115 as a biochemical tool compound in our T cell studies. Notably, APG115 targets the interaction between MDM2 and p53, and in turn increases MDM2 expression. Thus, the effect of APG115 depends on both MDM2 and p53. However, our data demonstrates that MDM2 can regulate T cell survival and function in p53-independent manner.

In response to this reviewer and to explicitly address a role of MDM2 in $CD8^+$ T cells in the context of p53, we implemented several approaches - including p53 transcriptional inhibitor, p53 knockdown with specific iRNA, and p53 knockout in $Cd4^{Cre}p53^{f/f}$ mice. These approaches allowed us to demonstrate that MDM2 regulates T cell survival and function in the absence of p53 (page 6, new Fig. 1r-w).

Furthermore, we expressed MDM2 and c-Cbl in p53-deficient T cells, and treated them with APG115. We validated that the protective effect of APG115 on T cell survival depends on p53 (page 15, new Fig. 6r-s).

Again, to demonstrate an MDM2-dependent role of STAT5 in regulation of $CD8^+$ T cell survival and function, we included in vitro and in vivo studied on T cells expressing a constitutive active STAT5 mutant, specific STAT5 siRNAs, and treated T cells with an STAT5 inhibition (page 13, new Fig. 6f-m, new Extended Data Fig. 6i-j).

In summary, MDM2 can regulate T cell immunity via the MDM2-c-Cbl-STAT5 axis in the absence of p53, whereas the anti-tumor role of APG115 depends on both MDM2 and p53 in T cells, but not tumor cells.

• The depletion of MDM2 in CD4 expressing cells doesn't exclude a possible role in CD4 T-cells. Would be interesting to explore this point? On this line, the work of Fang et al. in 2019 has shown that APG-115 treatment enhances CD4+ T- cell activation and, in combination with PD-1 blockade, increases the percentage of CD4+ T-cells which produce IFNγ.

Response: We agree that the depletion of MDM2 in CD4 expressing cells doesn't exclude a possible role in $CD4^+$ T cells. In response to this comment, we include new in vivo experiments with $CD4^+$ T cell depletion (page 12, new Extended Data Fig. 5c-d). As $CD4^+$ T cell depletion did not alter tumor read-out with or without APG115 (page 12, new Extended Data Fig. 5c-d), we focus our studies on $CD8^+$ T cells

Decision Letter, first revision:

Subject: Decision on Nature Immunology submission NI-A29928A

Message: Dear Weiping,

Your revised manuscript has been seen again by the Referees and we are happy to inform you that if you revise your manuscript appropriately in response to the referees' comments and our editorial requirements your manuscript should be publishable in Nature Immunology. Referees still request some clarifications to the text (see below) so could you please address this in a revision and resubmit. Note, in their comments to the Editors, Referee #1 voiced concerns that they were still not fully convinced with the broad significance of the findings because MDM2 or p53 modulators have not yet hit main stream clinical use. Some textual additions to the Intro/Discussion addressing this concern might be useful. Once we have this we'll proceed with the edits.

Please revise your manuscript according with the reviewers' comments and as outlined in your letter. At resubmission, please include a point-by-point response to the referees' comments, noting the pages and lines where the changes can be found in the revision. Please highlight the changes in the revised manuscript as well.

We are trying to improve the quality and transparency of methods and statistics reporting in our papers (please see our editorial in the May 2013 issue). Please update the Life Sciences Reporting Summary, and supplements if applicable, with any information relevant to any new experiments and upload it (as a Related Manuscript File) along with the files for your revision. If nothing in the checklist has changed, please upload the current version again.

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In recognition of the time and expertise our reviewers provide to Nature Immunology's editorial process, we would like to formally acknowledge their contribution to the external peer review of your manuscript entitled "MDM2 sustains STAT5 stability to control T cell-mediated anti-tumor immunity". For those reviewers who give their assent, we will be publishing their names alongside the published article.

When you are ready to submit your revised manuscript, please use the URL below to submit the revised version: [REDACTED]

We hope to receive your revised manuscript in 10 days, by 28th Dec 2020. Please let us know if circumstances will delay submission beyond this time. If you have any questions please do not hesitate to contact me.

Sincerely,

Zoltan Fehervari, Ph.D.
Senior Editor
Nature Immunology

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Fax: 212-696-9752
z.fehervari@nature.com

Reviewer #1 (Remarks to the Author):

This study provides evidence on the importance of MDM2 signaling in anti-tumor T cells. The data have implications for cancer immunotherapy as well as therapeutic MDM2 modulators. The authors have addressed my criticisms in full. The manuscript has greatly improved by the additional experimentation and explanation. It seems technically robust and more detail has been included in the Methods section. In response to my previous comment #3 on Cd4-cre mice, the authors should explicitly state in the first paragraph of the Results section that genes will be floxed out from both CD4s and CD8s in these mice. Most readers will not know this and it will help justify why these mice were used to study CD8 T cells.

Reviewer #2 (Remarks to the Author):

The revised manuscript addresses most of my concerns and improves the clarity. This intriguing new action of MDM2 may provide critical foundation for interventions targeting this pathway. I only have two comments for this revised manuscript.

1. In the abstract, the author should modify the description to "clearly" point out only APG115 treatment requires both MDM2 and p53. The current description is relatively confusing.

2. The last sentence in page 6 is wrong. It should be "at 6-9 months of age in CD4crep53f/f mice", not CD4crep53+/+ mice.

Author Rebuttal, first revision:

nature research

Zoltan Fehervari, Ph.D.
Senior Editor
Nature Immunology

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z.fehervari@nature.com

December 15, 2020

Dear Dr. Fehervari,

Thank you for your editorial decision regarding our manuscript entitled "MDM2 sustains STAT5 stability to control T cell-mediated anti-tumor immunity (NI-A29928-T)." Please see our point-by-point responses.

Editorial comment: because MDM2 or p53 modulators have not yet hit main stream clinical use. Some textual additions to the Intro/Discussion addressing this concern might be useful.

Response: As you suggested, we have included some textual additions to the revised discussion section. Please see page 3: "In spite of these efforts, no therapy targeting MDM2 has been approved. Therefore, we reason the frustration of cancer therapeutic application exists due to a gap in the substantial knowledge of the p53 and MDM2 pathway in the fields of tumor biology and oncology, and the limited understanding of the p53 and MDM2 pathway in T cells in the context of tumor immunology".

Reviewer #1 (Remarks to the Author):

This study provides evidence on the importance of MDM2 signaling in anti-tumor T cells. The data have implications for cancer immunotherapy as well as therapeutic MDM2 modulators. The authors have addressed my criticisms in full. The manuscript has greatly improved by the additional experimentation and explanation. It seems technically robust and more detail has been included in the Methods section. In response to my previous comment #3 on Cd4-cre mice, the authors should explicitly state in the first paragraph of the Results section that genes will be floxed out from both CD4s and CD8s in these mice. Most readers will not know this and it will help justify why these mice were used to study CD8 T cells.

Response: We reported in the revised result section that MDM2 and P53 are floxed out from both $CD4^+$ and $CD8^+$ T cells in $CD4^{Cre}MDM2^{+/+}$ mice and $CD4^{Cre}P53^{+/+}$ mice, respectively.

Reviewer #2 (Remarks to the Author):

The revised manuscript addresses most of my concerns and improves the clarity. This intriguing new action of MDM2 may provide critical foundation for interventions targeting this pathway. I only have two comments for this revised manuscript.

1. In the abstract, the author should modify the description to "clearly" point out only APG115 treatment

requires both MDM2 and p53. The current description is relatively confusing.

Response: We clarified this point in the revised abstract.

2. The last sentence in page 6 is wrong. It should be "at 6-9 months of age in CD4crep53f/f mice", not CD4crep53+/+ mice.

Response: We have made this correction in the revision.

I am looking forward to your evaluation.

Decision Letter, second revision:

Subject: Nature Immunology - NI-A29928B pre-edit

Message: Our ref: NI-A29928B

8th Jan 2021

Dear Weiping,
Apologies for the delay getting back to - the holidays and being swamped with COVID manuscripts have really slowed things

Thank you for your patience as we've prepared the guidelines for final submission of your Nature Immunology manuscript, "MDM2 sustains STAT5 stability to control T cell-mediated anti-tumor immunity" (NI-A29928B). Please follow the instructions provided here and in the attached files, as the formal acceptance of your manuscript will be delayed if these issues are not addressed.

When you upload your final materials, please include a point-by-point response to the points below. We won't be able to proceed further without this detailed response.

General formatting:
1. Our standard word limit is 4500 words for the Introduction, Results and Discussion. Your current manuscript exceeds this limit by please cut accordingly.

2. Please include a separate "Data availability" subsection at the end of your Online Methods. This section should inform our readers about the availability of the data used to support the conclusions of your study and should include references to source data, accession codes to public repositories, URLs to data repository entries, dataset DOIs, and any other statement about data availability. We strongly encourage submission of source data (see below) for all your figures. At a minimum, you should include the following statement: "The data that support the findings of this study are available from the corresponding author upon request", mentioning any restrictions on availability. If DOIs are provided, these should be included in the Reference list (authors, title, publisher (repository name), identifier, year). For more guidance on how to write this section please see: http://www.nature.com/authors/policies/data/data-availability-statements-data-citations.pdf.

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4. Your abstract must be fewer than 150 words and should not include citations.

5. As a guideline, Articles allow up to 50 references in the main text. An additional 20 references can be included in the Online Methods. Only papers that have been published or accepted by a named publication or recognized preprint server should be in the numbered list. Published conference abstracts, numbered patents and research data sets that have been assigned a digital object identifier may be included in the reference list.

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7. Genes must be clearly distinguished from gene products (e.g., "gene Abc encodes a kinase," not "gene Abc is a kinase"). For genes, provide database-approved official symbols (e.g., NCBI Gene, http://www.ncbi.nlm.nih.gov/gene) for the relevant species the first time each is mentioned; gene aliases may be used thereafter. Italicize gene symbols and functionally defined locus symbols; do not use italics for proteins, noncoding gene products and spelled-out gene names.

Figures and Tables:

8. All figures and tables, including Extended Data, must be cited in the text in numerical order.

9. Figure legends should be concise. Begin with a brief title and then describe what is presented in the figure and detail all relevant statistical information, avoiding inappropriate methodological detail.

10. All relevant figures must have defined error bars.

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12. Cropping of gel and/or blot images must be mentioned in the figure legend. Gel pieces should be separated with white space (do not add borders). Please ensure that all blots and gels are accompanied by the locations of molecular weight/size markers; at least one marker position must be present in all cropped images. Please also supply full scans of all the blots and gels as Source Data, as instructed below.

13. All bar graphs should be converted to a dot-plot format or to a box-and-whisker format to show data distribution. All box-plot elements (center line, limits, whiskers, points) should be defined.

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All Extended Data must be called you in your manuscript and cited as Extended Data 1, Extended Data 2, etc. Additional Supplementary Figures (if permitted) and other items are not required to be called out in your manuscript text, but should be numerically numbered, starting at one, as Supplementary Figure 1, not SI1, etc.

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We ask that you aim to return your revised paper within 7 days. If you have any further questions, please feel free to contact me.

Best regards,

Zoltan Fehervari, Ph.D.
Senior Editor
Nature Immunology

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z.fehervari@nature.com

Final Decision Letter:

Subject: Decision on Nature Immunology submission NI-A29928C

Message: In reply please quote: NI-A29928C

Dear Dr. Zou,

I am delighted to accept your manuscript entitled "The ubiquitin ligase MDM2 sustains STAT5 stability to control T cell-mediated anti-tumor immunity" for publication in an upcoming issue of Nature Immunology.

The manuscript will now be copy-edited and prepared for the printer. Please check your calendar: if you will be unavailable to check the galley for some portion of the next month, we need the contact information of whom will be making corrections in your stead. When you receive your galleys, please examine them carefully to ensure that we have not inadvertently altered the sense of your text.

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Sincerely,

Zoltan Fehervari, Ph.D.
Senior Editor
Nature Immunology

The Macmillan Building
4 Crinan Street
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z.fehervari@nature.com