Reviewers' comments:

Reviewer #1 (Remarks to the Author):

Huang et al present their hypothesis -and good experimental evidence- that IFNg (derived from effector immune cells) may induce tumor dormancy via IDO-kynurenine-AhR metabolic circuitry in several tumor models. Manipulation of this signaling can block this. The work is conceptually important and experimentally justified. It will stimulate interesting discussion. My specific comments are as follows:

1. The concept that immune cells can endow stem-like properties have been tested and reported in human cancer literature. For example, human Th22 cells (Immunity, 2014) and MDSCs (Immunity, 2013) can induce cancer stemness via distinct molecular mechanisms in colon cancer and ovarian cancer respectively. The authors may include discussion on these important and relevant findings. Nonetheless, the authors now found that effector T cells and NK cells may induce tumor stemness via IFNg. This extends previous studies to effector immune cells. It is surprising but interesting.

2. Concentrations of IFNg and mechanisms: The authors may logically test and/or at least discuss why different concentrations of IFNg are needed, and why IFNg may play differential roles in different tumors (maybe different stages). In addition to IDO, are PD-L1 and arginase and iNOS involved? These pathways are all activated by IFNg and should be primarily tested and/or at least discussed.

3. PD-L1/PD-1 blockade resistance and cancer dormancy (stemness): I understand that the authors wish to link the clinical PD-L1/PD-1 blockade resistance to potential cancer dormancy (stemness). However, there is no evidence in the literature and the current work. The cited work (Ref50 and Ref51) (page 22) did not talk about this at all. The authors may refer recent authority review on this topic by Drs. Zou, Wolchock, and Chen (Science TM, 2016).

Reviewer #2 (Remarks to the Author):

In this manuscript entitled: "Blockade of IDO-kynurenine-AhR metabololic circuitry abrogates INF-g-induced immunologic dormancy of tumor-repopulating cells" the authors show that INF-gamma induces dormancy by activating the IDO-AhR-p27 pathway which reduces the STAT1-induced apoptosis signal only in B16 TRCs and also in human melanoma-derived TRCs.
The authors show that B16 TRCs are insensitive to INF-gamma killing effect and actually they undergo cell growth arrest (in vitro experiments). These in vitro experiments are well-designed and high quality controlled. The use of statistics is appropriated.
Finally, the authors conclude from the in vivo experiments that the therapeutic combination or INFgamma and IDO1 or AhR inhibitor directly target dormant TRCs. These in vivo experiments are not entirely accurate in demonstrating if TRCs undergo dormancy after INFgamma treatments or if they are killed by INFgamma/1-MT/DMF.
The manuscript is well-written and of great clarity. The references to the literature seem adequate and the technical details are explained clearly for the most part.
Although, the results of this manuscript are original and of high relevance for the field of minimal residual disease there is not one experiment that shows in vivo if TRC actually activate the INFgamma-induced dormancy pathway proposed by the authors. Thus, this manuscript should be accepted for publication in Nat Comm. only after addressing the following concerns:

Suggested improvements for possible revision:

-It is speculated from the in vivo experiments in figure 7 and supplementary figure 7 that treatment of proliferating TRC (3 days after injection) or tumors of 7x7mm with all the described drugs: INFgamma, or anti-INFgamma ab (for syngenic mice) or INFgamma+1-MT/DMF, affects the dormancy/survival behavior of TRC. However, this is never demonstrated. Instead, the authors show us tumor growth curves and survival experiments after treatment with these drugs that, as shown by the authors, induce apoptosis (more than 50% of non-TRC population) and thus this could account for the observed results. Thus, this concern should be addressed by IF using specific markers for TRC in the tumors (for example after 10 days treatment as shown in Fig 7 e) or sorting by FACs (using CD133, nestin, Bmi-1 and c-kit markers as shown by this group previously) this TRC population and doing a direct analysis of cell cycle arrest.
In addition, the authors show that cells recovered from INFgamma-treated tumors have more sphere-forming capacity than control groups arguing for dormancy induction of TRC while in the tumor mass. However, these results could be interpreted in a different manner, for instance that dormancy phase of TRC was not affected by IFNgamma but rather that the non-TRC population was killed and therefore there are more TRC forming more spheres or that INFgamma treatment increased the sphere-forming capacity of TRC. Thus, the authors need: 1- to count number of TRC isolated from control or INFgamma-treated tumors, 2- to seed the same number of TRC in sphere conditions and count number of sphere in both groups. Here is important to use specific makers for the TRC population that the authors consider are responsible for the whole phenomenon.
Lastly, the author show in figure 7a, c or e that tumors still grow upon INFg/1-MT/DMF/shIDO1 suggesting that either a TRC population is left behind or these are differentiated cells. This should be addressed.

- Regarding the experiments of INFgamma plus DMF or 1-MT (Figure 6c), what would happen if you first add IFNg every 2 days for 20 days after 3 days post-injections of B16 TRCs and then stop INFgamma and add DMF or 1-MT for another 20 days? Would the number of recovered TRC be less in treated groups than control? Would the number of colonies formed by recovered TRCs be less in treated animals than PBS control? The authors tried to address this in Figure 6 C but only after 3 days of treatment with both INFgamma plus 1-MT or DMF and interestingly, the recovered TRC formed the same number of colonies than PBS-treated mice. Thus, Isn't this result suggesting that blocking the IDO pathway did not kill TRC? Thus, knowing the number of TRC after these treatments should be more informative.

-Why is that 1-MT has an effect on tumor growth in figure 7d? On the contrary, DMF has not effect on tumor growth as seen in figure 7e. These results are showing us that without addition of INFgamma B16 cells grew normally (TRC and non-TRC) and only after adding INFgamma tumor growth decreased. So how is that 1-MT alone is affecting growth? Is inducing apoptosis of spontaneous dormant TRCs? Is affecting non-TRCs?

--The authors should address if AhR staining signal comes from TRC or non-TRC upon INFgamma treatment in figure 6a.

-In a model of spontaneous dormancy of TRC what would happen after addition of DMF or 1-MT? Would metastasis formation be reduced? (examples of spontaneous dormancy of TRC assays will be: after removal of primary tumor treat animals with 1-MT or DMF and then measure onset of metastatic foci in secondary organs. You will need to know the average time to metastasis in the chosen system and verify that disseminated TRC are in a dormant state before treatment; or a GEM). This experiment is crucial and it will support the concept that these agents are actually affecting spontaneous dormancy of disseminated tumor cells (as found in patients) and their clinical relevance as potential therapeutic drugs as proposed by the authors in the discussion. And it will also validate the activation of INFgamma-IDO-AhR-p27 pathway in dormant TRC. Because if spontaneous dormant disseminated cancer cells are not eliminated by DMF or 1-MT drugs that would imply that they don't activate the INFgamma-IDO-AhR-p27 and STAT1 pathway by themselves. This reviewer understands that this experiment may require longer time to get it done

therefore it is not required for acceptance for publication at this moment.

Minor Concerns:
-In each colony size graph, the authors should clarify in the "y" axis what is the relative size compare to? It is compared to day 1 after seeding cells? when do the authors consider a colony is formed (size, number of cells, day)?
- In Figure 7 d and e which mice background are those?
-Figure 8 J and K have no legends.
-in several figures the legends are shown in green but graph bars are blue.
-The majority of the colony size experiments are done by starting treatments at day 2-3 after seeding. Maybe worth a try to test the effect of INFgamma in solitary TRC as it would occur in cellular dormancy phenomenon?

Reviewer #3 (Remarks to the Author):

This study focuses on mechanisms that cause tumor stem cell (TRC) dormancy in the tumor microenvironment (TME), a topical issue of high clinical importance due to the potential for dormant tumor cells to cause tumor relapse after treatment. The major claim is that tumor cell dormancy is due to elevated oxidative tryptophan (Trp) catabolism mediated by the enzyme indoleamine 2,3 dioxygenase, IDO, which is induced by IFN type 2 (IFNγ) in the TME. The authors' further claim that increased IDO activity stimulates aryl hydrocarbon receptor (AhR) signaling to activate the cell cycle regulatory factor p27. Data presented supports these claims that identify a novel metabolic feature of the TME leading to tumor cell dormancy, and suggest that IDO pathway inhibitors currently under scrutiny in several cancer clinical trials may target tumor dormancy directly, as well as enhancing anti-tumor immunity by alleviating T cell suppression in the TME. Though the manuscript is well presented several concerns should be addressed to increase rigor, novelty and significance of the study, as detailed below.

Major Points:

1. IDO degrades Trp to generate several catabolites, of which kynurenine (Kyn) is just one. The authors claim that tumor cell dormancy is mediated by Kyn binding to AhR to induce p27 is supported by the data presented. However, potential contributions from other pathways downstream of IDO are not excluded rigorously. The authors suggest that the GCN2-dependent integrated stress response (ISR) to amino acid withdrawal (in this case Trp) is excluded from consideration because their data identifies Kyn/AhR as the key pathway that prevents tumor cell dormancy (line 199-200). This premise should be tested, not just assumed. Several approaches could be used to test if Trp depletion promotes tumor cell dormancy, including (but not limited to) adding excess Trp to assays (to prevent Trp depletion but not Kyn production) and monitoring ISR responses in tumor cells and the TME.

2. Linked to the point above, several Trp catabolites (not just Kyn) have been reported to have anti-tumor effects due to their ability to alleviate T cell suppression directly and by reducing regulatory T cell (Treg) functions. Some of these compounds may also be produced in the TME and may activate the AhR (or other) pathways to induce tumor cell dormancy. This point should be addressed as there is some controversy about Kyn-mediated AhR activation because AhR has low relatively affinity for Kyn and because Kyn is removed rapidly from tissue microenvironments and excreted.

3. Data identifying the cell cycle regulator p27, as a downstream response factor to Kyn-AhR

signaling should be reported in this study (line 218).

4. The authors suggest that differentiated tumor cells and TRCs have distinct responses to IFNγ mediated by JAK-STAT1 or IDO/Kyn/AhR signaling, respectively (lines 432-434). However, these are not alternative signaling pathways since considerable evidence shows that IDO induction occurs via the JAK-STAT1 pathway acting on GAS elements in the IDO gene promoter. While it is certainly intriguing that the response to IFNγ appears different in differentiated tumor cells and TRCs the authors should revise their interpretation to consider how JAK-STAT1 signaling might be modified in these cell types to generate distinctive downstream responses that explain the phenomena under study.

5. Data identifying IFNβ as an inducer of tumor cell dormancy should be reported (line 414-416). This is important because the authors suggest that the effects they observe are not dependent on adaptive immune cells, eliminating a potential source of IFNγ in the TME that could cause tumor cell dormancy. Moreover, recent reports identify DNA as a potent anti-tumor adjuvant due to STING/IFNβ signaling in the TME that activates dendritic cells to prime CTL responses. Thus natural or vaccine adjuvants may incite tumor cell dormancy via IFNβ signaling. Hence, data supporting this possibility should be reported in this study.

Minor Points:

1. It is unclear why Kyn-induced AhR translocation in TRCs is described as 'remarkable' (line 204), as several studies have documented previously that Kyn mediates AhR signaling.
2. Several typographical errors should be corrected.

RESPONSES TO REVIEWERS

We would like to express our sincere thanks to all three reviewers for their critical and constructive comments. We have performed substantial additional experiments to address their concerns. We respond point-by-point to each of their comments and criticisms. We feel that their comments have helped us on significantly improving and strengthening the manuscript and clarifying some issues. We hope that the revision has addressed their major concerns.

RESPONSE TO REVIEWER #1:

The concept that immune cells can endow stem-like properties have been tested and reported in human cancer literature. For example, human Th22 cells (Immunity, 2014) and MDSCs (Immunity, 2013) can induce cancer stemness via distinct molecular mechanisms in colon cancer and ovarian cancer respectively. The authors may include discussion on these important and relevant findings. Nonetheless, the authors now found that effector T cells and NK cells may induce tumor stemness via IFNg. This extends previous studies to effector immune cells. It is surprising but interesting.

Response:

Despite killing tumor cells, the interaction between immune elements and tumor cells may also result in immunoediting of the tumor which either molds the cancer into a dormant state or fosters tumor immune evasion, even promoting tumor stemness. We appreciated the reviewer constructive suggestion. And according to the reviewer's suggestion, we added the below information in the revised Discussion section, page 22 line 13.

"Recently, the concept that immune cells can endow stem-like properties of tumor cells have been evidenced from both human Th22 and MDSCs that can induce cancer stemness via distinct molecular mechanisms in human colon and ovarian cancer, respectively (Immunity. 2013;39:611-21; Immunity. 2014;40:772-84). Nonetheless, our present study indicates that effector T cells and NK cells may also induce tumor stemness via IFN-γ-mediated TRC dormancy, which thus extends previous studies to antitumor effector immune cells."

Concentrations of IFNg and mechanisms: The authors may logically test and/or at least discuss why different concentrations of IFNg are needed, and why IFNg may play differential roles in different tumors (maybe different stages).

Response:

We thank the reviewer for this insightful question. In our in vitro experiment, we found that

only high concentration (>50 ng/ml) of IFN-γ is capable of inducing TRC dormancy. In general, physiological IFN-γ probably cannot reach such high concentration in vivo, however during the direct interaction of effector T or NK cells with TRCs, high concentration of IFN-γ might be formed at the local immune synapse site, leading to TRC dormancy. Generation of high levels of IFN-γ at the local synapse site might be regulated by the stages of tumor. Since tumors at their early stage generate much less immunosuppressive factors, effector T or NK cells could be optimally activated and produce amount of IFN-γ. By contrast, tumors at their late stage generate much more immunosuppressive factors, which impede the production of IFN-γ by T or NK cells. However, low levels of IFN-γ can stimulate immune cells such as macrophages and DCs to upregulate the expression of IDO1 and PD-L1 to mediate immunosuppression (J Clin Immunol. 2012;32:837-47; Blood. 2006 ;107:2846-54; Science. 2016;353: 399-403).

According to the reviewer's suggestion, we added the above information in the revised Discussion section, page 24 line 20.

In addition to IDO, are PD-L1 and arginase and iNOS involved? These pathways are all activated by IFNg and should be primarily tested and/or at least discussed.

Response:

We thank the reviewer's constructive suggestion. To address whether PD-L1, arginase and iNOS were also involved in regulating TRC dormancy by IFN-γ, we treated B16 TRCs and the control differentiated B16 tumor cells cultured on rigid plastic with IFN-γ for 24h. The real-time PCR analysis showed that the expression of IDO1 in B16 TRCs was strikingly upregulated. Unexpectedly, we found that IFN-γ strongly stimulated the upregulation of PD-L1 in differentiated B16 cells but not in TRCs. Despite the above effect, IFN-γ seemed to have no effect on the expression of arginase 1 in B16 TRCs. In addition, the expression of iNOS in TRCs was also not regulated by IFN-γ. Based on these results, PD-L1, arginase 1 and iNOS might not be involved in regulating TRC dormancy by IFN-γ. To further confirm this, we used siRNAs to knock down PD-L1 and iNOS, respectively. Under such condition, IFN-γ was still able to induce B16 TRC dormancy, suggesting that IFN-γ selectively mobilizes IDO1 pathway to mediate TRC dormancy.

According to the reviewer's suggestion, we added these new data in the revised manuscript, page 10 line 23 and revised supplementary Fig 2g, h.

PD-L1/PD-1 blockade resistance and cancer dormancy (stemness): I understand that the authors wish to link the clinical PD-L1/PD-1 blockade resistance to potential cancer dormancy (stemness). However, there is no evidence in the literature and the current work. The cited work (Ref50 and Ref51) (page 22) did not talk about this at all. The authors may refer recent authority review on this topic by Drs. Zou, Wolchock, and Chen (Science TM, 2016).

Response:

We appreciated the reviewer's constructive comment. In our original manuscript, the Ref50 reported the recurrence of cancer patients after PD-1 antibody treatment and the Ref51 indicated that IDO1 inhibitor was in clinical trials. These two works did not link the clinical PD-L1/PD-1 blockade resistance to potential cancer dormancy (stemness), but had some relevance to our current work. The review (Sci Transl Med, 2016) by Zou et al. indeed elegantly discussed the relationship between PD-L1/PD-1 blockade resistance to potential cancer dormancy (stemness).

In the revised manuscript, we added the information "Notably, recent review by Zou et al. emphasized the link of clinical PD-L1/PD-1 blockade resistance to potential cancer dormancy (stemness), further suggesting the significance of targeting TRC dormancy in tumor immunotherapy (Sci Transl Med. 2016;8:328rv4.)." to page 25 line 16.

RESPONSE TO REVIEWER #2:

-It is speculated from the in vivo experiments in figure 7 and supplementary figure 7 that treatment of proliferating TRC (3 days after injection) or tumors of 7x7mm with all the described drugs: INFgamma, or anti-INFgamma ab (for syngenic mice) or INFgamma+1-MT/DMF, affects the dormancy/survival behavior of TRC. However, this is never demonstrated. Instead, the authors show us tumor growth curves and survival experiments after treatment with these drugs that, as shown by the authors, induce apoptosis (more than 50% of non-TRC population) and thus this could account for the observed results. Thus, this concern should be addressed by IF using specific markers for TRC in the tumors (for example after 10 days treatment as shown in Fig 7 e) or sorting by FACs (using CD133, nestin, Bmi-1 and c-kit markers as shown by this group previously) this TRC population and doing a direct analysis of cell cycle arrest.

Response:

We appreciate the reviewer's pertinent comment and constructive suggestion. In the revised manuscript, we conducted new experiments to provide more evidence to show that combining IFN-γ and an IDO or AhR inhibitor indeed affects the dormancy/survival behavior of TRCs. Firstly, we sorted $CD133^{high}$ tumor cells from B16 melanoma, and seeded these positive B16 cells to the soft 3D fibrin gels. We found that most cells could grow colonies, suggesting these B16 melanoma cells belong to TRCs. By contrast, most $CD133^{-}$ B16 cells could not grow colonies in the soft 3D fibrin gels. We thus used $CD133^{high}$ B16 cells as TRCs and analyzed their cell cycle status in B16 melanoma-bearing mice with different treatments. The result showed that IFN-γ treatment did not reduce the number of $CD133^{high}$ B16 cells but resulted in most of them entering G0/G1 cell cycle arrest; however, IFN-γ plus

1-MT (IDO inhibitor) or DMF (AhR inhibitor) significantly reduced CD133^{high} B16 cells. In addition, we also used CD133 antibody and Ki67 (a cell proliferating marker) to stain melanoma tissue. Consistently, we found that IFN-γ treatment caused much less Ki67 staining in CD133^{high} tumor cells, compared to CD133^{-} tumor cells, suggesting CD133^{high} tumor cells stay at a cell cycle arrest state. We also found that in the IFN-γ/1-MT or DMF treatment group, the density of CD133^{high} tumor cells markedly decreased, suggesting that TRCs are abrogated by the combined treatment.

According to the reviewer's suggestion, we added these new data in the revised manuscript, page 18 line 2 and revised supplementary Fig 7d, 8a, b, c.

In addition, the authors show that cells recovered from INFgamma-treated tumors have more sphere-forming capacity than control groups arguing for dormancy induction of TRC while in the tumor mass. However, these results could be interpreted in a different manner, for instance that dormancy phase of TRC was not affected by IFNgamma but rather that the non-TRC population was killed and therefore there are more TRC forming more spheres or that INFgamma treatment increased the sphere-forming capacity of TRC. Thus, the authors need: 1- to count number of TRC isolated from control or INFgamma-treated tumors, 2- to seed the same number of TRC in sphere conditions and count number of sphere in both groups. Here is important to use specific makers for the TRC population that the authors consider are responsible for the whole phenomenon.

Response:

We thank the reviewer's constructive suggestion. In the revised manuscript, we injected 1×10^5 B16 cells into C57BL/6 mice. When tumors grew to 5×5 mm, mice were treated with PBS and IFN-γ, respectively, once daily for 3 days. The total tumor cells were isolated from tumor mass and CD133^{high} melanoma cells were counted by flow cytometry. The result showed that there was the similar number of CD133^{high} melanoma cells between PBS and IFN-γ groups. Then, we seeded CD133^{high} tumor cells in the soft 3D fibrin gels, and found that there was no significant difference in colony formation between PBS and IFN-γ groups. As evidenced above, >90% CD133^{high} B16 cells belong to TRCs, thus these data together suggest that IFN-γ has no effect on the sphere-forming capacity of TRCs.

According to the reviewer's suggestion, we added these new data in the revised manuscript, page 15 line 20 and revised supplementary Fig 7c, d, e.

Lastly, the author show in figure 7a, c or e that tumors still grow upon INFg/1-MT/DMF/shIDO1 suggesting that either a TRC population is left behind or these are differentiated cells. This should be addressed.

Response:

In this study, we show that IFN-γ induced STAT1 signaling in TRCs is diverted to the IDO1-AhR-p27 pathway, leading to the activation of dormancy program while suppressing the process of cell death. Thus, blockade of the IDO1-AhR pathway may recover STAT-1 signaling, which mediates IFN-γ-induced cell death. Therefore, a key issue for the combination of IFN-γ and 1-MT or DMF lies in that tumor cells are able to access to IFN-γ. In our treatment model, we intratumorally injected IFN-γ (20 μg/day in PBS). This may produce high concentration of IFN-γ at the local injection site, but probably resulted in low concentration even absence of IFN-γ at the distant tumor site. This unequal distribution of IFN-γ might cause the unavailable IFN-γ to some tumor cells. So those tumor cells could still grow. Alternatively, although IFN-γ receptor is generally expressed by all live cells, some tumor cells including TRCs might be deficient of IFN-γ signaling pathway, due to the high heterogeneity of tumor cells, leading to TRCs escape of the combined treatment.

According to the reviewer's concern, we added this information in the revised Discussion section, page 26 line 8.

- Regarding the experiments of INFgamma plus DMF or 1-MT (Figure 6c), what would happen if you first add IFNg every 2 days for 20 days after 3 days post-injections of B16 TRCs and then stop INFgamma and add DMF or 1-MT for another 20 days? Would the number of recovered TRC be less in treated groups than control? Would the number of colonies formed by recovered TRCs be less in treated animals than PBS control? The authors tried to address this in Figure 6 C but only after 3 days of treatment with both INFgamma plus 1-MT or DMF and interestingly, the recovered TRC formed the same number of colonies than PBS-treated mice. Thus, Isn't this result suggesting that blocking the IDO pathway did not kill TRC? Thus, knowing the number of TRC after these treatments should be more informative.

Response:

We thank the reviewer's constructive suggestion. We understood the reviewer's questions on the recovered TRCs forming the similar number of colonies between the PBS group and IFN-γ/1-MT or DMF group in Fig. 6c. In our original Fig. 6b, we analyzed the cell cycle status, and also indicated the apoptosis in different groups. The result clearly showed that IFN-γ treatment effectively increased the proportion of cells with G0/G1arrest (changed from 15% to 34%), however the combined treatment strikingly decreased the proportion of cells with G0/G1 arrest (changed from 15% to 7-9%) and dramatically increased cell apoptosis (>90%) and the most live cells were in G0/G1 arrest. Based on these results, we further performed the experiment of Fig. 6c and tried to further confirm that blockade of IDO-Kyn-AhR cascade plus IFN-γ disrupts dormant TRCs in vivo. After three days treatment, the tumor size was actually reduced much. The tumor weight in the PBS group was 0.2 ± 0.015 g, which became to 0.12 ± 0.01 g in the IFN-γ group and further decreased

to 0.02 ±0.001g in the IFN-γ/1-MT group and 0.03 ±0.001g in the IFN-γ/DMF. Since we took same number of tumor cells from each group to seed them into the soft 3D fibrin gels, these tumor cells from each group theoretically were equal to the similar tumor weight/size. Thus, the result of Fig. 6c did not reflected the total TRCs in whole tumor mass. We appreciated the reviewer's comment on "knowing the number of TRC after these treatments should be more informative". In the revised manuscript, we revised the original Fig. 6c instead to reflect the total TRCs in each group.

Regarding to the reviewer's question of "what would happen if you first add IFN-γ every 2 days for 20 days after 3 days post-injections of B16 TRCs and then stop INF-γ and add DMF or 1-MT for another 20 days", we conducted the experiment. C57BL/6 mice were s.c. injected with 5×10^4 B16 TRCs. Three days later, mice were treated with IFN-γ (intratumoral injection of 20 μg/day, once every two days) for 20 days, and then treated with 1-MT (5 mg/ml in drinking water, 3-4 ml/mouse/day) or DMF for another 20 days. $CD133^{high}$ tumor cells from the whole tumor were sorted and counted. The result showed that there was a slight decrease in the number of $CD133^{high}$ tumor cells, but no significant difference between the PBS and IFN-γ groups. However, 1-MT or DMF treatment significantly decreased the number of $CD133^{high}$ tumor cells. Then, we seeded tumor cells into soft 3D fibrin gels, and found that the number of colony between the PBS and IFN-γ group had no difference, while the colony number remarkably decreased from 1-MT or DMF group. This unusual result might be due to the endogenous production of IFN-γ after 20 days' IFN-γ treatment. As a result, the administration of 1-MT or DMF would combine such endogenous IFN-γ to disrupt TRCs.

According to reviewers' suggestion, we added these new data in the revised manuscript, page 16 line 17 and revised supplementary Fig. 7i, j.

-Why is that 1-MT has an effect on tumor growth in figure 7d? On the contrary, DMF has not effect on tumor growth as seen in figure 7e. These results are showing us that without addition of INFgamma B16 cells grew normally (TRC and non-TRC) and only after adding INFgamma tumor growth decreased. So how is that 1-MT alone is affecting growth? Is inducing apoptosis of spontaneous dormant TRCs? Is affecting non-TRCs?

Response:

Indoleamine 2, 3-dioxygenase (IDO) is an inducible enzyme that catalyzes the metabolism of tryptophan. Originally, IDO was identified to play an immunosuppressive function in pregnancy (Science. 1998;281:1191-3). Later, its promoting cancer immune evasion was confirmed (Nat Med. 2003;9:1269-74). Recent preclinical data have showed that IDO inhibitor can delay tumor growth by immune-mediated mechanisms (Clin Cancer Res. 2009;15(2):641-9; Cancer Res. 2007; 67(2):792-801; Nat Med. 2005; 11(3):312-9). Consistently, we found that 1-MT treatment also inhibited tumor growth, as shown in Fig. 7d. In addition, we found that IDO1 knockdown affected neither B16 TRC growth in soft 3D

fibrin gels (Supplemental Fig. 4c) nor B16 tumor cell growth in conventional rigid plastic. According to the reviewer's concern, we added the above information in the revised manuscript, page 17 line 23.

--The authors should address if AhR staining signal comes from TRC or non-TRC upon INFgamma treatment in figure 6a.

Response:

We thank the reviewer's constructive suggestion. In the revised manuscript, we used anti-AhR and anti-CD133 for immuno-staining. The result showed that AhR translocated into the nucleus of $CD133^+$ tumor cells upon IFN-γ treatment. We added this new result in the revised manuscript, page 15 line 8 and revised supplementary Fig. 7a.

-In a model of spontaneous dormancy of TRC what would happen after addition of DMF or 1-MT? Would metastasis formation be reduced? (examples of spontaneous dormancy of TRC assays will be: after removal of primary tumor treat animals with 1-MT or DMF and then measure onset of metastatic foci in secondary organs. You will need to know the average time to metastasis in the chosen system and verify that disseminated TRC are in a dormant state before treatment; or a GEM). This experiment is crucial and it will support the concept that these agents are actually affecting spontaneous dormancy of disseminated tumor cells (as found in patients) and their clinical relevance as potential therapeutic drugs as proposed by the authors in the discussion. And it will also validate the activation of INFgamma-IDO-AhR-p27 pathway in dormant TRC. Because if spontaneous dormant disseminated cancer cells are not eliminated by DMF or 1-MT drugs that would imply that they don't activate the INFgamma-IDO-AhR-p27 and STAT1 pathway by themselves. This reviewer understands that this experiment may require longer time to get it done therefore it is not required for acceptance for publication at this moment.

Response:

We appreciate the reviewer's comment and constructive suggestions regarding to the model of spontaneous dormancy of TRCs. At this moment, we did not find existing model of spontaneous dormancy. Developing and validating such model will take a considerable time. Therefore, such experiments are beyond the scope of our current investigation. However, the spontaneous dormancy model is undoubtedly critical for us to better understand the concept of INF-γ-IDO-AhR-p27 mediating TRC dormancy and disruption of TRCs by the combined treatment and to develop a potential strategy to target spontaneous dormant disseminated cancer cells.

Regarding to the effect of 1-MT or DMF treatment on spontaneous dormancy of TRCs, we think such treatment might abrogate dormant TRCs upon how much IFN-γ available locally. Without immune surveillance, TRCs may rapidly grow and proliferate and do not enter dormancy. Therefore, spontaneous dormancy of TRCs might imply local IFN-γ availability.

However, if the IFN-γ level is too low, 1-MT or DMF treatment may have weak effect on dormant TRCs. On the other hand, spontaneous dormancy of TRCs might not necessitate immune surveillance, since extracellular matrix structure may be also capable of inducing TRC dormancy, which has been demonstrated in our another study (unpublished data). In this case, sole 1-MT or DMF treatment might not affect spontaneous dormancy of TRCs. However, if we provide exogenous IFN-γ, 1-MT or DMF treatment may work well.

-In each colony size graph, the authors should clarify in the "y" axis what is the relative size compare to? It is compared to day 1 after seeding cells? when do the authors consider a colony is formed (size, number of cells, day)?

Response:

We thank the reviewer's suggestion. In the revised manuscript, we added the information on the relative size in the "y" axis and other description for each Figure in the figure legends.

In addition, regarding to the colony issue, the reply was below:

We cultured tumor cells in soft fibrin gels (90-Pa) for 5 days to select them in our previous study (Nat Mater. 2012;11(8):734-41). At that time, we say that these cells not only have to survive the soft fibrin matrix but also have to proliferate in that soft mechanical environment. The reason that we waited for 5 days is to get more cells from a single colony.

Then we find that their Sox2 expression is tightly controlled by the softness of the matrix and it is time-dependent (Nat Commun. 2014;5:4619). Their mechanical stiffening and Sox2 expression all decreased to that of the control 2D cells after being plated on rigid plastic for 3 days. So it is at least 2 days to be called a TRC colony.

So, a cell that can proliferate and generate a colony from a single cell in soft (90-Pa) fibrin matrix is called a TRC and the colony it generates is a TRC colony.

- In Figure 7 d and e which mice background are those?

Response:

The mice used in figure 7d and e were C57BL/6 background. We added this information in the revised Figure legend of Fig. 7.

-Figure 8 J and K have no legends.

Response:

We appreciate the reviewer indicating this error. In the revised manuscript, we added the figure legends for figure 8j, k.

-in several figures the legends are shown in green but graph bars are blue.

Response:

In the revised manuscript, we corrected this error and changed the "green" to "blue".

-The majority of the colony size experiments are done by starting treatments at day 2-3 after seeding. Maybe worth a try to test the effect of INFgamma in solitary TRC as it would occur in cellular dormancy phenomenon?

Response:

We thank the reviewer's inspiring suggestion. We seeded B16 melanoma into soft 3D fibrin gels. Four days later, single TRCs were isolated from spheroids and seeded into the gel again in the presence of IFN-γ. As a result, most TRCs didn't grow an overt colony in the soft 3D fibrin gel. We showed the result below:

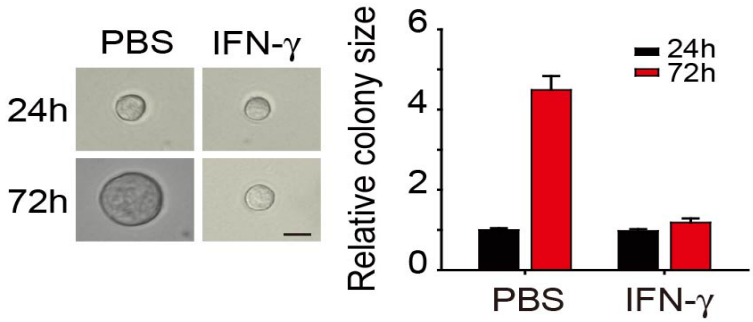

Figure B16 cells were seeded in soft 3D fibrin gels for 4 days. Then, single B16 TRCs were isolated, which were seeded to new 3D fibrin gels. Four hours later, these single TRCs were treated with IFN-γ (100 ng/ml) for indicated time. The relative colony size was calculated by comparing the colony size in other groups with the colony size in the group of PBS (24h), which was set to 1. The data represent mean ± SEM. Bar, 50 μm.

RESPONSE TO REVIEWER #3:

IDO degrades Trp to generate several catabolites, of which kynurenine (Kyn) is just one. The authors claim that tumor cell dormancy is mediated by Kyn binding to AhR to induce p27 is supported by the data presented. However, potential contributions from other pathways downstream of IDO are not excluded rigorously. The authors suggest that the GCN2-dependent integrated stress response (ISR) to amino acid withdrawal (in this case Trp) is excluded from consideration because their data identifies Kyn/AhR as the key pathway that prevents tumor cell dormancy (line 199-200). This premise should be tested, not just assumed. Several approaches could be used to test if Trp depletion promotes tumor cell dormancy, including (but not limited to) adding excess Trp to assays (to prevent Trp depletion but not Kyn production) and monitoring ISR responses in tumor cells and the TME.

Response:

We appreciated the reviewer's constructive suggestion on potential contributions from other pathways downstream of IDO, which was addressed in the second point-by-point answer. The reviewer also raised the question on exclusion of GCN2 contributing to the dormancy pathway. GCN2 is a critical kinase to sense the deprivation of amino acids. Under conditions of essential amino acid limitation, GCN2 phosphorylates eIF2α, inhibiting protein translation. Meanwhile, eIF2α phosphorylation also increases the translation of ISR transcription factor ATF4 that regulates the stress response gene expression. To verify this, we constructed stable knockdown GCN2-B16 cell lines by CRISPR-Cas9 technology. GCN2-SGGFP-B16 cells (control), GCN2-SGRNA1-B16 cells or GCN2-SGRNA2-B16 cells were seeded in 3D soft fibrin gels for 2 days, and then treated with IFN-γ for 4 days. The result showed that GCN2 knockdown did not affect the colony size of B16 TRCs, compared to the control group, suggesting that GCN2 is not involved in the process of IFN-γ-induced TRCs dormancy.

According to the reviewer's suggestion, we added this new result in the revised manuscript, page 10 line 22 and revised supplementary Fig. 2f.

Linked to the point above, several Trp catabolites (not just Kyn) have been reported to have anti-tumor effects due to their ability to alleviate T cell suppression directly and by reducing regulatory T cell (Treg) functions. Some of these compounds may also be produced in the TME and may activate the AhR (or other) pathways to induce tumor cell dormancy. This point should be addressed as there is some controversy about Kyn-mediated AhR activation because AhR has low relatively affinity for Kyn and because Kyn is removed rapidly from tissue microenvironments and excreted.

Response:

Kyn is a key element of tryptophan metabolism; it is enzymatically converted by kynurenine aminotransferase II to kynurenic acid (KYNA). It has been reported that KYNA inhibits proliferation of several cancer cell lines including colon cancer (Amino Acids. 2014;46(10):2393-401), renal cancer (Amino Acids. 2012; 43(4):1663-70) and glioblastoma cells (Pharmacol Rep. 2014;66(1):130-6). In addition, food tryptophan can be catalyzed to indole by bacterial tryptophanase in the gut and indole can be further metabolized by the liver into indoxyl sulfate. Both KYNA and indoxyl sulfate can act as endogenous ligand to activate AhR (Toxicol Sci. 2010; 115:89–97, Biochemistry. 2010; 49:393–400; Nat Rev Cancer. 2014; 14(12): 801–814). We therefore treated B16 TRCs with different dose of KYNA or indoxyle sulfate for 4 days and found that neither KYNA nor indoxyle sulfate could inhibit B16 TRCs growth even at high concentrations. Besides KYNA and indoxyl sulfate, tryptophan can also be converted to serotonin (a neurotransmitter) and the latter can be further converted to melatonin (a hormone). Melatonin has been shown to exert anti-tumor effect on many kinds of tumor by regulating immune responses (Cancer Res. 2006

15;66(20):9789-93; Recent Pat Endocr Metab Immune Drug Discov. 2011;5(2):109-23). Consistently, we found that serotonin and melatonin treatment did not induce TRC into dormancy. We understood the reviewer's concern on the low relatively affinity and excretion of Kyn. For this issue, the concentration might be very important. In our study, we actually found that TRCs expressed much higher IDO1 and AhR than differentiated tumor cells, leading to much higher levels of Kyn within TRCs (Supplemental Fig. 3b). Together, these results suggest that kynurenine might be a unique tryptophan metabolite participating AhR-regulated TRC dormancy.

According to the reviewer's suggestion, we added the new results in the revised manuscript, page 10 line 13 and revised supplementary Fig. 2c, d, e.

Data identifying the cell cycle regulator p27, as a downstream response factor to Kyn-AhR signaling should be reported in this study (line 218).

Response:

According to the reviewer's suggestion, we included the data that identified p27 as the downstream molecular in response to IFN-γ-Kyn-AhR signaling. We determined the expression of cell cycle-related genes such as cyclin D1, cyclin D3, cyclin A2, cyclin E, CDK2, p18, p21, p27 and p53 by western blot and found that only p27 expression was highly upregulated by IFN-γ. Moreover, we constructed stable knockdown AhR B16 cells. B16 cells (AhR-Sramble, AhR-Sh1 or AhR-Sh2) were seeded into soft 3D fibrin gels for 2 days and then treated with IFN-γ for 3 days. The result showed that p27 expression significantly decreased in AhR knockdown B16 TRCs, further confirming that p27 is indeed the downstream factor of IFN-γ-Kyn-AhR pathway.

According to the reviewer's suggestion, we added these new results in the revised manuscript, page 12 line 3 and revised supplementary Fig. 3e, f.

4. The authors suggest that differentiated tumor cells and TRCs have distinct responses to IFNγ mediated by JAK-STAT1 or IDO/Kyn/AhR signaling, respectively (lines 432-434). However, these are not alternative signaling pathways since considerable evidence shows that IDO induction occurs via the JAK-STAT1 pathway acting on GAS elements in the IDO gene promoter. While it is certainly intriguing that the response to IFNγ appears different in differentiated tumor cells and TRCs the authors should revise their interpretation to consider how JAK-STAT1 signaling might be modified in these cell types to generate distinctive downstream responses that explain the phenomena under study.

Response:

We appreciate this pertinent comment by the reviewer. In line with the notion indicated by the reviewer that IDO induction occurs via the JAK-STAT1 pathway acting on GAS elements in the IDO gene promoter, we actually found that complete knockdown of STAT1

through Crispr/Cas9 technology blocked IFN-γ-induced upregulation of IDO1 in TRCs. Notwithstanding these IFN-γ-induced signaling pathways, differentiated tumor cells prefer to choose JAK-STAT1 signaling but TRCs bias to IDO-Kyn-AhR signaling. Such difference might be due to that a very potential activity to metabolize tryptophan pre-exists in TRCs. Compared to differentiated tumor cells, TRCs expressed much higher IDO1 and AhR (see the original Fig. 4a and b and supplemental Fig. 3a). In addition, we also found that compared to differentiated tumor cells, TRCs expressed much higher levels of tryptophan transporter on their membrane surface that transports tryptophan into the cytosol of TRCs (unpublished data). Another reason might be the aforementioned intracellular Kyn concentration. Due to the low relatively affinity and excretion of Kyn, cells have to metabolize enough tryptophan to produce abundant Kyn, thus leading to activating AhR. In fact, as shown in our original supplemental Fig. 4b, we found that the Kyn level was much higher in TRCs than in differentiated cells in the presence or absence of IFN-γ treatment. Although IFN-γ can upregulate the expression of IDO1 in differentiated tumor cells, due to the relative low levels of tryptophan transporter (unpublished data), IDO1 and AhR, there is no enough Kyn to activate AhR. By contrast, when IFN-γ stimulates TRCs, the phosphorylated STAT1 can rapidly upregulate the expression of both IDO1 and AhR, leading to the production of high levels of Kyn and subsequent activation of AhR. Interestingly, recent study showed that the activation of Kyn-AhR signaling pathway only requires IFN-γ at the beginning (J Immunol. 2016;197(3):962-70.).
According to the reviewer's suggestion, we added the above information in the revised manuscript, page 23 line 15.

Data identifying IFNβ as an inducer of tumor cell dormancy should be reported (line 414-416). This is important because the authors suggest that the effects they observe are not dependent on adaptive immune cells, eliminating a potential source of IFNγ in the TME that could cause tumor cell dormancy. Moreover, recent reports identify DNA as a potent anti-tumor adjuvant due to STING/IFNβ signaling in the TME that activates dendritic cells to prime CTL responses. Thus natural or vaccine adjuvants may incite tumor cell dormancy via IFNβ signaling. Hence, data supporting this possibility should be reported in this study.
Response:
We thank the reviewer's constructive suggestion. Recently, we have completed the study on IFN-β and TRC dormancy, which is preparing for submission. In the IFN-β study, although we found that IFN-β was capable of inducing TRCs into dormancy, the molecular pathway was some different from IFN-γ. To avoid data duplication, we did not add IFN-β in this manuscript. On the other hand, the addition of IFN-β data might make the IFN-γ topic decentral. To make the theme more focus, we deleted the sentence "In addition, besides IFN-γ showed in this study, we also found in a separate study that IFN-β could induce TRC dormancy and seemed to be a better inducer than IFN-γ (data not shown)." in the revised

manuscript. We hope the reviewer could understand the difficulty for us to add the data of IFN-β in this manuscript.

It is unclear why Kyn-induced AhR translocation in TRCs is described as 'remarkable' (line 204), as several studies have documented previously that Kyn mediates AhR signaling.

Response:

We thank the reviewer for indicating this inappropriate wording. We chose "remarkable" to emphasize that Kyn can activate AhR for its nuclear translocation. Actually, we cited the reference (Kyn mediates AhR signaling) just before the word "remarkably". In the revised manuscript, we changed the word "Remarkably" to "Here, we found that" to make the wording more accurate, page 11 line 11.

Several typographical errors should be corrected.

Response:

We thank the reviewer's constructive suggestion. We carefully read the manuscript and corrected any typographical error in the revised manuscript.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The authors have addressed previous comments.

Reviewer #2 (Remarks to the Author):

The authors have made a great effort answering all the concerns form all the 3 reviewers. However, based on the new evidence provided by the authors there are some conclusions that are not accurate and should be changed.
- One of them is the conclusion in lane 4 page 9: "suggesting that the majority of B16 TRCs are induced to enter dormancy by INFgamma". Based on the new data offered by the authors (Suppl. 7 d, e, i, j, Supp. Fig 8a, b) the number of TRC (CD133+ cells) does not change upon IFNg treatment and the number of colonies produced by these TRCs is the same in treated vs untreated tumors. The fact that the authors showed differences in the number of colonies in Figure 2c is because INFg is killing the majority of tumor cells (non TRC) and leaving TRC unaffected. But from these results (figure 2c) you cannot conclude that TRC are entering dormancy upon in vivo treatment with INFg. I would suggest removing these results (Figure 2c, 2i,) from the paper because they are misinterpreted. The only figure that shows that actually CD133+ cells are entering cell cycle arrest is Suppl. Fig. 8a. Thus, Suppl. 8a should be added together with Figure 2a, because the suppl. figure 8a is actually addressing cell cycle arrest in the TRC population and this is the main message of the paper. In addition, this same type of analysis should be done in the other cell lines used in this manuscript.

- The staining for CD133 and ki67 (Supplementary figure 8c) are not convincing. CD133+ is not well appreciated from those images. Maybe a zoom in picture will help and the IgG control as well. In any case these images need to be quantified, showing just one field is not correct. At least 300 TRC CD133+ cells/tissue section (at least 5 non-consecutive sections) need to be counted and from those how many are Ki67+ in untreated- or INFg-treated mice. Why do the nuclei in INFg look smaller than in PBS or even INFg/DMF or/1-MT?

- The staining for CD133 and Ahr in Supplementary figure 7 a are inconclusive. It is hard to see CD133+/Ahr nuclear cells in those images. Also, this figure needs quantification and better resolution of the images.

-The conclusion in page 17, line 3 and 4 should be changed. The authors concluded that these results "are unusual". Honestly, these results are expected as the addition of INFg alone for 20 days induced first dormancy on TRC and killed the rest of the non-TRC cells. Then, the addition of 1-MT and DMF kill the dormant TRC cells. This is exactly what the authors have proposed, thus why are suddenly "unusual" these results? Even more, when the authors performed this strategy (first INFg then DMF) the number of TRC was almost zero (Supp. Figure 8i). This strategy was even more robust than combining both INFg +DMF at the same time (Fig. 6c). Furthermore, the strategy of treating tumors first with INFg followed by DMF or 1MT might be more attractive from a clinical perspective.

Minor suggestions/comments:

Remove figure 2d from principal figures (move to supplementary?) because it does not address which population (if TRC) is the one entering G0/G1.

The legend of figure 6c still says these are total tumor cells but according to the rebuttal letter these should be TRC CD133+ cells, right?

Reviewer #3 (Remarks to the Author):

The scope and impact of this study is improved substantially by the authors' robust responses to initial review and extensive new data added. My original concerns were addressed adequately, as were (in my opinion) the concerns of the other two reviewers.

RESPONSES TO REVIEWERS

We would like to express our sincere thanks to all three reviewers for their critical and constructive comments. We have performed substantial additional experiments to address the concerns by the reviewer #2. We respond point-by-point to each of the comments and criticisms. We feel that the comments have helped us on significantly improving and strengthening the manuscript and clarifying some issues. We hope that the revision has addressed the reviewer's concerns.

RESPONSE TO REVIEWER #1:

The authors have addressed previous comments.

RESPONSE TO REVIEWER #2:

The authors have made a great effort answering all the concerns form all the 3 reviewers. However, based on the new evidence provided by the authors there are some conclusions that are not accurate and should be changed.

One of them is the conclusion in lane 4 page 9: "suggesting that the majority of B16 TRCs are induced to enter dormancy by INFgamma". Based on the new data offered by the authors (Suppl. 7 d, e, i, j, Supp. Fig 8a, b) the number of TRC (CD133+ cells) does not change upon IFNg treatment and the number of colonies produced by these TRCs is the same in treated vs untreated tumors. The fact that the authors showed differences in the number of colonies in Figure 2c is because INFg is killing the majority of tumor cells (non TRC) and leaving TRC unaffected. But from these results (figure 2c) you cannot conclude that TRC are entering dormancy upon in vivo treatment with INFg. I would suggest removing these results (Figure 2c, 2i,) from the paper because they are misinterpreted. The only figure that shows that actually CD133+ cells are entering cell cycle arrest is Suppl. Fig. 8a. Thus, Suppl. 8a should be added together with Figure 2a, because the suppl. figure 8a is actually addressing cell cycle arrest in the TRC population and this is the main message of the paper. In addition, this same type of analysis should be done in the other cell lines used in this manuscript.

Response:

We appreciate the reviewer's constructive suggestion. In the revised manuscript, we redid the Supplementary Figure 8a experiment by injecting IFN-γ to B16 tumor-bearing mice. The $CD133^{+}$ tumor cells were then used for cell cycle analysis. The result showed that most of them are arrested in G0/G1 phase upon IFN-γ treatment.

For the *in vivo* dormancy section, B16 and OVA-B16 melanoma cell lines and murine H22 hepatocarcinoma cell line were used. Although we previously did a lot of studies on H22 tumor cells, neither we nor other labs clearly figured out the surface marker to identify stem-like H22 cells or TRCs. Currently, we could not conduct the same analysis on H22 TRCs due to no proper surface marker. However, we used OVA-B16 tumor cells to repeat the experiment. We sorted $CD133^+$ OVA-B16 tumor cells to perform cell cycle analysis and found that most of them are arrested in G0/G1 phase.

According to the reviewer's suggestion, we removed the results of figure 2c and figure 2i and added the new data in the revised manuscript, page 9 line 1, revised figure 2b and supplementary figure 1d.

The staining for CD133 and ki67 (Supplementary figure 8c) are not convincing. CD133+ is not well appreciated from those images. Maybe a zoom in picture will help and the IgG control as well. In any case these images need to be quantified, showing just one field is not correct. At least 300 TRC CD133+ cells/tissue section (at least 5 non-consecutive sections) need to be counted and from those how many are Ki67+ in untreated- or INFg-treated mice. Why do the nuclei in INFg look smaller than in PBS or even INFg/DMF or/1-MT?

Response:

We thank the reviewer for the constructive suggestion. Previously, we used Biolegend anti-CD133 antibody (Cat# 141201). In the revised manuscript, we changed the antibody provider and used Invitrogen anti-CD133 (Cat# PA5-38014). In the new immunostainings, $Ki67^+CD133^+$ cells are clearly distinguishable. The resolution is much improved. We also counted the number of cells with $Ki67^+CD133^+$ from 500 $CD133^+$ cells using 10 non-consecutive sections. As expected, much less $Ki67^+CD133^+$ cells were found in IFN-γ-treated mice, compared to the control.

As for the nuclear size issue in the IFN-γ-treated group, it might have been due to that it was from a different part of tissue sections, since the result was derived from histological sections. In the new immunostainings, the nuclear size in the IFN-γ treated group was of a similar size, maybe even slightly larger.

According to the reviewer's suggestion, we made a new staining with a better antibody and updated supplementary figure 8c in the revised manuscript. We also added the counted TRC numbers (quantified the result) in the revised manuscript, page 18 line 10.

The staining for CD133 and Ahr in Supplementary figure 7 a are inconclusive. It is hard to see CD133+/Ahr nuclear cells in those images. Also, this figure needs quantification and better resolution of the images.

Response:

We thanked the reviewer's constructive suggestion. In the revised manuscript, we used a better anti-CD133 antibody (Invitrogen, Cat# PA5-38014), instead of the one from Biolegend (Cat# 141201), to perform the immunostaining of anti-CD133 and anti-AhR on the tissue sections. We also counted the number of cells with $CD133^+$ and AhR nuclear localization from 500 $CD133^+$ cells using 10 non-consecutive sections. The result showed that AhR translocated into the nucleus of $CD133^+$ tumor cells upon IFN-γ treatment. We replaced the original images with high resolution ones and added the quantification result in the revised supplementary figure 7a.

The conclusion in page 17, line 3 and 4 should be changed. The authors concluded that these results "are unusual". Honestly, these results are expected as the addition of INFg alone for 20 days induced first dormancy on TRC and killed the rest of the non-TRC cells. Then, the addition of 1-MT and DMF kill the dormant TRC cells. This is exactly what the authors have proposed, thus why are suddenly "unusual" these results? Even more, when the authors performed this strategy (first INFg then DMF) the number of TRC was almost zero (Supp. Figure 8i). This strategy was even more robust than combining both INFg +DMF at the same time (Fig. 6c). Furthermore, the strategy of treating tumors first with INFg followed by DMF or 1MT might be more attractive from a clinical perspective.

Response:

We thanked the reviewer's great help in identifying this inappropriate wording. In the revised manuscript, we deleted the word "unusual".

Remove figure 2d from principal figures (move to supplementary?) because it does not address which population (if TRC) is the one entering G0/G1.

Response:

We thank the reviewer's suggestion. In the revised manuscript, we moved figure 2d to the supplementary materials. It is now supplementary figure 1c.

The legend of figure 6c still says these are total tumor cells but according to the rebuttal letter these should be TRC $CD133^+$ cells, right?

Response:

In figure 6c, we seeded the same number tumor cells into 3D fibrin gels. According to the number of colonies formed in the gels, we calculated the entire colony numbers for each group. Although we demonstrated that $CD133^{high}$ B16 melanoma cells belong to TRCs, in Figure 6c, the colony numbers were derived from the whole tumor cells.

RESPONSE TO REVIEWER #3:

The scope and impact of this study is improved substantially by the authors' robust responses to initial review and extensive new data added. My original concerns were addressed adequately, as were (in my opinion) the concerns of the other two reviewers.

REVIEWERS' COMMENTS:

Reviewer #2 (Remarks to the Author):

Main concern:
As much as I try to see the CD133 staining this is still difficult to see. I have checked in the literature and the staining should show at least a consistent membrane pattern (Check: A novel fixative for immunofluorescence staining of CD133-positive glioblastoma stem cells. Jonathan H. Shermana,*, Gerard T. Redpathb, Jan A. Redickc, et.al. J Neurosci Methods. 2011 May 15; 198(1): 99–102. doi:10.1016/j.jneumeth.2011.03.003.). In the pictures shown here is hard to see this pattern. And still no IgG control is shown. Therefore, with the data presented the conclusions are based on unreal results. Which cells are the authors counting as positive cells? A key of what they consider positive or negative staining should be shown. For example, supplementary Fig. 7a in PBS control there is basically no cell stained for CD133, what is that the authors want to show with that picture? Even more, the authors went on and quantified the % of nuclear ARH in these supposedly "positive" cells. The same in Supplementary Figure 8c: none of those cells look positive for CD133.
Since the authors have done a lot of work on this manuscript my suggestion would be to eliminate any data with IF staining for CD133 and focus on the FACs data for CD133.

Additional concerns:
-Supplementary Figure 8a- Could you add the percentage of CD131+ cells in G0, as shown in Supp. Fig. 1c?

RESPONSES TO REVIEWERS

We would like to express our sincere thanks to reviewer #2 for her or his critical and constructive comments.

RESPONSE TO REVIEWER #2:

Main concern:

As much as I try to see the CD133 staining this is still difficult to see. I have checked in the literature and the staining should show at least a consistent membrane pattern (Check: A novel fixative for immunofluorescence staining of CD133-positive glioblastoma stem cells. Jonathan H. Shermana,, Gerard T. Redpathb, Jan A. Redickc, et.al. J Neurosci Methods. 2011 May 15; 198(1): 99–102. doi:10.1016/j.jneumeth.2011.03.003.). In the pictures shown here is hard to see this pattern. And still no IgG control is shown. Therefore, with the data presented the conclusions are based on unreal results. Which cells are the authors counting as positive cells? A key of what they consider positive or negative staining should be shown. For example, supplementary Fig. 7a in PBS control there is basically no cell stained for CD133, what is that the authors want to show with that picture? Even more, the authors went on and quantified the % of nuclear ARH in these supposedly "positive" cells. The same in Supplementary Figure 8c: none of those cells look positive for CD133.*

Since the authors have done a lot of work on this manuscript my suggestion would be to eliminate any data with IF staining for CD133 and focus on the FACs data for CD133.

Response:

We thank the reviewer for the comment and help. We read carefully the cited paper as the reviewer referenced. In that paper, the authors used the established glioblastoma stem cell lines to immunostaining CD133, which are different from the tissue sections of melanoma we used in our experiment. In order not to make confusion, we took the reviewer's suggestion and removed the data with IF staining for CD133 in the revised manuscript.

Additional concerns:

-Supplementary Figure 8a- Could you add the percentage of CD131+ cells in G0, as shown in Supp. Fig. 1c?

Response:

We thank the reviewer for this constructive suggestion. According to the reviewer's suggestion, we add the percentage of $CD133^{+}$ cells in G0/G1 phase.