nature portfolio

Peer Review File

VSV$^{\Delta M51}$ drives CD8^{+} T cell-mediated tumour regression through infection of both cancer and non-cancer cells

Corresponding Author: Dr Douglas Mahoney

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In the current work, the authors examine the potential contribution of oncolytic VSV's infection of non-tumor cells on therapy. They attempt to show that systemically injected VSV retains therapeutic efficacy and immunological impacts even in tumor models where the tumor cells themselves are not directly infected. To this end, they identify direct viral infection of cells in the spleen and the draining lymph nodes as potentially serves to enhance activation and trafficking of dendritic cells from sites of inflammation. Overall, the direction of the study is viewed as extremely interesting. There is an increasing body of literature suggesting that oncolytic infection of tumor cells is not the only mechanism through which these treatments function and the mechanistic work contained in the manuscript, particularly the work done with the FitC painting model which truly precludes infection of tumor cells, is viewed as extremely attractive. However, many of the authors conclusions are also viewed as overstated in regards to the ability of their tumor models to truly study treatment in the complete absence of malignant cell infection. Nevertheless, the work is viewed as a potentially excellent addition to the overall body of literature if the authors can either provide additional support that their models truly lack infection or alter the language of their conclusions to be more in line with their provided data.

Specific Comments:
One of the major conclusions of the current paper is that VSV can induce tumor regressions and anti-tumor T cells responses in the complete absence of tumor cell infection. However, the data provided is not viewed as sufficient to support this conclusion. Indeed, most of the tumor models used appear to show some evidence of viral infection based on titers at early time points (Fig 1A) and the genetically resistant tumor models created (F7 and M3-9-Mres1 and res2) appear to replicate VSV almost as well as their parental counterparts (based on replication kinetics shown in Figs S2C and S2N). This data is therefore viewed as more clearly supporting the conclusion that VSV does not replication WELL in these tumor models (or that it is unable to spread within the tumors which also blunts resulting titers/g) as opposed to it being truly UNABLE to replicate. Because of this, many of the authors conclusions are viewed as too strong. As just a few examples:
On line 132 "that infection of non-cancer cells has the capacity to elicit an anti-tumour immune response".
On line 165 "Taken together, these data demonstrate that cancer cell infection is not a strict requirement or immune-mediated tumour regression after VSVΔM51 treatment"
On line 239 "Given that VSVΔM51 can generate antitumour activity in the absence of cancer cell infection"
On line 258 "This suggests that non- ancer cell infections promote DC activation and their migration from the tumour to the TdLN."
On line 129 the authors claim that "CT-26CL25, EMT-6, and M3-9-M tumours also underwent significant regression", however, only EMT6 tumors appears to actually regress. The other tumors simply display a delay in tumor growth (which is not the same as regression). The authors should clarify their language in this section.
The data shown is Fig 2K-N is slightly difficult to interpret. Some of these mice have 2 different types of tumors (M3-9-Msen and Res), however, there appears to only be a single average tumor volume shown in 2L and 2M. this makes it impossible to determine if the two types of tumors responded identically or differently to treatment. It would be helpful if the data was displayed differently to allow the reader to assess the individual growth of Sen and Res tumors on a mixed mouse.
I'm not sure that the experiments with FTY-720 show what the authors claim. Data in Fig S3H appears to show that FTY-720 reduced the efficacy of VSV treatment in infection sensitive tumors and does not appear significantly different that the impact of FY-720 in infection resistant tumors (Fig 3B). This appears to be more of an issue of the different models displaying intrinsically different responses to VSV treatment than a real mechanistic difference in where the T cells are derived from as the authors claim.

The data shown in Fig 6E regarding the reduction in recruitment of CD103+ DC's to the dLN following splenectomy is not convincing. The statistics applied here do not directly compare recruitment of these cells to the dLN between sham and splenectomy but instead compare recruitment with and without virus in each group. This comparison assumes that splenectomy has no impact on CD103+ DC's in the dLN in the absence of virus which does not appear to be true since splenectomized mice have higher baseline rates of CD103+ DC's even with no virus treatment in this experiment. The authors need to show that there is a statistical difference in the numbers of CD103+ DC's in the dLN of virally treated mice given sham surgery or splenectomy.
The data showing that splenectomized mice do not mount a therapeutic response following VSV treatment does not prove that this is due to splenic infection. These mice could easily mount a lower immune response due to the removal of the spleen. To draw this conclusion, the authors need to show that splenectomy does not impact therapeutic outcomes following treatment in which the spleen is not infected (such as direct intratumoral injection of virus).
I do believe that the authors data shows that VSV infection of non-cancerous cells can have immunological impacts, which is highly mechanistically interesting. However, most of their data is still consistent with viral infection of tumor cells being the primary driver of oncolytic efficacy since their most profound tumor regressions are still seen in tumor models that display high viral replication. The authors should discuss the potential relative impacts of tumor cell vs non-tumor cell infection on oncolytic outcomes in their discussion.

Reviewer #2

(Remarks to the Author)
In their submitted manuscript ,J Rajwani and co-workers provide extensive evidence that oncolytic viruses do not only debulk tumor mass by direct infection, and provide tumors antigens from ICD of cancer cells that can elicit an antitumor immune response, but in addition, that the oncolytic virus VSVdM51 can induce the production of Type I INFs which activate DCs and stimulate their migration to the draining LN. This latter effect alone can elicit permanent cure in immunogenic tumors resistant to VSVdM51 infection. These conclusions are supported by multiple complementary, carefully designed experiments. The manuscript, although not the shortest, is very well written.

A few points however, have not convinced me completely. The evidence that the spleen is the major if not only source of these IFNs is not that sound. The splenectomy experiments in mice shown in Figure 6H are the only evidence for this. However here it seems as if splenectomy alone slows down tumor growth of the controls and sham surgery already reduces the therapeutic effect of VSVdM51 considerably, which together reduce the validity of the data and the conclusions drawn by the authors.

In addition, it does not become clear, whether the effect is largely dependent on Type I IFNs alone, or if TNFa also plays a crucial role, as the authors state. In Figure 5 k e.g.: is the difference between TNFalpha neutralization and VSV alone significant? It seems marginal. This issue could be resolved if the immunogenic tumors that are not permissive for VSVdM51 would respond to the treatment of Type I INF alone to a similar degree as to VSVdM51 and if the additional application of TNFalpha would not significantly enhance this IFN only effect. This experiment would round up the story nicely.

Minor points:
In the bilateral tumor models: do the immunogenic resistant tumors indeed regress faster if the contralateral tumor is sensitive to VSV replication and not also resistant? This is addressed In Figure 2 M, i.e. is the difference in the tumor growth in the VSV treated animals between uniform and mixed model significant?

The summary of the data at the end of the introduction is unusually long and could be shortened if possible.

Taken together this paper provides important data that considerably enhance our understanding of the mode of action of oncolytic viruses.

Reviewer #3

(Remarks to the Author)
Title: VSVΔM51 drives CD8+ T cell-mediated tumour regression through
infection of both cancer and non-cancer cells

Major Comments:

As stated in the abstract, the new finding in this paper is "that while cancer cell infections induce viral oncolysis and antitumor immunity, infection of non-cancer cells in secondary lymphoid organs alone is sufficient to elicit durable cures. This occurs through the systemic production of TNFα and type I interferons, which promote activation of dendritic cells (DCs) in mouse and human tumors, as well as their ability to cross-present tumor antigen and migrate to the lymph node. Together, this results in enhanced CD8+ T cell activation, which generates tumor regression in a manner that is complementary with PD-1 blockade."

To my reading, the data does not support this broad claim. The paper does not present cancer models with definitive absence of any tumor infection that are cured by virus. The models have reduced replication but not clear evidence of absence of infection. The Galvio paper that they cite has already shown that a single-cycle VSV can be equally effective as

a fully replication-competent VSV in a mouse model of melanoma. In addition, the paper has no data showing direct infection of inflammatory, endothelial or other non-tumor cells producing cytokines which generate an anti-tumor response in the absence of viral infection of tumor.

“FITC painting” of the gastrocnemius following by IV virus enhanced the migration of FITC+ DCs (CD11c+ and CD103+) to the dLN and increased expression of the co-stimulatory markers, CD80 and CD86, on FITC+ DCs, perhaps related to infection of CD169+ macrophages and mediated by expression of secretion of TNFα and type 1 IFNs. Virus infection is clearly an immune stimulant, but the paper does not show that this non-specific immune stimulation has a therapeutic anti-tumor effect. In fact, Fig. 4J suggests that it does not because IV virus which can access the tumor produced therapeutic benefit but subcutaneous virus which accesses the draining LN but not the tumor, did not.

The study uses scientifically sound methods and materials, is presented clearly, and makes useful points. It shows that VSVΔM51 elicits tumor regression and durable cures in the infection-resistant, though not infection-absent, tumor models though it must be noted that cures occur in 100% of infection-sensitive compared with 20-40% of infection resistant tumors. The study also finds that OV infection increases the number of CD11c+ DCs and CD103+ classical DC1s (cDC1s) in the TdLN, as well as their expression of the costimulatory markers, CD80 and CD86.

The paper uses a male mouse derived tumor to demonstrate that tumor immunogenicity is a critical factor for responsiveness to systemic VSVΔM51 therapy in vivo. This raises the important question whether non-specific immune effects beneficial in an implantable tumor model translates to the clinical situation, but this issue confounds almost all animal models of cancer.

Minor Comments:

M3-9-M, ldlr knockout, is said to be infection resistant but no in vivo data is presented. Indeed, the papers shows that these resistant cells genetically engineered to express mCherry, have significantly increased numbers of mCherry DC in the TdLN following viral treatment, suggesting that the virus did infect the tumor and release mCherry which was taken up by the DC and transported to the LN.

Fig. 5K does not convincingly show that TNFα and type 1 IFNs are important mediators of the viral anti-tumor effect.

Fig. 6H does not convincingly show that virus infection of spleen helps the therapeutic response because the benefit in the positive control is marginal, and the sham negative control has the same result as the experimental group.

The figures are too small and resist interpretation in print but can be magnified on a computer screen.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Overall, this work is still viewed as an excellent exploration of an extremely challenging mechanistic question in OV. As such, the conclusions drawn in the revision are much more in line with the data shown (which is always going to be more gray areas than black and white), however, I feel that in a few places the statements provided are still slightly too strong (see specific comments below). I feel that these issues can certainly be addressed by minor textual changes, however, without the need for additional experimentation.

Line 41: the word alone should be removed
Line 75: the word live should be removed (viruses are not technically alive)
Line 77: The first approved OV was H101 in china in 2006 not T-vec.
Line 270: this should also reference Fig 1d since it draws a comparison between the M3-9-M res tumors (shown in Fig 1h) and the WT tumors (shown in Fig 1d).
Line 300-303: The authors claim that
Taken together, these data demonstrate that productive virus replication within cancer cells is not a strict requirement for immune-mediated tumour regression after VSVΔM51 treatment,
As with the previous critique, this statement is still not supported by the data. All the data presented by the authors suggests that VSV CAN replicate in all of the tested cell lines (even the ‘resistant’ ones). Figs S1B, S3C, and S3N actually show robust viral replication in all the cell lines used in the study and the ‘resistant’ clones (with the exception of a reduction in the F7 clone) actually display virtually identical replication curves to WT cells. The difference is that the virus can’t spread well in the resistant clones after it produces new progeny.
Similarly, Fig S2H shows that virus can at least initiate infection in M3-9-M tumors (or at least express sufficient CRE transgene to flox out the stop codon) if the virus is delivered IT. The data therefore seems more consistent with the idea that IV delivery of VSV is very inefficient and, in the absence of robust spread, the infections are below the limit to detection for

IVM and BLI (which are not very sensitive assays). The indicated statement therefore should be revised to indicate that high level infections of tumor cells are not required.
Figure 2F appears to be missing the yellow line that should link M3sen#1 dots.
Line 427: The statement that “Given that VSVΔM51 can generate anti-tumour activity without productively infecting cancer cells” is not supported by the data and should be changed to indicate something about not having ‘high levels’ of infection in cancer cells.
Line 426-439: This paragraph contains numerous statements that are not supported by the data. For example,
it is not clear whether infection of non-cancer cells ‘elicits’ CD8 T cell responses (line 428) or whether it amplifies existing responses (the latter seems more likely). This wording should be changed accordingly.
It is not definitively shown that in the M3-9-Mres model contain only no cancer cells are infected making the statement “To begin, we asked whether non-cancer cell infections lead to anticancer CD8+ T-cell accumulation in tumours.” an over interpretation of the data.
It is unclear whether VSV infection in these models induces or enhances CD8 T cell response making the statement “This suggests that virus replication within non-cancer cells generates anticancer immunity by acting predominantly on CD8+ T cells in the TdLN.” an over interpretation of the data.
Line 446: As above, the following statement “This suggests that non-cancer cell infections promote DC activation and their migration from the tumour to the TdLN.” an over interpretation of the data.
Lines 483-485: Same comment as above about an over interpretation of the data.
The data in Fig S8C does not show viral titers as the text indicates (it shows BLI). It would be beneficial (although probably not required if the authors adjust the language) if the authors could show actual viral titers in the LN’s following IV injection of SQ injection of 1e8 PFU.
Line 569: The statement “LN infections alone were insufficient to provide a survival benefit” should be changed to “SQ viral injections alone…”
Lines 692-694: I appreciate the revisions to the text here, however, I think the statement that “this recruitment was blunted” is probably not accurate since the same number of CD103+ DC’s appear to be in the LN’s of splenectomized mice (the data is not significant because the background is higher in these mice not because the recruitment is less).
There appears to be a typo or deletion of text on line 763…

Reviewer #2

(Remarks to the Author)
All comments of the reviewers have been carefully addressed and the additional experiments suggested were performed.

REVIEWER COMMENTS Manuscript NCOMMS-23-48454-T

Reviewer #1 (Remarks to the Author):

In the current work, the authors examine the potential contribution of oncolytic VSV's infection of non-tumor cells on therapy. They attempt to show that systemically injected VSV retains therapeutic efficacy and immunological impacts even in tumor models where the tumor cells themselves are not directly infected. To this end, they identify direct viral infection of cells in the spleen and the draining lymph nodes as potentially serves to enhance activation and trafficking of dendritic cells from sites of inflammation. Overall, the direction of the study is viewed as extremely interesting. There is an increasing body of literature suggesting that oncolytic infection of tumor cells is not the only mechanism through which these treatments function and the mechanistic work contained in the manuscript, particularly the work done with the FitC painting model which truly precludes infection of tumor cells, is viewed as extremely attractive. However, many of the authors conclusions are also viewed as overstated in regards to the ability of their tumor models to truly study treatment in the complete absence of malignant cell infection. Nevertheless, the work is viewed as a potentially excellent addition to the overall body of literature if the authors can either provide additional support that their models truly lack infection or alter the language of their conclusions to be more in line with their provided data.

We thank the reviewer for providing valuable feedback on our work. We have attempted to address all concerns that the reviewer has raised, which we have detailed below.

Specific Comments:

- One of the major conclusions of the current paper is that VSV can induce tumor regressions and anti-tumor T cells responses in the complete absence of tumor cell infection. However, the data provided is not viewed as sufficient to support this conclusion. Indeed, most of the tumor models used appear to show some evidence of viral infection based on titers at early time points (Fig 1A) and the genetically resistant tumor models created (F7 and M3-9-Mres1 and res2) appear to replicate VSV almost as well as their parental counterparts (based on replication kinetics shown in Figs S2C and S2N). This data is therefore viewed as more clearly supporting the conclusion that VSV does not replication WELL in these tumor models (or that it is unable to spread within the tumors which also blunts resulting titers/g) as opposed to it being truly UNABLE to replicate. Because of this, many of the authors conclusions are viewed as too strong. As just a few examples:
- On line 132 "that infection of non-cancer cells has the capacity to elicit an anti-tumour immune response".
- On line 165 "Taken together, these data demonstrate that cancer cell infection is not a strict requirement or immune-mediated tumour regression after VSVΔM51 treatment"
- On line 239 "Given that VSVΔM51 can generate antitumour activity in the absence of cancer cell infection"
- On line 258 "This suggests that non- ancer cell infections promote DC activation and their migration from the tumour to the TdLN."

We thank the reviewer for these comments and agree that the original data presented in the manuscript better supports the conclusion that VSV$^{\Delta M51}$ does not replicate *well* in the tumour, rather than that it is unable to replicate. Indeed, it might be possible that when we measure replication by assessing transgene expression (either luminescence from VSV$^{\Delta M51\text{-}Fluc}$, or GFP from VSV$^{\Delta M51\text{-}GFP}$), we miss time points at which there may be some replication, or the level of transgene expression is below the threshold of detection. To address this, we devised a cre-inducible system to more reliably detect cells that have supported virus replication within the tumour. In this system, delivery of cre-expressing

VSV$^{\Delta M51}$ (VSV$^{\Delta M51\text{-creGFP}}$) permanently labels cells in which the virus has replicated (Supplementary Fig. 2a). We first tested this by delivering VSV$^{\Delta M51\text{-creGFP}}$ intravenously to mice in which all cells encode TdTomato downstream of a floxed stop codon. We found that even once GFP was no longer detectable in tissues such as the spleen and lymph node, cells remained TdTomato positive (Supplementary Fig. 2b,c). We thus engineered the M3-9-M cell line to inducibly express mCherry with VSV$^{\Delta M51\text{-creGFP}}$ infection (Supplementary Fig. 2d), which we show permanently marks cells *in vitro* (Supplementary Fig. 2e-g), even after GFP expression from the virus begins to decrease. We also validated this system *in vivo* and showed that tumour cells became permanently mCherry+ post intra-tumoral delivery of VSV$^{\Delta M51\text{-creGFP}}$ (Supplementary Fig 2h). Nonetheless, despite thoroughly imaging tumours after intravenous delivery of VSV$^{\Delta M51\text{-creGFP}}$, we very rarely observed mCherry+ cancer cells in the tumour microenvironment (Supplementary Fig 2i). This further supports our conclusion that VSV$^{\Delta M51}$ does not replicate well in the tumour. We have compiled these data in Supplementary Figure 2, which we have added to the revised manuscript.

We have also adjusted the language in our manuscript as per the reviewer's suggestions. Thus, instead of making strong conclusions that VSV$^{\Delta M51}$ elicits tumour regression in the *complete absence* of cancer cell infection, we now state that VSV$^{\Delta M51}$ can elicit tumour regression *without measurable productive infection and spread* through the tumour. These have been noted with track changes in the manuscript document.

Finally, we produced data to address the reviewer's specific comment that high viral titers in the tumour at early time points may be evidence of virus infection. We believe that these titers are not progeny virions from productive infection, but rather, represent residual virus particles from the initial intravenous administration. Indeed, we have found in M3-9-M that tumour titers are highest at 15 minutes post virus administration, and steadily decline over time. We have included this new data as Supplementary Figure 1g.

- On line 129 the authors claim that "CT-26CL25, EMT-6, and M3-9-M tumours also underwent significant regression", however, only EMT6 tumors appears to actually regress. The other tumors simply display a delay in tumor growth (which is not the same as regression). The authors should clarify their language in this section.

We agree with this comment, and thus have rephrased the sentence to indicate that only mice bearing EMT-6 and M3-9-M tumours experienced significant regression (as several mice cured); CT-26.CL25 bearing mice experienced delayed tumour growth. This change in phrasing is found on page 6, lines 217-218.

- The data shown is Fig 2K-N is slightly difficult to interpret. Some of these mice have 2 different types of tumors (M3-9-Msen and Res), however, there appears to only be a single average tumor volume shown in 2L and 2M. this makes it impossible to determine if the two types of tumors responded identically or differently to treatment. It would be helpful if the data was displayed differently to allow the reader to assess the individual growth of Sen and Res tumors on a mixed mouse.

We appreciate this being brought to our attention. The average tumour volume in Figure 2l is only for the M3-9-M^{Sens1} tumours. Thus, even when mice carry both M3-9-M^{Sens1} and M3-9-M^{Res1} tumours (labeled "mixed bilateral"), Fig 2l only shows the average tumour growth of M3-9-M^{Sens1} tumours. Similarly, Figure 2m only shows average tumour growth of infection resistant (M3-9-M^{Res1}) tumours. These data show that after VSV$^{\Delta M51}$ treatment, M3-9-M^{Res1} tumours experience greater tumour regression when there is a contralateral infection sensitive tumour (as in the mixed bilateral model) as

opposed to when there is another infection resistant tumour (as in the M3-9-M^{Res1} uniform bilateral model). To clarify this, we have changed the description of this experiment in the figure legend as well as in the main text (page 10, 393-399). We have also modified the subfigures by adding a schematic to 2l and 2m so that the reader knows which tumour is being measured in each subfigure.

- I'm not sure that the experiments with FTY-720 show what the authors claim. Data in Fig S3H appears to show that FTY-720 reduced the efficacy of VSV treatment in infection sensitive tumors and does not appear significantly different that the impact of FY-720 in infection resistant tumors (Fig 3B). This appears to be more of an issue of the different models displaying intrinsically different responses to VSV treatment than a real mechanistic difference in where the T cells are derived from as the authors claim.

We agree that the intrinsically different responses of M3-9-M^{Res1} and M3-9-M^{Sens1} tumours to $VSV^{\Delta M51}$ make it challenging to compare these two models. We have thus amended the manuscript to remove the comparison between the two models. We simply highlight that in M3-9-M^{Res1} tumours, $VSV^{\Delta M51}$ no longer elicits durable cures with FTY-720 treatment, suggesting that T cell recruitment from the lymph nodes is required to generate cures in this model. This change in phrasing can be found on page 11, lines 434-439.

- The data shown in Fig 6E regarding the reduction in recruitment of CD103+ DC's to the dLN following splenectomy is not convincing. The statistics applied here do not directly compare recruitment of these cells to the dLN between sham and splenectomy but instead compare recruitment with and without virus in each group. This comparison assumes that splenectomy has no impact on CD103+ DC's in the dLN in the absence of virus which does not appear to be true since splenectomized mice have higher baseline rates of CD103+ DC's even with no virus treatment in this experiment. The authors need to show that there is a statistical difference in the numbers of CD103+ DC's in the dLN of virally treated mice given sham surgery or splenectomy.

We thank the reviewer for this comment. What we intended to ask with this experiment was whether $VSV^{\Delta M51}$ still recruited DCs to the lymph node in splenectomized mice. We believe that the comparison should thus be carried out between control and $VSV^{\Delta M51}$ treatment in splenectomized mice. This also accounts for changes that splenectomy surgery may have on $CD103^{+}$ DC contexture in the lymph node. Our non-significant p-value suggests that $VSV^{\Delta M51}$ treatment does not significantly increase $CD103^{+}$ DC recruitment to the lymph node, suggesting that $VSV^{\Delta M51}$ infection in the spleen contributes to DC recruitment from the tumour to the lymph node.

We have amended the language in the manuscript to better describe the statistical analyses that were carried out. These changes can be found on page 17, lines 692-694.

- The data showing that splenectomized mice do not mount a therapeutic response following VSV treatment does not prove that this is due to splenic infection. These mice could easily mount a lower immune response due to the removal of the spleen. To draw this conclusion, the authors need to show that splenectomy does not impact therapeutic outcomes following treatment in which the spleen is not infected (such as direct intratumoral injection of virus).

We very much appreciate this suggestion. To test whether splenectomized mice are able to mount a functional immune response, we treated tumour-bearing mice that underwent no surgery, sham surgery, or splenectomy with anti-PD-1. We chose this treatment over intratumoral VSV for several reasons; first, anti-PD1 treatment is a systemic therapy (as is i.v. VSV) that enhances antitumour T cell activity

primarily by acting in the tumour and the TdLN rather than the spleen; second, in the case of intratumoral VSV delivery, we are unsure whether virus drains from the tumour bed to the spleen, and whether this may impact tumour regression.

We found that in all groups, mice achieved significant tumour regression and durable cures with anti-PD1 treatment. Thus, splenectomy alone does not impact the ability of mice to mount an antitumour immune response. We have added this data to the manuscript as Supplementary Fig 10, and described it in the manuscript on page 18, lines 723-726.

- I do believe that the authors data shows that VSV infection of non-cancerous cells can have immunological impacts, which is highly mechanistically interesting. However, most of their data is still consistent with viral infection of tumor cells being the primary driver of oncolytic efficacy since their most profound tumor regressions are still seen in tumor models that display high viral replication. The authors should discuss the potential relative impacts of tumor cell vs non-tumor cell infection on oncolytic outcomes in their discussion.

We appreciate these suggestions and have amended the discussion to address these comments.

Reviewer #2 (Remarks to the Author):

- In their submitted manuscript ,J Rajwani and co-workers provide extensive evidence that oncolytic viruses do not only debulk tumor mass by direct infection, and provide tumors antigens from ICD of cancer cells that can elicit an antitumor immune response, but in addition, that the oncolytic virus VSVdM51 can induce the production of Type I INFs which activate DCs and stimulate their migration to the draining LN. This latter effect alone can elicit permanent cure in immunogenic tumors resistant to VSVdM51 infection. These conclusions are supported by multiple complementary, carefully designed experiments. The manuscript, although not the shortest, is very well written.

We appreciate the feedback provided on our work and have attempted to address all concerns raised by the reviewer, which we describe below.

- A few points however, have not convinced me completely. The evidence that the spleen is the major if not only source of these IFNs is not that sound. The splenectomy experiments in mice shown in Figure 6H are the only evidence for this. However here it seems as if splenectomy alone slows down tumor growth of the controls and sham surgery already reduces the therapeutic effect of VSVdM51 considerably, which together reduce the validity of the data and the conclusions drawn by the authors.

We thank the reviewer for this comment. We agree that the spleen is not the only source of IFNs after VSV infection. However our results suggest that splenic infections nonetheless contribute to systemic interferon production (Fig 6C), consistent with previous literature[1,2]. We have amended the manuscript to better reflect the spleen's role in the production of interferons. These changes can be found on page 17, lines 686-688 and page 18, lines 727-729.

We also agree with the reviewer's evaluation of the splenectomy overall survival experiments. It is true that the effect of VSV$^{\Delta M51}$ is reduced in mice that underwent sham surgery, which could possibly be attributed to a wound healing response that is immunosuppressive[3]. There is also a trend towards decreased interferons in the serum of mice that underwent sham surgery compared to control (no surgery mice) after VSV$^{\Delta M51}$ treatment, which may also have led to attenuated responses (Fig 6c). This might be enough immunosuppression to decrease the response to VSV$^{\Delta M51}$, as this therapy only generates survival in approximately 20-40% of control (no surgery) mice. It does not seem to be enough immunosuppression to affect the response to a more potent immunotherapy, such as immune checkpoint blockade (as this therapy generated near 100% survival in control mice, as well as mice that underwent sham surgery, data that is newly generated and can now be found in Supplementary Figure 10). Further, as the reviewer stated, splenectomy alone slows down tumour growth. We believe this could be because the spleen contributes immunosuppressive myeloid derived suppressor cells (MDSCs) to the tumour, particularly in the M3-9-M tumour model (as shown by Dr. Crystal Mackall's group[4]). We suspect that it is because of these opposing activities – the increase in tumour growth with sham surgery and the decrease in growth with splenectomy – that it is difficult to measure potential differences in overall survival between sham and splenectomy mice after VSV$^{\Delta M51}$ treatment. Indeed, we believe that it is promising that we are still able to observe a statistically significant delay in tumour growth after VSV$^{\Delta M51}$ treatment of mice that received sham surgery, and that this effect is lost in splenectomized mice.

Although beyond the scope of this study, we hope to eventually design other methods to block VSV$^{\Delta M51}$ infection in the spleen. This likely will require knowing which cell types are infected in order to design specific strategies for blockade.

- In addition, it does not become clear, whether the effect is largely dependent on Type I IFNs alone, or if TNFa also plays a crucial role, as the authors state. In Figure 5 k e.g.: is the difference between TNFalpha neutralization and VSV alone significant? It seems marginal. This issue could be resolved if the immunogenic tumors that are not permissive for VSVdM51 would respond to the treatment of Type I INF alone to a similar degree as to VSVdM51 and if the additional application of TNFalpha would not significantly enhance this IFN only effect. This experiment would round up the story nicely.

We appreciate the reviewer's comment and have added the appropriate statistical comparisons to subfigure 5k. As can be seen, the difference between $VSV^{\Delta M51}$ and $VSV^{\Delta M51}$ + aTNFa is not significant ($p = 0.4687$). Thus, neutralizing TNFa alone is not sufficient to abrogate $VSV^{\Delta M51}$-induced tumour regression. Neutralizing both TNFa and type I IFNs completely abrogates VSV-induced antitumour immunity, which our data suggests may be due to impaired antigen presentation (Fig 5d) and T cell activation (Fig 5j). Interestingly, neutralizing Type I IFNs alone is also sufficient to nullify $VSV^{\Delta M51}$-induced tumour regression. This may be because IFN has other roles in promoting antitumour immunity – such as the stimulation of NK cells, or promotion of MHC I expression.

We also thank the reviewer for their experimental suggestion. We agree that assessing the response of tumour bearing mice to TNFa, Type I IFNs or both would be a valuable addition to our study. Given the dose dependent differences in the activity of these cytokines, and the potential for toxicity at high doses, we chose to treat mice with 1µg of cytokines, as this dose achieves similar serum concentrations to the peak $VSV^{\Delta M51}$ response and has been used in previous mouse studies[5–8]. Interestingly, we found that these cytokines alone were insufficient to induce tumour regression, suggesting either that other factors are involved in the $VSV^{\Delta M51}$ response, or that the more continuous production of cytokines from $VSV^{\Delta M51}$-infected cells is required over the discrete delivery of cytokines. We have added this data to the manuscript as Fig 5l.

- Minor points:
- In the bilateral tumor models: do the immunogenic resistant tumors indeed regress faster if the contralateral tumor is sensitive to VSV replication and not also resistant? This is addressed In Figure 2 M, i.e. is the difference in the tumor growth in the VSV treated animals between uniform and mixed model significant?

We thank the reviewer for this question. Yes, we found that after $VSV^{\Delta M51}$ treatment, M3-9-M^{Res1} (infection resistant) tumours experienced greater tumour regression when there was a contralateral infection sensitive tumour (as in the mixed bilateral model) as opposed to when there was another infection resistant tumour (uniform bilateral model). The p-values on Figure 2m indicate that these differences in average tumour growth are significant. We have adjusted this subfigure (as well as the corresponding figure legend and description in the main text) to better clarify the results.

- The summary of the data at the end of the introduction is unusually long and could be shortened if possible.

Thank you for this suggestion, we have shortened the introduction.

- Taken together this paper provides important data that considerably enhance our understanding of the mode of action of oncolytic viruses

Reviewer #3 (Remarks to the Author):

Title: VSVΔM51 drives CD8+ T cell-mediated tumour regression through infection of both cancer and non-cancer cells

Major Comments:

- As stated in the abstract, the new finding in this paper is "that while cancer cell infections induce viral oncolysis and antitumor immunity, infection of non-cancer cells in secondary lymphoid organs alone is sufficient to elicit durable cures. This occurs through the systemic production of TNFα and type I interferons, which promote activation of dendritic cells (DCs) in mouse and human tumors, as well as their ability to cross-present tumor antigen and migrate to the lymph node. Together, this results in enhanced CD8+ T cell activation, which generates tumor regression in a manner that is complementary with PD-1 blockade."
- To my reading, the data does not support this broad claim. The paper does not present cancer models with definitive absence of any tumor infection that are cured by virus. The models have reduced replication but not clear evidence of absence of infection. The Galvio paper that they cite has already shown that a single-cycle VSV can be equally effective as a fully replication-competent VSV in a mouse model of melanoma. In addition, the paper has no data showing direct infection of inflammatory, endothelial or other non-tumor cells producing cytokines which generate an anti-tumor response in the absence of viral infection of tumor.

This comment is similar to one made by reviewer 1, and we are thankful that both reviewers brought this to our attention. We agree that the original data presented in the manuscript suggests that VSV$^{\Delta M51}$ is unable to productively replicate in the tumour, as opposed to there being a complete absence of cancer cell infection. We have adjusted the language in our manuscript to better reflect this, and hope that this addresses both reviewer 1 and 3's concerns.

We also appreciate the reviewer discussing the Galivo paper, in which Galivo et al showed that single cycle replication of VSV$^{\Delta M51}$ is as effective as replication-competent VSV$^{\Delta M51}$ in a mouse model of melanoma[9]. In this paper, VSV$^{\Delta M51}$ was rendered replication-defective by deleting the glycoprotein gene (ΔG- VSV$^{\Delta M51}$). In theory, this virus should still be able to express transgenes in infected cells, despite being unable to spread throughout the tumour. In all our tumour models except for CT-26^{IFNmut}, we were unable to detect virus transgene expression (by bioluminescence imaging and intravital microscopy), suggesting that VSV$^{\Delta M51}$ is not productively replicating within tumour cells.

However, we agree that there are limitations to the ability of the methods used in our manuscript to detect infected cells – namely that they rely on identification of cells based on transient viral transgene expression. To address this, we devised a cre-inducible system to identify infected cancer cells, in which tumour cells are engineered to become permanently mCherry+ upon infection with cre-expressing VSV$^{\Delta M51}$ (VSV$^{\Delta M51\text{-creGFP}}$). More details about this system and its validation can be found in our answer to reviewer 1's first 'specific comment,' as well as in Supplementary Figure 2 of the revised manuscript. In short, however, we found that mCherry+ cancer cells were rarely observed after i.v. delivery of VSV$^{\Delta M51\text{-creGFP}}$ (Supplementary Figure 2i). This further supports our conclusion that VSV$^{\Delta M51}$ does not replicate well in the tumour.

Finally, to address the reviewer's comments about non-cancer cell infections, we have shown in several subfigures that VSV$^{\Delta M51}$ infects secondary lymphoid organs after intravenous delivery (Fig. 4f, Fig. 6a, Supplementary Fig. 2b,c, Supplementary Fig. 8a,b). These non-cancer cell infections do indeed produce inflammatory cytokines after intravenous VSV$^{\Delta M51}$ administration, as shown in Supplementary figure 9b

(serum harvested from non-tumour bearing mice). This suggests that VSV$^{\Delta M51}$ can elicit a robust cytokine response in the absence of cancer cell infections. We apologize that this was unclear and have amended the figure legend of Supplementary Figure 9b to clarify that the cytokines/chemokines being measured are from non-tumour bearing mice.

We acknowledge that other cell types in the tumour microenvironment have been reported to be infected by VSV$^{\Delta M51}$ and have been studied elsewhere. However, we found that the majority of non-cancer cell infections after systemic delivery of VSV$^{\Delta M51}$ were in secondary lymphoid organs, and thus focused our investigation on the impact of these infections. Nonetheless, we have listed the lack of exploration of non-cancer cell infections in the TME as a limitation to our study in the discussion.

- "FITC painting" of the gastrocnemius following by IV virus enhanced the migration of FITC+ DCs (CD11c+ and CD103+) to the dLN and increased expression of the co-stimulatory markers, CD80 and CD86, on FITC+ DCs, perhaps related to infection of CD169+ macrophages and mediated by expression of secretion of TNFα and type 1 IFNs. Virus infection is clearly an immune stimulant, but the paper does not show that this non-specific immune stimulation has a therapeutic anti-tumor effect. In fact, Fig. 4J suggests that it does not because IV virus which can access the tumor produced therapeutic benefit but subcutaneous virus which accesses the draining LN but not the tumor, did not.

We thank the reviewer for this comment. In experiments where we interrogate the role of non-cancer cell infections (Figures 3,4,5,6), we use infection-resistant M3-9-M tumours in all experiments. To the best of our abilities, this limits confounding effects that may be caused by productive virus replication within cancer cell. Results from these experiments show that non-cancer cell infections induce non-specific immune stimulation through the systemic production of inflammatory cytokines (TNFa and type I IFNs), and that neutralization of these cytokines reduces antigen presentation in the TdLN, antitumour T cell activity, and ultimately tumour regression and overall survival (Fig 5)

In figure 4 specifically, we asked whether VSV$^{\Delta M51}$ infection of the tumour draining lymph node (TdLN) alone could induce tumour regression. We found that while TDLN infections enhanced co-stimulatory marker expression on dendritic cells, this did not lead to improved survival. We thus focused our attention on other non-cancer cell infections (namely in the spleen).

- The study uses scientifically sound methods and materials, is presented clearly, and makes useful points. It shows that VSVΔM51 elicits tumor regression and durable cures in the infection-resistant, though not infection-absent, tumor models though it must be noted that cures occur in 100% of infection-sensitive compared with 20-40% of infection resistant tumors. The study also finds that OV infection increases the number of CD11c+ DCs and CD103+ classical DC1s (cDC1s) in the TdLN, as well as their expression of the costimulatory markers, CD80 and CD86.

- The paper uses a male mouse derived tumor to demonstrate that tumor immunogenicity is a critical factor for responsiveness to systemic VSVΔM51 therapy in vivo. This raises the important question whether non-specific immune effects beneficial in an implantable tumor model translates to the clinical situation, but this issue confounds almost all animal models of cancer.

Minor Comments:

- M3-9-M, ldlr knockout, is said to be infection resistant but no in vivo data is presented. Indeed, the papers shows that these resistant cells genetically engineered to express mCherry, have significantly increased numbers of mCherry DC in the TdLN following viral treatment, suggesting that the virus did infect the tumor and release mCherry which was taken up by the DC and transported to the LN.

We appreciate the reviewer bringing the lack of *in vivo* data showing infection resistance to our attention. We originally did not have *in vivo* data showing that M3-9-M LDLR knockout cell lines do not support VSV$^{\Delta M51}$ infection, since parental M3-9-M tumours also do not show measurable infection *in vivo*. However, we agree with the reviewer that these data should be included. We have generated a subfigure showing that M3-9-M resistant lines do not demonstrate productive infection with intravenous administration of VSV$^{\Delta M51\text{-}Fluc}$, (measured using bioluminescence). This is now Supplementary Fig 3u in the manuscript.

Given this, we believe that the increase in mCherry+ DCs in the lymph node reflects virus-induced migration of DCs to the lymph node, rather than virus infection of cancer cells. Indeed, it is not necessary to treat mice with virus to measure mCherry+ DCs in the lymph node. We have shown mCherry+ DCs in the lymph nodes of mice treated with PBS (Fig 3i). Similarly, other studies have shown mCherry+ (or fluorescent protein+) DC populations in the tumour draining lymph nodes of treatment naïve mice[10]. Thus, we believe that the increase in mCherry+ DCs in the lymph nodes of VSV$^{\Delta M51}$-treated mice reflects enhanced migration of tumour antigen bearing DCs to the tumour draining lymph node. Congruent with this interpretation is the fact that DCs in the tumour upregulate the lymph node-homing receptor CCR7 after VSV$^{\Delta M51}$ treatment (Figure 3h) and that total DC populations also increase in the tumour draining lymph node following VSV$^{\Delta M51}$ treatment (as shown in Figure 3d).

- Fig. 5K does not convincingly show that TNFα and type 1 IFNs are important mediators of the viral anti-tumor effect.

This is a similar comment made by reviewer 2 – to address both reviewers, we have amended the statistical comparisons in subfigure 5k to make the conclusions clearer. As can be seen, the difference between VSV$^{\Delta M51}$ and VSV$^{\Delta M51}$ + aTNFa is not significant ($p = 0.4687$). Thus, neutralizing TNFa alone is not sufficient to abrogate VSV$^{\Delta M51}$-induced tumour regression. Neutralizing both TNFa and type I IFNs completely abrogates VSV$^{\Delta M51}$-induced antitumour immunity, likely due to impaired antigen presentation and T cell activation. Interestingly, neutralizing Type I IFNs alone is also sufficient to nullify VSV-induced tumour regression. This may be because IFN has other roles in promoting antitumour immunity – such as the stimulation of NK cells, or promotion of MHC I expression.

- Fig. 6H does not convincingly show that virus infection of spleen helps the therapeutic response because the benefit in the positive control is marginal, and the sham negative control has the same result as the experimental group.

This is similar to a concern raised by reviewer 2 (which we have addressed above) – we are grateful to both reviewers for bringing this up. It is true that compared to no surgery, the effect of VSV$^{\Delta M51}$ is reduced in mice that underwent sham surgery, likely because the wound healing response is immunosuppressive[3]. However, there is still a significant delay in tumour growth with VSV$^{\Delta M51}$ treatment in these mice. This is not true for mice that underwent splenectomy surgery, as VSV$^{\Delta M51}$ treatment did not improve overall survival compared to the control group. Thus, we believe that splenic infections do play a role in VSV$^{\Delta M51}$-induced tumour regression. We provide a more detailed explanation for why we believe our sham and splenectomy surgery yielded the results they did in our response to reviewer 2 (bulleted comment #2).

Although beyond the scope of this study, we hope to eventually design other methods to block VSV$^{\Delta M51}$ infection in the spleen. This likely will require knowing which cell types are infected in order to design specific strategies for blockade.

- The figures are too small and resist interpretation in print but can be magnified on a computer screen.

Thank you for mentioning this – we have increased the minimum font size on all main and supplementary figures (but kept within the font range allowed by Nature Communications).

References

1. Barchet, W. *et al.* Virus-induced interferon alpha production by a dendritic cell subset in the absence of feedback signaling in vivo. *J. Exp. Med.* **195**, 507–516 (2002).
2. Iannacone, M. *et al.* Subcapsular sinus macrophages prevent CNS invasion on peripheral infection with a neurotropic virus. *Nature* **465**, 1079–1083 (2010).
3. Antonio, N. *et al.* The wound inflammatory response exacerbates growth of pre-neoplastic cells and progression to cancer. *EMBO J.* **34**, 2219–2236 (2015).
4. Highfill, S. L. *et al.* Disruption of CXCR2-mediated MDSC tumor trafficking enhances anti-PD1 efficacy. *Sci. Transl. Med.* **6**, 237ra67 (2014).
5. Biesmans, S. *et al.* Peripheral Administration of Tumor Necrosis Factor-Alpha Induces Neuroinflammation and Sickness but Not Depressive-Like Behavior in Mice. *BioMed Res. Int.* **2015**, 716920 (2015).
6. van Holten, J. *et al.* Treatment with recombinant interferon-beta reduces inflammation and slows cartilage destruction in the collagen-induced arthritis model of rheumatoid arthritis. *Arthritis Res. Ther.* **6**, R239-49 (2004).
7. Skelly, D. T., Hennessy, E., Dansereau, M.-A. & Cunningham, C. A Systematic Analysis of the Peripheral and CNS Effects of Systemic LPS, IL-1B, TNF-α and IL-6 Challenges in C57BL/6 Mice. *PLOS ONE* **8**, e69123 (2013).
8. Stifter, S. A. *et al.* Visualizing the Selectivity and Dynamics of Interferon Signaling In Vivo. *Cell Rep.* **29**, 3539-3550.e4 (2019).
9. Galivo, F. *et al.* Single-cycle viral gene expression, rather than progressive replication and oncolysis, is required for VSV therapy of B16 melanoma. *Gene Ther.* **17**, 158–170 (2010).
10. Salmon, H. *et al.* Expansion and Activation of CD103(+) Dendritic Cell Progenitors at the Tumor Site Enhances Tumor Responses to Therapeutic PD-L1 and BRAF Inhibition. *Immunity* **44**, 924–938 (2016).

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

Overall, this work is still viewed as an excellent exploration of an extremely challenging mechanistic question in OV. As such, the conclusions drawn in the revision are much more in line with the data shown (which is always going to be more gray areas than black and white), however, I feel that in a few places the statements provided are still slightly too strong (see specific comments below). I feel that these issues can certainly be addressed by minor textual changes, however, without the need for additional experimentation.

- Thank you for these comments, we appreciate your thorough feedback.

Line 41: the word alone should be removed

- We have removed the word “alone” as requested.

Line 75: the word live should be removed (viruses are not technically alive)

- Thank you for this comment, we have removed the word “live” as requested.

Line 77: The first approved OV was H101 in china in 2006 not T-vec.

- We appreciate the reviewer pointing this out. We have adjusted the introduction so that it mentions both Oncorine (H101) and T-Vec.

Line 270: this should also reference Fig 1d since it draws a comparison between the M3-9-M res tumors (shown in Fig 1h) and the WT tumors (shown in Fig 1d).

- We have amended this sentence so that it also references Fig 1d.

Line 300-303: The authors claim that
Taken together, these data demonstrate that productive virus replication within cancer cells is not a strict requirement for immune-mediated tumour regression after VSVΔM51 treatment,
As with the previous critique, this statement is still not supported by the data. All the data presented by the authors suggests that VSV CAN replicate in all of the tested cell lines (even the ‘resistant’ ones). Figs S1B, S3C, and S3N actually show robust viral replication in all the cell lines used in the study and the ‘resistant’ clones (with the exception of a reduction in the F7 clone) actually display virtually identical replication curves to WT cells. The difference is that the virus can’t spread well in the resistant clones after it produces new progeny.
Similarly, Fig S2H shows that virus can at least initiate infection in M3-9-M tumors (or at least express sufficient CRE transgene to flox out the stop codon) if the virus is delivered IT. The data therefore seems more consistent with the idea that IV delivery of VSV is very inefficient and, in the absence of robust spread, the infections are below the limit to detection for IVM and BLI (which are not very sensitive assays). The indicated statement therefore should be revised to indicate that high level infections of tumor cells are not required.

- We agree with the reviewer that the cell lines are not completely refractory to virus infection *in vitro*, suggesting that viral entry is still possible using additional mechanisms when cells are placed directly in contact with high MOIs of virus. However, *in vivo*, we believe that several factors account for the substantial decrease in infection. First, interferons within the tumour microenvironment allow cells to mount an antiviral state even before the administration of virus. And second, as the reviewer mentioned, i.v.

delivery likely does not efficiently deposit virions into the tumour microenvironment (although certainly some infectious particles do reach the tumour bed, as we observed when titering tumours 15 minutes post virus delivery, as seen in Supplementary Fig 1g). Thus, we believe it is difficult to use *in vitro* data to determine whether cancer cells can support productive infection *in vivo* – this has also been demonstrated by Liu et al (2013)[1].

- Further, in all methods that we used to detect infection (titers, IVM, BLI, cre-inducible model), we did not detect any indication of infection *in vivo*, which is why we believe that VSV was not productively infecting cancer cells *in vivo*. Indeed, if VSV had replicated within even a few cells and produced a large number of progeny that were released into the tumour microenvironment, we should have detected this by viral titers, but this was never seen. We also think it is encouraging that VSV did not spread in the F7 tumour model – despite this model being deficient in interferon signaling, as it is derived from the CT-26^{IFNmut} line[2]. We believe that if there were even low levels of infection permitted in some cells, the virus would have spread through the tumour – but we did not observe this. This suggests that VSV was not productively infecting cancer cells. Finally, it would be difficult to amend our statement to say that high levels of virus infection are not required within the tumour, as that would require arbitrarily defining "high" and "low" levels of infection.
- For these reasons, we believe it is more correct to say that cancer cells in our models do not support productive virus infection. However, to soften the statement, we have amended the phrasing to say that the "the data suggest" rather than "the data demonstrate."

Figure 2F appears to be missing the yellow line that should link M3sen#1 dots.

- Thank you for noticing this, we have amended the subfigure to include the line.

Line 427: The statement that "Given that VSVΔM51 can generate anti-tumour activity without productively infecting cancer cells" is not supported by the data and should be changed to indicate something about not having 'high levels' of infection in cancer cells.

- We have amended the statement to say "Given that VSVΔM51 can generate anti-tumour activity without productively infecting and spreading through the tumour" – we believe this may be more accurate, as it is difficult to define what "low" vs "high" levels of infection are in the tumour, as mentioned above.

Line 426-439: This paragraph contains numerous statements that are not supported by the data. For example,
it is not clear whether infection of non-cancer cells 'elicits' CD8 T cell responses (line 428) or whether it amplifies existing responses (the latter seems more likely). This wording should be changed accordingly.

- Thank you for this comment. We have adjusted the text so that the statements are better supported by the data. For example, in line 428, we intended for the statement to ask whether non-cancer cell infections elicit tumour regression that is CD8 T cell mediated (rather than asking whether non-cancer cell infections elicit CD8 T cell responses). We have thus made this clearer.

It is not definitively shown that in the M3-9-Mres model contain only no cancer cells are infected making the statement "To begin, we asked whether non-cancer cell infections lead to anticancer CD8+ T-cell accumulation in tumours." an over interpretation of the data.

- We amended the statement to say “To begin, we asked whether productive infection of non-cancer cells leads to anticancer CD8+ T-cell accumulation in tumours.” We believe this is more in line with the data, as we believe our methods show that VSV does not productively infect within cancer cells (as we described above).

It is unclear whether VSV infection in these models induces or enhances CD8 T cell response making the statement “This suggests that virus replication within non-cancer cells generates anticancer immunity by acting predominantly on CD8+ T cells in the TdLN.” an over interpretation of the data.

- We agree with the reviewer and have amended the statement to replace the word “generates” with “enhances”.

Line 446: As above, the following statement “This suggests that non-cancer cell infections promote DC activation and their migration from the tumour to the TdLN.” an over interpretation of the data.

- Thank you for this comment. We have softened the language such that we now state “This suggests that VSV∆M51 infection of predominantly non-cancer cells can promote DC activation and their migration from the tumour to the TdLN.”

Lines 483-485: Same comment as above about an over interpretation of the data.

- As above, we have softened the statement.

The data in Fig S8C does not show viral titers as the text indicates (it shows BLI). It would be beneficial (although probably not required if the authors adjust the language) if the authors could show actual viral titers in the LN’s following IV injection of SQ injection of 1e8 PFU.

- We appreciate the reviewer catching this error, we have made the language more accurate by stating “LN infection” rather than “LN titers.”

Line 569: The statement “LN infections alone were insufficient to provide a survival benefit” should be changed to “SQ viral injections alone…”

- Thank you for this comment, we have adjusted the wording as suggested.

Lines 692-694: I appreciate the revisions to the text here, however, I think the statement that “this recruitment was blunted” is probably not accurate since the same number of CD103+ DC’s appear to be in the LN’s of splenectomized mice (the data is not significant because the background is higher in these mice not because the recruitment is less).

- We agree with the reviewer that the background of CD103+ DCs in the LN is greater in the splenectomized mice. However, we still believe that the recruitment of DCs to the LN is blunted, as VSV treatment in splenectomized mice no longer significantly induces DC recruitment to the LN when compared to control treatment. We have amended the text to make it clear that the comparison should be carried out within the splenectomized group alone.

There appears to be a typo or deletion of text on line 763…

- Thank you for catching this! We have corrected the text.

Reviewer #2 (Remarks to the Author):

All comments of the reviewers have been carefully addressed and the additional experiments suggested were performed.

Thank you, we appreciate your comments and believe that it has improved the quality of the paper.

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References

1. Liu, Y.-P., Suksanpaisan, L., Steele, M. B., Russell, S. J. & Peng, K.-W. Induction of antiviral genes by the tumour microenvironment confers resistance to virotherapy. Sci. Rep. 3, 2375 (2013).

2. Ruotsalainen, J. J. et al. Clonal variation in interferon response determines the outcome of oncolytic virotherapy in mouse CT26 colon carcinoma model. Gene Ther. 22, 65–75 (2015).