

Peer Review File

Sculpting the tumour microenvironment by combining radiotherapy and ATR inhibition for curative-intent adjuvant immunotherapy



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REVIEWER COMMENTS

Reviewer #1 (expert in head and neck cancer and radiation oncology):

This series of preclinical studies evaluates the combination of PD-1 directed therapy and anti-NKG2A therapy following radiation and ATR inhibition. These experiments build on prior work and suggest a pathway for clinical translation and further correlative investigations. The major limitation is several initial experiments do not provide a comparison with immune activation engendered by radiation without ATRi followed by immunotherapy. This makes it more difficult to assess what the ATRi might be adding to the overall treatment regimen and subsequent immune response.

Other minor suggestions for improvement are below.

Introduction:

- For a general audience unfamiliar with head and neck cancer treatment, it would be helpful to mention that ICB is effective in both HPV+ and – head and neck tumors.
- Was the timing of administration of NKG2A/PD-1 therapy after ATRi/RT informed by prior preclinical work? Or the clinical failure of concurrent RT/ICB approaches?

Results

- TCGA analyses are likely overpowered looking for correlations between KLRC1 and PD1/L1 and $R < 0.6$ are relatively weak, particularly for PD-L1
- In the human scRNAseq data, were there any differences in the KLRC1/PDCD1 population according to HPV status?
- Figure 2B also demonstrates increases in PD1+ / NKG2A negative T cells following ATRi/RT – do these T cells also demonstrate an activated signature? Also how do the increases in PD1+ / NKG2A populations differ after treatment with RT alone versus RT/ATRi?
- Figure 2E and F – is subsequent immune therapy able to compensate for treatment with RT only versus RT/ATRi?
- Figure 5 is overpowered to detect correlations and the R range from weak correlations to more convincing with PD1

Discussion

- The authors suggest these data may lead to clinical translation but the radiation ATRi regimen used is not standard. Can the hypofractionated regimen used in combination with ATRi be translated to standard radiation / chemoradiation with the expectation that impacts on immune activation will be similar? The same question applies to combinations with chemotherapy or elective nodal radiation.

Reviewer #2 (expert in head and neck cancer and immunotherapy):

This manuscript presents interesting data regarding the impact of ATR inhibition and radiation on NKG2A/PD-L1 positive T cells in animal models of HNSCC. The authors go on to show that addition of NKG2A and PD-L1 inhibition after ATRi and radiation improved tumor control, and that this effect is dependent on CD40 signaling.

Concerns regarding the significance of this work arise from considerations of the models employed, both with regard to the number of models, the immunocompetent mEER HPV-driven carcinoma model, and the hypofractionated radiation schedule. As HPV+ and HPV- HNSCC are distinct disease entities, with different radiosensitivity and different immune microenvironments, it is not appropriate to study them as a single entity. For this reason, it would strengthen the current manuscript to have 2 models for each of these distinct diseases. For HPV- HNSCC, additional syngeneic models such as MOC 2 are available. AS MOC2 is more immunotherapy resistant, approaches to eliciting an immune response here would hold greater promise for translation. For HPV+ HNSCC, the mEER model may fail to recapitulate immune silencing in poorer prognosis human HPV+ HNSCC. As an alternative, some investigators have turned to PDX in mice with reconstituted human immune populations. A significant concern with the radiation schedule employed here is that numerous human studies have failed to recapitulate predictions of

immunotherapy sensitivity, abscopal effect, and other immune modulation documented in animal systems employing hypofractionated schedules to the point where the ongoing relevance of this model is questionable. The authors also do not address how they mimicked radiation to tumor-draining lymph nodes, as a potential cause of reduced T cell response in human HNSCC radiation. Safety data are not presented.

Response to reviewers

We would like to thank all the reviewers for their time, positive comments, suggestions and critical feedback on our manuscript “Sculpting the tumour microenvironment by combining radiotherapy and ATR inhibition for curative-intent adjuvant immunotherapy”. Please find below our point-by-point responses to reviewer comments. Collectively, these revisions (7 new Supplementary Figures), have provided important clarification of data within our manuscript. We have also re-written some parts of the Results, to streamline the text, focus on the key findings and bring in aspects of the requested revisions. Changes to text in the manuscript have been highlighted in yellow. Requested analyses and data are shown below and where we have included them within the revised manuscript, we have further outlined this in red. We trust that the additional data we provide and our detailed explanations will be satisfactory and that our manuscript can now be accepted for publication.

Reviewer #1 (expert in head and neck cancer and radiation oncology):

This series of preclinical studies evaluates the combination of PD-1 directed therapy and anti-NKG2A therapy following radiation and ATR inhibition. These experiments build on prior work and suggest a pathway for clinical translation and further correlative investigations. The major limitation is several initial experiments do not provide a comparison with immune activation engendered by radiation without ATRi followed by immunotherapy. This makes it more difficult to assess what the ATRi might be adding to the overall treatment regimen and subsequent immune response.

Other minor suggestions for improvement are below.

Introduction:

- For a general audience unfamiliar with head and neck cancer treatment, it would be helpful to mention that ICB is effective in both HPV+ and – head and neck tumors.

We have now added this important information to the introduction section (line 7).

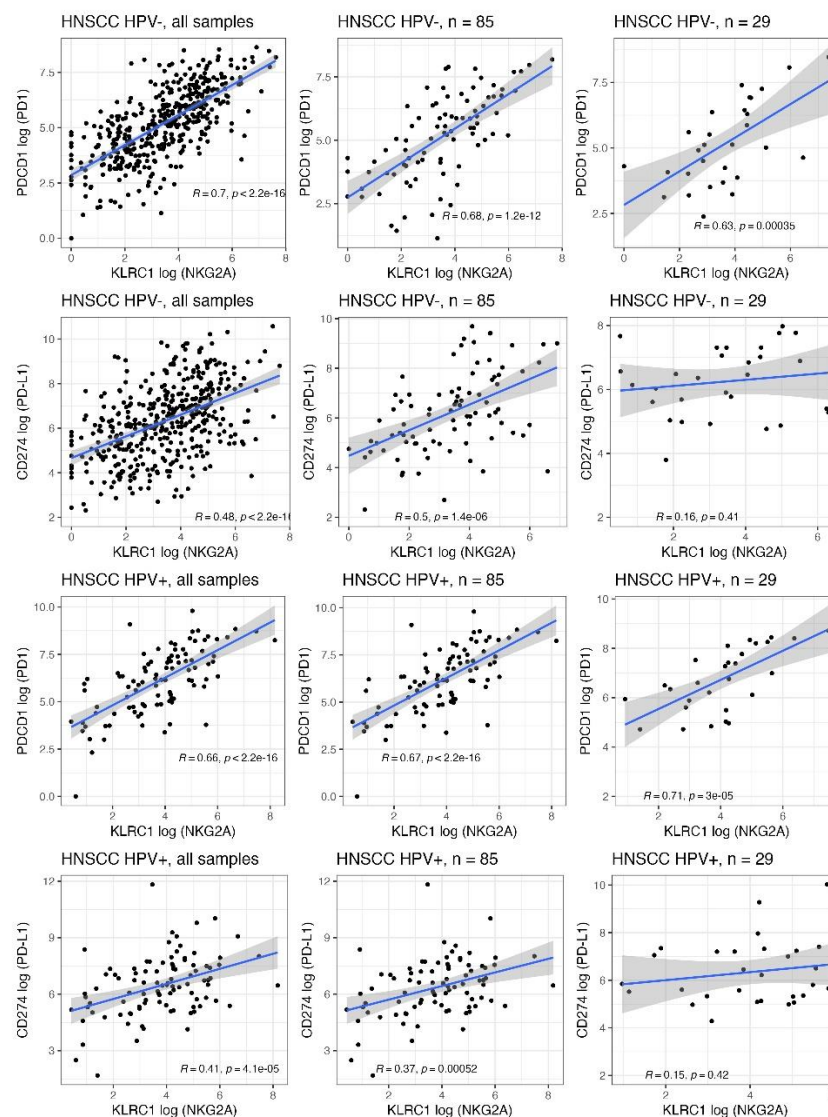
- Was the timing of administration of NKG2A/PD-1 therapy after ATRi/RT informed by prior preclinical work? Or the clinical failure of concurrent RT/ICB approaches?

Timing of administration of anti-NKG2A/PD-L1 immunotherapy was based on our previous preclinical studies (Patin EC et al., 2022, Journal for Immunotherapy of Cancer) and also according to clinical failure of concomitant RT/ICB approaches cited in the introduction.

Results

- TCGA analyses are likely overpowered looking for correlations between KLRC1 and PD1/L1 and $R < 0.6$ are relatively weak, particularly for PD-L1

Based on correlation analysis we performed using full TCGA HNSCC data (HPV- $n = 419$, HPV+ $n = 97$), we expect at least a moderate effect size of $r = 0.3$ or large effect size of $r = 0.5$ (effect size criteria suggested by Cohen (1988)). For these effect sizes, significance level of 0.05 and expected power of 0.8, the required sample sizes are 85 and 29, respectively (calculated using pwr R package, v1.3-0). We downsampled TCGA data to $n = 85$ and $n = 29$ and re-ran correlation analysis. The results obtained are consistent with our previous analysis using all samples (see fig attached).



- In the human scRNAseq data, were there any differences in the KLRC1/PD1 population according to HPV status?

Further analysis showed that there was no noticeable difference in the *KLRC1/PDCD1*-double positive population between HPV- and HPV+ status (see figure S2). This information has been added to the text (lines 69-70).

A

cluster_man	n cells			n DP cells			% DP cells		
	HPV-	HPV+	Total	HPV-	HPV+	Total	HPV-	HPV+	Total
T CD8+	15,840	9,892	25,732	496	335	831	3.1%	3.4%	3.2%
T CD4+	12,313	12,554	24,867	31	27	58	0.3%	0.2%	0.2%
Macro/Mono	14,829	6,827	21,656	27	7	34	0.2%	0.1%	0.2%
Tregs	4,322	2,386	6,708	13	17	30	0.3%	0.7%	0.4%
B cells	2,149	7,058	9,207	2	2	4	0.1%	0.0%	0.0%
NK	3,342	2,323	5,665	2	1	3	0.1%	0.0%	0.1%
Epithelial	3,983	9,970	13,953	1		1	0.0%	0.0%	0.0%
pDC	621	394	1,015	1		1	0.2%	0.0%	0.1%
Endothelial	6,327	798	7,125				0%	0%	0%
T cells	1	28	29				0%	0%	0%
Mast	214	76	290				0%	0%	0%
Pericytes	674	26	700				0%	0%	0%
Fibroblasts	3,377	495	3,872				0%	0%	0%
Grand Total	67,992	52,827	120,819	573	389	962	0.8%	0.7%	0.8%

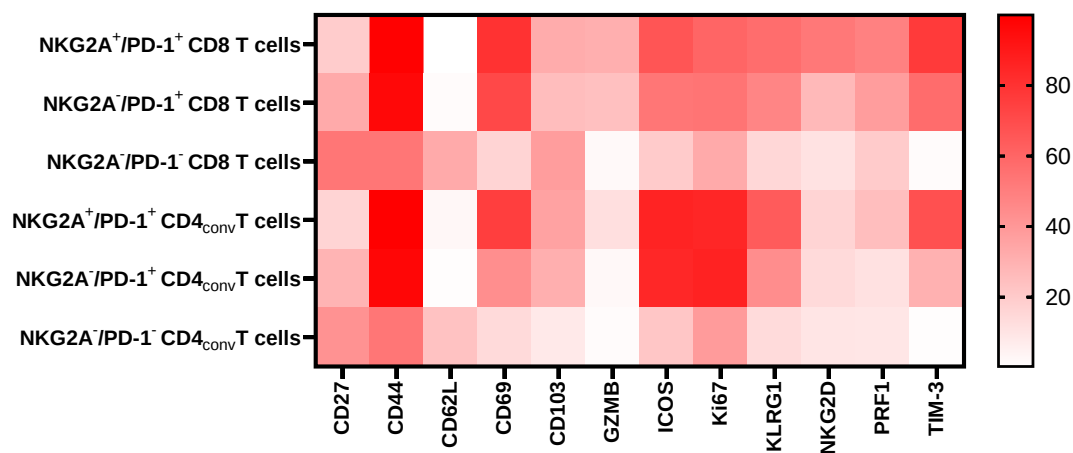
Figure S2

B

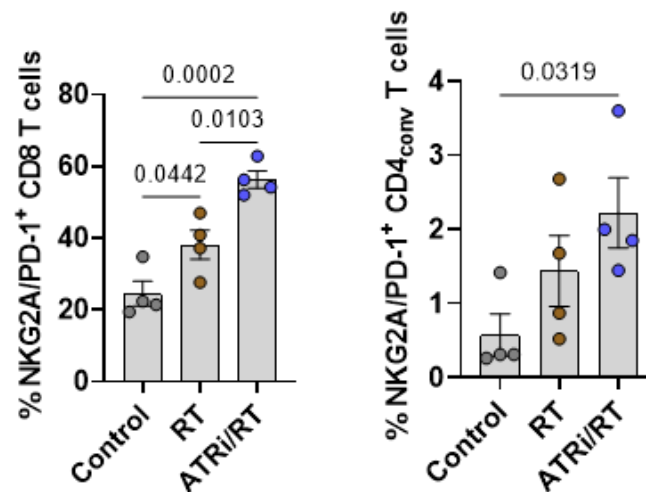
cluster_man	n cells			n DP cells			% DP cells		
	HPV-	HPV+	Total	HPV-	HPV+	Total	HPV-	HPV+	Total
T CD8+	22,269	9,468	31,737	644	333	977	2.9%	3.5%	3.1%
T CD4+	30,458	11,868	42,326	79	27	106	0.3%	0.2%	0.3%
Tregs	1,735	687	2,422	16	22	38	0.9%	3.2%	1.6%
Macro/Mono	20,988	4,758	25,746	24	3	27	0.1%	0.1%	0.1%
DC	1,717	501	2,218	12	3	15	0.7%	0.6%	0.7%
B cells	11,537	5,690	17,227	7	2	9	0.1%	0.0%	0.1%
pDC	859	337	1,196	3	0	3	0.3%	0.0%	0.3%
NK	5,445	2,436	7,881	1	1	2	0.0%	0.0%	0.0%
Mast	262	58	320	0	0	0	0%	0%	0%
Grand Total	95,270	35,803	131,073	786	391	1,177	0.8%	1.1%	0.9%

- Figure 2B also demonstrates increases in PD1+ / NKG2A negative T cells following ATRi/RT – do these T cells also demonstrate an activated signature? Also how do the increases in PD1+ / NKG2A populations differ after treatment with RT alone versus RT/ATRi?

PD-1+/NKG2A- T cells already displayed an activated signature. However, NKG2A/PD-1 double-positive populations displayed generally a higher activation status than the PD-1 single-positive populations, (figure 2C). This information was added to the text (line 90-92).

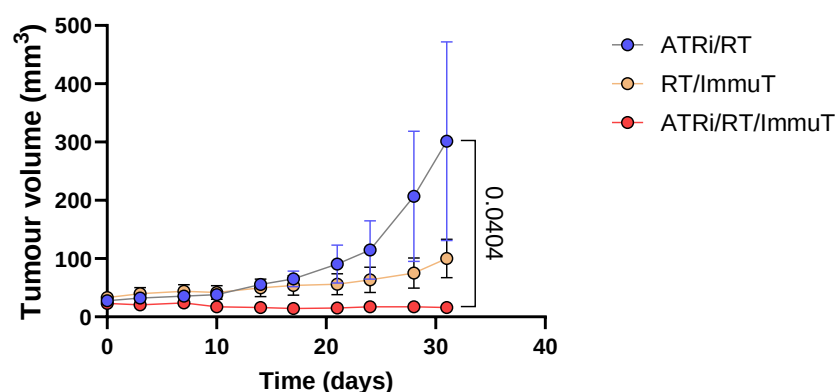


Furthermore, an additional flow cytometry experiment showed that administration of ATRi to RT significantly increased the infiltration of NKG2A/PD-1⁺ double-positive CD8 and CD4 T cells in the TME when compared with the other treatment groups (figure S4A). The text has been changed accordingly (lines 87-88).



- Figure 2E and F – is subsequent immune therapy able to compensate for treatment with RT only versus RT/ATRi?

An additional experiment revealed that administration of ATRi significantly increased the efficacy of immunotherapy following RT (figure S4B). This information has been added to the text (lines 103-104).



- Figure 5 is overpowered to detect correlations and the R range from weak correlations to more convincing with PD1.

Please, see answer to the first results section comment above.

Discussion

- The authors suggest these data may lead to clinical translation but the radiation ATRi regimen used is not standard. Can the hypofractionated regimen used in combination with ATRi be translated to standard radiation / chemoradiation with the expectation that impacts on immune activation will be similar? The same question applies to combinations with chemotherapy or elective nodal radiation.

We have analysed surface expression of both NKG2A and PD-1 in PBMCs of six head and neck cancer patients enrolled in the phase I PATRIOT clinical trial (NCT02223923) but could not include those data due to Nature Communication publication policies. [REDACTED]

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[REDACTED] [TEXT REDACTED]

[IMAGE REDACTED]



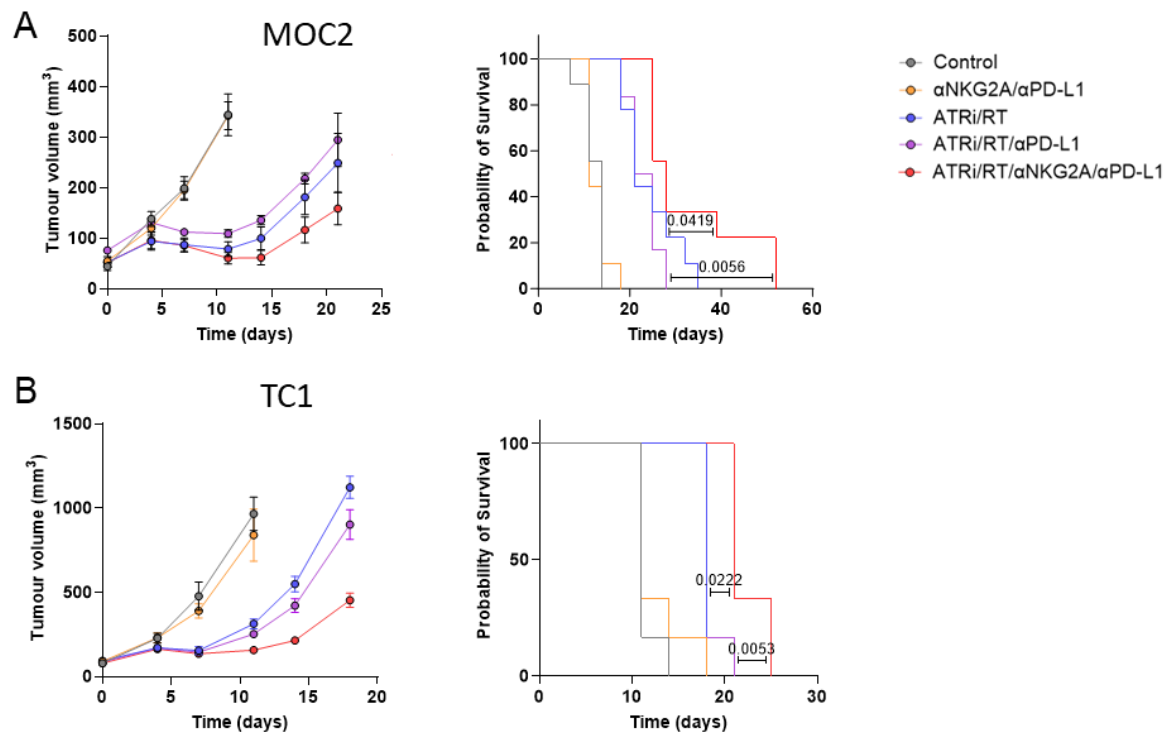
Reviewer #2 (expert in head and neck cancer and immunotherapy):

This manuscript presents interesting data regarding the impact of ATR inhibition and radiation on NKG2A/PD-L1 positive T cells in animal models of HNSCC. The authors go on to show that addition of NKG2A and PD-L1 inhibition after ATRi and radiation improved tumor control, and that this effect is dependent on CD40 signaling.

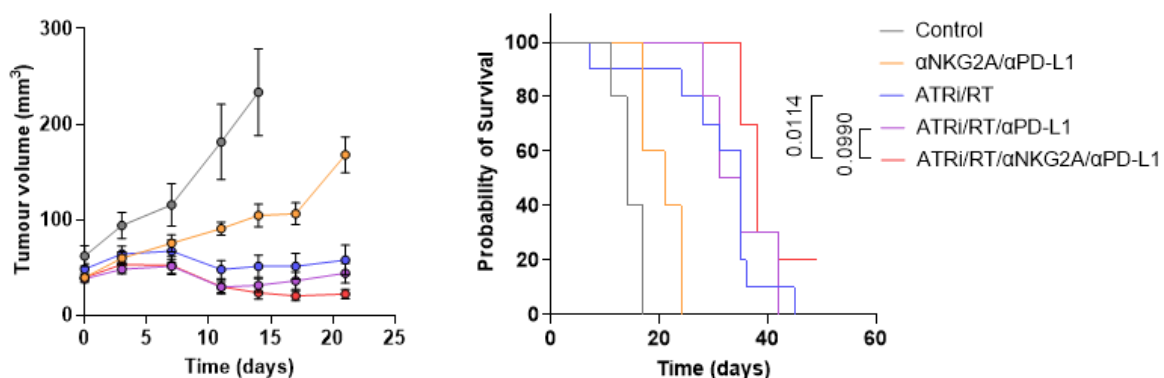
Concerns regarding the significance of this work arise from considerations of the models employed, both with regard to the number of models, the immunocompetent mEER HPV-driven carcinoma model, and the hypofractionated radiation schedule. As HPV+ and HPV- HNSCC are distinct disease entities, with different radiosensitivity and different immune microenvironments, it is not appropriate to study them as a single entity. For this reason, it would strengthen the current manuscript to have 2 models for each of these distinct diseases. For HPV- HNSCC, additional syngeneic models such as MOC 2 are available. As MOC2 is more immunotherapy resistant, approaches to eliciting an immune response here would hold greater promise for translation. For HPV+ HNSCC, the mEER model may fail to recapitulate immune silencing in poorer prognosis human HPV+ HNSCC. As an alternative, some investigators have turned to PDX in mice with reconstituted human immune populations.

In response to reviewer concerns, we have now added supplementary ectopic and orthotopic models to strengthen the findings of this manuscript and increase its translational relevance. We have also performed additional RNAseq and depletion experiment in one ectopic HPV- model. We do not have humanised murine competent animal models in place and feel that establishing such systems would be beyond the reasonable scope of these revisions.

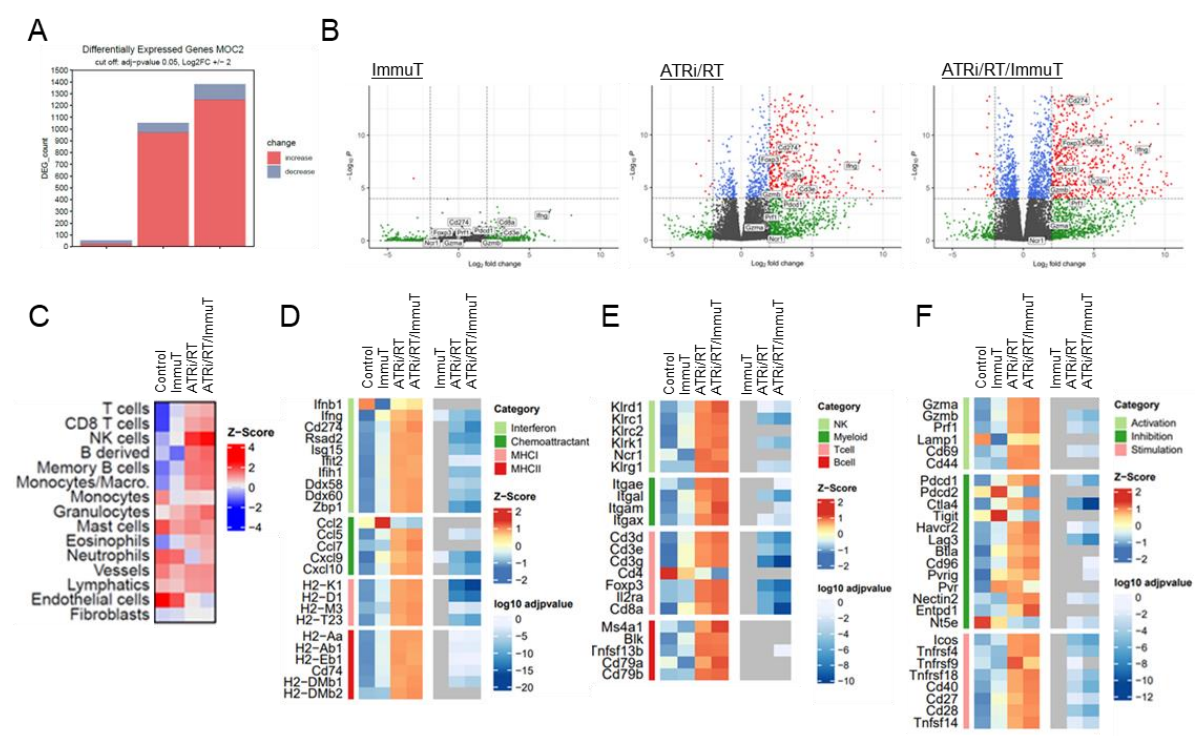
Our additional experiments showed that, in both MOC2 (HPV-) and TC1 (HPV+) ectopic models, full combination therapy ATRi/RT/anti-NKG2A/anti-PD-L1, brought therapeutic benefits when compared with the other treatment groups (figure S5). This information has been added to the text (lines 98-101).



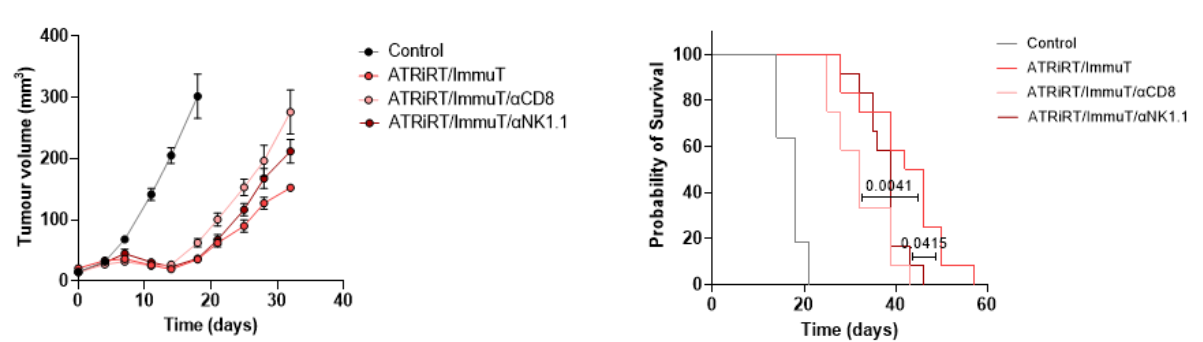
The therapeutic benefit of the full combination treatment was also confirmed in the additional experiment using the mEER (HPV+) orthotopic model (figure S3F). This information has been added to the text (lines 103-107).



To strengthen the manuscript, we also performed additional RNAseq in the MOC2 ectopic model (figure S9). Overall, the data were in line with the other HPV- model MOC1, including more interferon signalling, chemoattraction, MHCI- and MHCII-related gene expression as well as a gene signature characteristic of T cell activity. The corresponding results section was changed to include those new pieces of information (lines 125-149).



Depletion experiment in the MOC2 model also confirmed the importance of CD8 T cells in mediating full treatment efficacy (figure S9G). This information has been added to the text (lines 170-176).

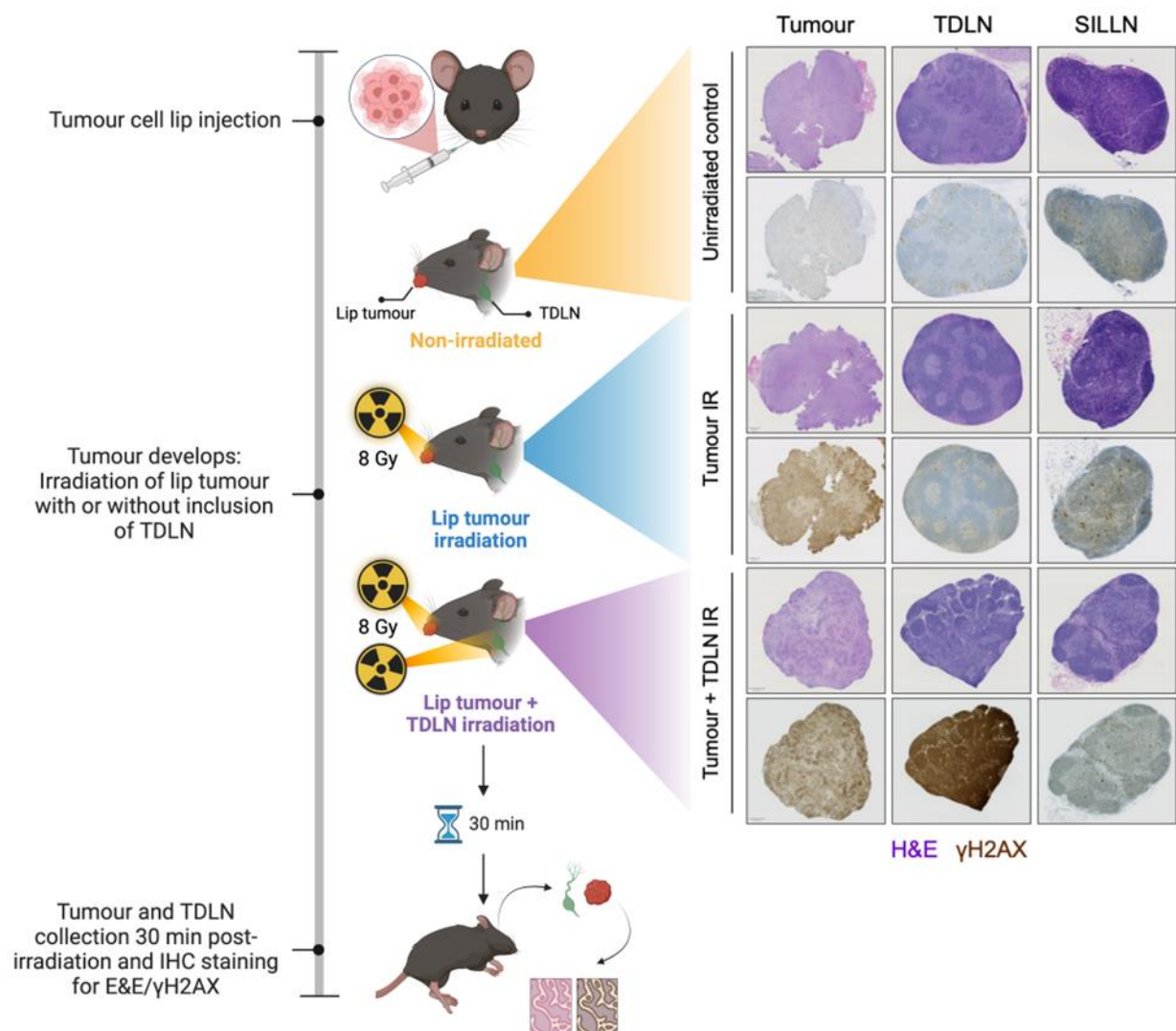


A significant concern with the radiation schedule employed here is that numerous human studies have failed to recapitulate predictions of immunotherapy sensitivity, abscopal effect, and other immune modulation documented in animal systems employing hypofractionated schedules to the point where the ongoing relevance of this model is questionable.

We understand the reviewer's concerns about the dose-fractionation schedule that we have employed but emphasise the difficulty in fully recapitulating the clinical fractionation schedules in in vivo animal models. By conducting experiments in multiple tumour types, both HPV+ and HPV-, in both ectopic and orthotopic sites, employing techniques to demonstrate both biological and therapeutic efficacy, we trust that our models give an acceptable level of confidence in the applicability to the human system. In addition, as highlighted in our response to reviewer 1, the clinical data (under submission) using standard dose-fractionation are consistent with the data from our animal models.

The authors also do not address how they mimicked radiation to tumor-draining lymph nodes, as a potential cause of reduced T cell response in human HNSCC radiation.

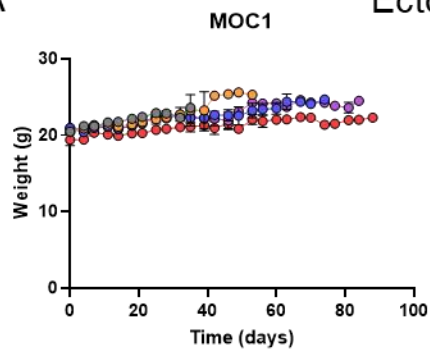
In response to the reviewer's concern, we have now performed additional experiments where we used γ -H2AX staining to confirm that the radiotherapy delivery in our lip orthotopic pre-clinical studies is tumour site-specific. Unirradiated tissues showed marginal γ -H2AX staining when compared to the tumour irradiation group, where only tumours, and not TDLNs or uninvolved LNs, showed high levels of γ -H2AX staining. Irradiation of both tumours and TDLNs in separate radiation fields yielded high levels of γ -H2AX staining in both the tumour and TDLN with no signal present in the distant, unirradiated sub-iliac LN (figure S7). This information has been added to the text (line 110-117).



Safety data are not presented.

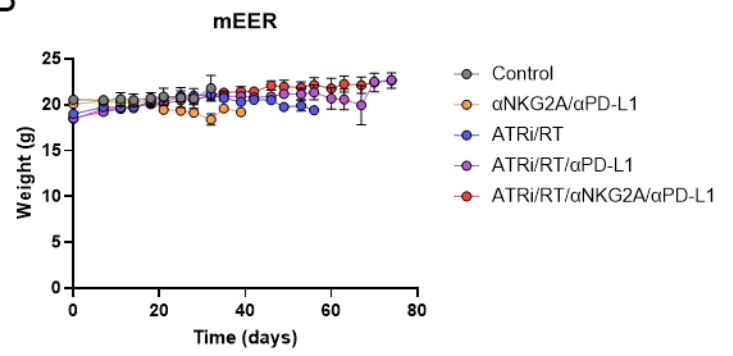
Body weights for all models have now been added to the text (line 108-109) and shown in the supplementary figure S6.

A

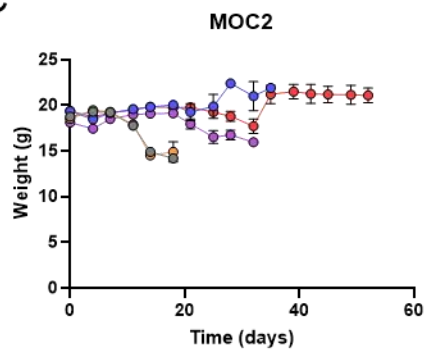


Ectopic

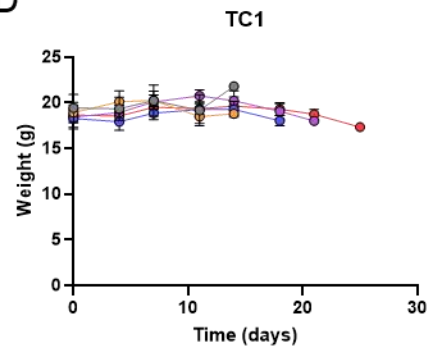
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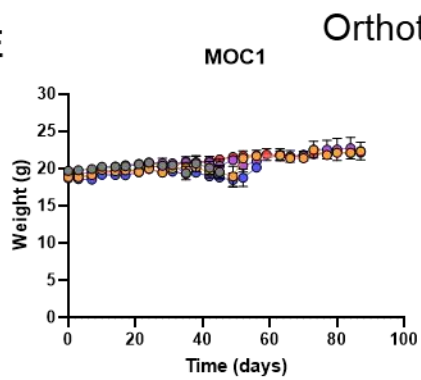
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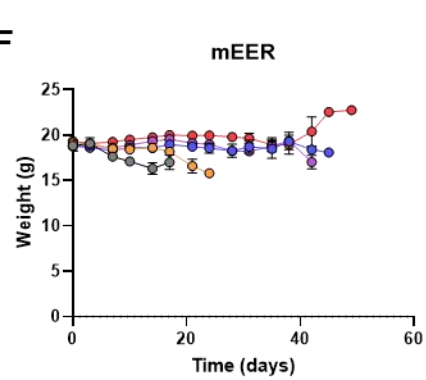


E



Orthotopic

F



REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

Previous comments addressed

Reviewer #2 (Remarks to the Author):

1. The manuscript is greatly improved by the inclusion of additional mouse models.
2. The revisions to address impact of radiation on TDLN in potentially lessening immune response in clinical (human) situation are not helpful. The finding that gamma H2AX is induced only in radiated tissues is not novel. The Methods section seems to indicate that TDLN were not radiated for the treatment experiments, in which case the translatability of the model to clinical testing would seem to be compromised. If effects were demonstrated despite radiation to TDLN, this would be worth emphasizing.

Response to reviewers

We would like to thank all the reviewers for their time, positive comments, suggestions and critical feedback on our manuscript “Sculpting the tumour microenvironment by combining radiotherapy and ATR inhibition for curative-intent adjuvant immunotherapy”. Please find below our point-by-point responses to reviewer comments. We trust that the additional data we provide and our detailed explanations will be satisfactory and that our manuscript can now be accepted for publication.

Reviewer #1 (Remarks to the Author):

Previous comments addressed

Reviewer #2 (Remarks to the Author):

1) The manuscript is greatly improved by the inclusion of additional mouse models.

We thank the reviewer for the positive comment.

2) The revisions to address impact of radiation on TDLN in potentially lessening immune response in clinical (human) situation are not helpful. The finding that gamma H2AX is induced only in irradiated tissues is not novel. The Methods section seems to indicate that TDLN were not irradiated for the treatment experiments, in which case the translatability of the model to clinical testing would seem to be compromised. If effects were demonstrated despite radiation to TDLN, this would be worth emphasizing.

In treating tumours in either ectopic or orthotopic sites, we attempted to treat only the tumour volume (i.e. we avoided unnecessary nodal irradiation). The new supplementary figure showing the distribution of γ H2AX foci when irradiating tumour and/or lymph node was provided to show the accuracy of our treatment, not to study advantages or deleterious effects of nodal irradiation. The question of whether or not the treatment effect is preserved with “elective” nodal irradiation is of course relevant to the clinical situation but beyond the scope of this manuscript. We aim to repeat such data in future publications.