nature portfolio

Peer Review File

Crosslinking of Ly6a metabolically reprograms CD8 T cells for cancer immunotherapy

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author): with expertise in cancer immuno-metabolism

Maliah and co-authors present interesting findings that chronic UVB exposure suppresses mouse skin-draining lymph node T cell killing in vivo, building upon current literature on UVB-induced tumour suppression. Mass cytometry profiling revealed increased Ly6a expression on UVB suppressed T cells and on non-UVB suppressed T cells in the tumour microenvironment. Anti-Ly6a antibodies convincingly improved tumour killing in vitro and in vivo, potentially through enhanced mitochondrial function.

Major concerns

1. A key part of this manuscript is that UVB exposure induces Ly6a T cell expression; however, the authors never test the potential of anti-Ly6a in the context of UVB induced tumour suppression in vivo.
2. The authors test the role of Ly6a in T cell function using anti-Ly6a antibodies and it would be good to consolidate these findings using a genetic approach e.g. for the killing assay and TMRE.
3. The flow cytometry data in figure 2O-P is not convincing and does not match the other flow plots showing Ly6a mean fluorescent intensity in figure 2G/I and figure 3B. Is this high population reflective of the in vivo Ly6a expression and is this finding reproducible? If you quantify MFI do you get the same results?

Minor concerns

1. Several of the panels are too small to read when printed on A4.
2. Grammatical/spelling errors throughout the manuscript.

Reviewer #2 (Remarks to the Author): with expertise in melanoma, cancer immunology

Through the studies of immune suppression mechanisms in chronic UVB exposure, the authors identified enrichment of a Ly6c+ CD8+ T cells in dLNs. This CD8 T cell population was also enriched in TILs. Amplification of Ly6c signaling using a mAb promoted T cell-mediated antitumor response. The author's finding might idenfy a therapeutic target in Ly6c for cancer immunotherapy, but at the very early stage. There are many main questions in

related to the role of Ly6c and its antibody in T cells. For example, the involvement of Ly6a in UVR-induced immunosuppression is not proved. What is the role of Ly6a on T cells? And other immune cells? The endogenous role for Ly6a in tumor immunity has not been tested. What would trigger Ly6c signaling in vivo. Thus, whether the Ly6a mAb used in vivo is antagonist or agonist is completely unknown.

Others:

1. In fig. 1c, the dosage of tumor cells for lung metastasis should be titrated. The negative result can be simply because that the dosage used is too higher to overwrite the potential difference.
2. Naïve OT-1 cells with primed are not good at killing tumor cells in 24 hours.
3. The assessment of the Ly6a level in the TILs between B16 and B16-OVA tumors is a good indicator for determining TCR dependency. The authors should examine the Ly6a level between OVA-tetramer-positive and -negative TILs of B16-OVA.

Reviewer #3 (Remarks to the Author): with expertise in cancer immuno-metabolism

This manuscript shows that Ly6a mediates UV exposure-induced sDLN T cell suppression. Outside the context of UV exposure, Ly6a crosslinking enhances T cell anti-tumoral cytotoxic activity and reprograms their mitochondrial metabolism. Treatment with anti-Ly6a antibody reduces tumor resistance to anti-PD1 therapy, leading to new immunotherapy treatment. The following points need to be further addressed:

Major:

1. Why did the authors specifically focus on Ly6a but not other upregulated molecules in T cells due to UVB exposure? What is the rationale? The analysis showed no marked difference in Ly6a expression induced by UVB exposure.

2. Besides T cells, it is unclear whether Ly6a is expressed in CD11b+ cells, including tumor-associated macrophages (TAMs) or dendritic cells. If so, why did the authors specifically

focus on Ly6a in T cells but ignore myeloid cells?

3. Given that the authors have shown that skin DLN CD11b+ cells specifically induce Ly6a expression in T cells (Fig. 2n), the mechanism is unclear to me. Additionally, is UVB exposure-induced Ly6a expression in T cells dependent on CD11b+ cells or independent of them?

4. Since Ly6a was highly induced in TILs, it is necessary to investigate Ly6a function in T cells through genetic Ly6a knockdown in CD8+ T cells for tumor cell killing and Ly6a x Cd8a cKO mice for tumor control.

5. Considering that chronic IFNa significantly increases Ly6a expression in CD8+ T cells, can IFNAR blockade phenocopy the results obtained with anti-Ly6a treatment?

6. Why did anti-Ly6a promote mitochondrial activity in T cells? Does Ly6a signaling control mitochondrial functions such as membrane potential, ROS generation, ATP production, and oxidative phosphorylation (OXPHOS)? If so, how?

7. In Fig. 4, does anti-Ly6a treatment affect dendritic cell migration to skin DLN? Does anti-Ly6a treatment reduce T cell exhaustion? Can anti-Ly6a treatment prolong the survival rate of tumor-bearing mice?

Minor:
In Fig. 2f, the label of row 3 should be L_CD8_3, not L_CD4_3. In Extended Fig. 1e and f, the figure legends do not match the figures."

Reviewer #4 (Remarks to the Author): with expertise in cancer immunology

The authors have identified that UV exposure induces immune suppression and expression of Ly6A in T cells, which is also confirmed to be present in tumor infiltrating T cells. Anti-Ly6A treatment affects metabolism of T cells and inhibited tumor growth in mice resistant to anti-PD-1 therapy. Comprehensive approaches were used to validate the findings.

However, overall the observations are rather preliminary due to the small number of mice used in the studies, and many observations are needed to be clarified, especially for the following:

1) It is unclear if UV induced ly6A expression in T cells also depends on IFN-a, if so, are CD11b+ cells in lymph nodes the source of it? Does anti-Ly6A antibody treatment reverse immune suppression induced by UV?

2) Although the authors show Ly6A can be induced by IFNa in vitro, it does not necessarily mean Ly6A is induced by IFNa in TME. Other factors such as IL-27 can also induce Ly6A. Antibody blocking experiments are needed to confirm it.

3) The mode of action of anti-Ly6A antibody is intriguing. How the authors conclude that these antibodies are doing cross-linking rather than blocking or depleting Ly6A+ cells in vivo?

4) The data are not rigorous. Very low numbers of mice (n=3-4) were used for essentially all in vivo experiments. It is also unclear how many times the experiments were repeated.

5) The human Ly6E appears to have very broad expression, it does not seem very relevant to Ly6A expression in mouse models.

Minor: There are quite some messed up labeling of Figures and figure legends. e.g., Figure 3 i, j, k, and extended data Figure 1.

Reviewer #5 (Remarks to the Author): with expertise in melanoma, UVB

This is a largely well-written manuscript that describes a novel finding that Ly6a-high T-cells in UV irradiated skin have reduced target cell killing activity and maybe a mechanism of UV-induced immunosuppression and resistance to checkpoint blockade immunotherapy. Similar Ly6a-high T-cells were found in melanoma tumor microenvironment, which seem to induce Ly6a expression in response to IFN-alpha. Treatment of tumor-bearing mice with anti-Ly6a

antibodies inhibited tumor growth, suggesting that this could be a viable immunotherapeutic strategy. These are interesting and novel results that would be of interest to the readers of Nature Communications.

However, there are several concerns that should be addressed to bring this manuscript up to par for publication:

Fig.1: It is not clear what Ret melanoma cell line was used, as no name has been mentioned. Please provide the name of the cell line, the source, and reference(s) for this cell line (Ref. 20 doesn't seem to be appropriate as it does not describe any Ret cell lines). The authors have not provided a rationale for why this cell line was preferred over some of the other readily available cell lines, e.g., the YUMM series of cell lines. Further details of the characteristics of this cell line should be included in the materials & methods section.

Fig. 1b: Only 3 mice per group have been used for this experiment, which is insufficient to draw any conclusions.

It is not clear what the authors mean by the following statement on page 3, in the context of a subcutaneous model: "Because the primary tumor volumes were significantly greater in UVB-treated mice, we reasoned that the number of circulating melanoma cells were higher and thus the number of cells available for metastasis formation were higher in the UVB-treated group compared to the control group."

Fig 1c: Only 4 mice have been used for this experiment, which is insufficient. Why is there a different number of mice shown for the same experiment in Suppl. Fig. 1c? The authors have failed to mention the data for liver mets shown in Suppl. Fig. 1c, where there seems to be a significant reduction in UVB-irradiated mice. Overall, all of these and related in vivo experiments throughout the manuscript have insufficient number of mice (as low as 2) included and robust conclusions cannot be made from these data.

The spleen and sDLN CD8+ cells have not been characterized for the expression of effector molecules such as IFN-gamma, granzyme B, perforin, etc. This is important to conclude that

UVB reduces CD8+ T cell activity.

There seems to be a disconnect between UVB-induced skin immunosuppression and IFN-alpha in an inflammatory tumor microenvironment, both leading to the induction of Ly6a expression. The authors have to address and explain these seemingly contrasting concepts.

The manuscript needs some editing to remove several typographical errors.

Point-by-point response.

Reviewer #1 (Remarks to the Author): with expertise in cancer immuno-metabolism

Maliah and co-authors present interesting findings that chronic UVB exposure suppresses mouse skin-draining lymph node T cell killing in vivo, building upon current literature on UVB-induced tumour suppression. Mass cytometry profiling revealed increased Ly6a expression on UVB suppressed T cells and on non-UVB suppressed T cells in the tumour microenvironment. Anti-Ly6a antibodies convincingly improved tumour killing in vitro and in vivo, potentially through enhanced mitochondrial function.

We thank the reviewer for the thoughtful insights and comments. Addressing the concerns raised has improved our manuscript.

Major concerns

1. A key part of this manuscript is that UVB exposure induces Ly6a T cell expression; however, the authors never test the potential of anti-Ly6a in the context of UVB induced tumour suppression in vivo.

In response to this suggestion, we performed a study in mice. C57BL/6 mice were exposed to UVB radiation or were mock irradiated for 5 days/week over 8 weeks (50 mJ up to 200 mJ, a gradual increase like that used in phototherapy). Post radiation, $2x10^5$ Ret melanoma cells were injected subcutaneously. Once tumors reached a palpable size, mice were injected intraperitoneally twice a week with 100 µg anti-Ly6a antibody (clone E13 161-7) or with anti-IgG2a as a control (n=7 mice in each condition). Treatment with anti-Ly6a antibody significantly inhibited tumor growth in mice that were exposed to UVB and to those that were mock irradiated (**new Figure 5c**). The inhibitory impact of the anti-Ly6a antibody on tumor growth after UVB treatment was associated with an elevation in activated CD8 cells within the tumor microenvironment (**new Figure 5d**). Further, this is in sharp contrast to the effect of anti-PD1 antibody, which did not reduce tumor growth following chronic exposure to UVB (original Figure 1g, h and **new Figure 1h** and **new extended data Figure 5e**).

In summary, our results demonstrate that anti-Ly6a treatment hinders the accelerating effect of UVB-induced immunosuppression on tumor growth.

2. The authors test the role of Ly6a in T cell function using anti-Ly6a antibodies and it would be good to consolidate these findings using a genetic approach e.g. for the killing assay and TMRE.

We apologize for not making it clearer in the initial version of the manuscript. We found that Ly6a induces T-cell activation through crosslinking and not through blocking, Therefore, we do not believe that genetic manipulation resulting in reduced Ly6a expression mimics the effects observed with anti-Ly6a treatment. In this revised version we further explored the molecular mechanism that governs Ly6a downstream signaling. Briefly, crosslinking of Ly6a activates the MAP kinase pathway resulting in phosphor-ERK induction and in nuclear localization of Myc (**new Figure 6i-j**). This results rescue of T-cells from exhaustion and deactivates mitochondrial de-activation (**new Figure 6g**). We also now report *ex vivo* and *in vivo* data that show that the

increase of Ly6a expression on T-cells is IFNα signaling dependent (**new Figure 3 a-e, new Figure 3k-l** and **new extended data Figure 3q-r**). Indeed, chronic activation of T-cells with IFNα induces Ly6a expression on almost 100 percent of the cells, thus endogenously, the system reaches 100% expression of Ly6a which is a benefit when ubiquitous phenotype is analyzed. We demonstrate that the impact of anti-Ly6a treatment on T-cell killing is increased when T-cells are concurrently treated with type I interferon (**new Figure 5c**). Notably, treatment with type I interferon alone does not enhance the T-cells' killing ability (**new Figure 5c**). This suggests a strong correlation between the activity of the anti-Ly6a antibody and the expression level of Ly6a.
Therefore, while we acknowledge that genetic manipulation could potentially contribute to a better understanding of Ly6a, we believe that our data concerning the effects of anti-Ly6a and the role of Ly6a as a marker for UVB-induced effects on T-cells holds independent significance.

3. The flow cytometry data in figure 2O-P is not convincing and does not match the other flow plots showing Ly6a mean fluorescent intensity in figure 2G/I and figure 3B. Is this high population reflective of the in vivo Ly6a expression and is this finding reproducible? If you quantify MFI do you get the same results?

This is a very important issue, and we apologize for not addressing this in our initial submission. In accordance with your suggestions, we re-performed this experiment. Unlike the previous approach, where all cells expressing CD11b were captured, in the new experiment, we have taken a more specific approach and isolated only dendritic cells (**new Figure 3k-l**). To assess the *in vivo* mechanism though which Ly6a is upregulated on $CD8^+$ T-cells, we isolated DCs from the skin, skin-draining lymph nodes, and spleens of mice chronically irradiated with UVB and incubated them with naïve splenic $CD8^+$ T-cells isolated from control mice. We found a significant increase of $Ly6a^{high}/CD8^+$ T-cells upon incubation with DCs isolated from the skin and skin-draining lymph nodes but not when DCs came from spleens (**new Figure 3k-l**). It is noteworthy that the effect of skin DCs on Ly6a expression on $CD8^+$ T-cells was much higher than that of skin-draining lymph node DCs.

To further dissect the mechanism by which DCs induce Ly6a expression on $CD8^+$ T-cells, we analyzed the skin-draining lymph by single-cell RNA-seq following UVB exposure. We found that $CD8^+$ and $CD4^+$ T-cells isolated from the skin-draining lymph nodes were significantly enriched with the type I interferon gene signature (**new Figure 3a-e** and **extended data Figure 3a-b**). Thus, we incubated naïve $CD8^+$ T-cells with DCs isolated from skin and skin-draining lymph nodes of UVB-treated mice in the presence of blocking antibodies for IFNAR-1 (BioCell, catalog #BE024) or with control IgG. There was a significant reduction in the ability of DCs to induce Ly6a expression on $CD8^+$ T-cells in the presence of anti IFNAR-1 (**new extended data Figure 3q-r**). Lastly, chronic exposure of naïve splenic $CD8^+$ T-cells to type I IFN induced Ly6a expression in the majority of cells. Taken jointly, our new data suggests that chronic exposure of $CD8^+$ T-cells to type I IFN predominantly secreted by UV-activated DCs induces Ly6a expression.

Minor concerns

1. Several of the panels are too small to read when printed on A4.

We replaced the panels with unreadable font with versions that are clearly readable when printed.

2. Grammatical/spelling errors throughout the manuscript.

The revised manuscript has been proofread by a professional scientific editor to ensure that there are no grammatical or spelling errors.

Reviewer #2 (Remarks to the Author): with expertise in melanoma, cancer immunology

Through the studies of immune suppression mechanisms in chronic UVB exposure, the authors identified enrichment of a Ly6c+ CD8+ T cells in dLNs. This CD8 T cell population was also enriched in TILs. Amplification of Ly6c signaling using a mAb promoted T cell-mediated antitumor response. The author's finding might idenfy a therapeutic target in Ly6c for cancer immunotherapy, but at the very early stage. There are many main questions in related to the role of Ly6c and its antibody in T cells.

We thank the reviewer for the thoughtful insights and comments. Addressing the concerns raised has improved our manuscript.

For example, the involvement of Ly6a in UVR-induced immunosuppression is not proved.

In response to this suggestion, we performed a study in mice. C57BL/6 mice were exposed to UVB radiation or were mock irradiated for 5 days/week over 8 weeks (50 mJ up to 200 mJ, a gradual increase similar to that used in phototherapy). Post radiation, $2x10^5$ Ret melanoma cells were injected subcutaneously. Once tumors reached a palpable size, mice were injected intraperitoneally twice a week with 100 µg anti-Ly6a antibody (clone E13 161-7) or with anti-IgG2a as a control (n=7 mice in each condition). Treatment with anti-Ly6a antibody significantly inhibited tumor growth in mice that were exposed to UVB and to those that were mock irradiated (**new Figure 5c**). The inhibitory impact of the anti-Ly6a antibody on tumor growth after UVB treatment was associated with an elevation in activated CD8 cells within the tumor microenvironment (**new Figure 5d**). Further, this is in sharp contrast to the effect of anti-PD1 antibody, which did not reduce tumor growth following chronic exposure to UVB (original Figure 1g, h and **new Figure 1h** and **new extended data Figure 5e**).

In summary, our results demonstrate that anti-Ly6a treatment hinders the accelerating effect of UVB-induced immunosuppression on tumor growth.

What is the role of Ly6a on T cells? And other immune cells? The endogenous role for Ly6a in tumor immunity has not been tested.

Consistent with previous reports[1,2], we found that expression of Ly6a is not limited to T-cells; it is expressed on other immune cells including B cells, DCs, and macrophages. Nonetheless, supervised CyTOF analyses indicated that upon UVB

treatment, Ly6a expression is increased on T-cells but not on other immune subsets (**new Fig. 2g** and **extended data Figure 2c**). In the revised version of our manuscript, we describe our further characterization of the changes in Ly6a expression induced in mice chronically exposed to UVB (50 mJ up to 200 mJ, a gradually increase similar to that employed in phototherapy). In addition, we have now characterized the signaling cascade induced by crosslinking of Ly6a in T-cells and its positive effect of T-cell cytotoxicity and activation. These results are shown in **new Figure 6 g, i** and **j**.

The role of Ly6a, which does not have a human homologue, is not well understood. Ly6a is a marker of hematopoietic stem cells[3], and it is detected on tissue-resident stem and progenitor cells of liver, muscle, and mammary gland (reviewed in[4]). Moreover, it is expressed on cancer stem cells[5]. Ly6a is a marker of $CD62L^{+}/CD44^{-}$ stem-like memory $CD8^{+}$ T-cells[6] and is increased in $CD4^{+}$ and $CD8^{+}$ T-cells in response to inflammatory cytokines[7]. Depending on the mouse system, *Ly6a* elimination results in significant increases in T-cells in response to stress[8] or to no distinguishable phenotype difference in response to viral infection[9]. This led to the suggestion that Ly6a is "although perhaps a useful marker of virus-specific memory T-cells, is not required for the regulation of T-cell quantity or quality" [9]. In previous work, anti-Ly6a crosslinking on T-cells led to either T-cell activation through IL-2 autocrine signaling[10,11] or to downregulation of IL-2 production by T-cells[11,12]. The contradictory data may be due to technical differences, or it may be that Ly6a function is context dependent. Our finding that anti-Ly6a antibodies inhibit tumor growth are supported by a previous study published in 1989[13], but, to our knowledge, no further studies of the mechanism of action or its human relevance have been undertaken.

Taken together, the role of Ly6a on T-cells, and on other immune cells in general, and in tumor immunity, in particular, was basically unknown. Our work showed that Ly6a is a marker of a subset of T-cells that result from chronic UV exposure and that are observed in the tumor microenvironment due to chronic exposure to IFNα. A paragraph that puts our findings into the context of this previous work has been added to the Discussion section.

What would trigger Ly6c signaling in vivo.

To address this important question, we isolated DCs from the skin, skin-draining lymph nodes, and spleens of mice chronically irradiated with UVB and incubated them with naïve splenic $CD8^{+}$ T-cells isolated from control mice. We found a significant increase of $Ly6a^{high}/CD8^{+}$ T-cells upon incubation with DCs isolated from the skin and skin-draining lymph nodes but not when incubated with DCs from spleen of UVB-irradiated mice (**new Figure 3k-l**). It is noteworthy that the effect of skin DCs on Ly6a expression on $CD8^{+}$ T-cells was much higher than that of skin-draining lymph node DCs.

To further dissect the mechanism that triggers Ly6a signaling in $CD8^{+}$ T-cells *in vivo* we conducted single-cell RNA-seq of $CD4^{+}$ and $CD8^{+}$ T-cells from skin-draining lymph nodes following UVB. There was a significant increase in Ly6a expression in certain clusters of $CD4^{+}$ and $CD8^{+}$ cells following UVB exposure (**new Figure 3a-e** and **extended data Figure 3a-b**). Enrichment analysis showed a notable and distinct impact of the type 1 interferon (IFN) response, in this clusters both in CD4 and in CD8. Notably, upregulation of the cytokines IFNα and IFNβ were the only cytokines identified

in this analysis (**new Figure 3b**). These findings indicate that type 1 IFN is important for the activation of Ly6a *in vivo*, and it suggests that Ly6a identifies a subset of interferon exposed $CD4^+$ and $CD8^+$ T-cells that exist also out of UVB context.

We also performed an experiment to block signaling through IFNAR-1. We incubated naïve $CD8^+$ T-cells with DCs from skin and from skin-draining lymph nodes in the presence of blocking antibodies for IFNAR-1 (BioXcell, catalog #BE024) or with control IgG. There was a significant reduction in the ability of skin DCs to induce Ly6a expression on $CD8^+$ T-cells in the presence of anti IFNAR-1 (**extended data fig 3q-r**). Moreover, chronic exposure of naïve splenic $CD8^+$ T-cells to type I IFN induced Ly6a expression in the majority of cells. Taken jointly, our new data suggests that chronic exposure of $CD8^+$ T-cells to type I IFN predominantly secreted by UV-activated DCs induces Ly6a expression.

Thus, whether the Ly6a mAb used in vivo is antagonist or agonist is completely unknown.

Since the endogenous ligand of Ly6a is not known, it is not easy to experimentally address this question. Nonetheless, several of our findings strongly suggest that anti-Ly6a antibody affects the biological activity of T-cells through crosslinking of Ly6a, rather than blocking its interactions with a ligand. First, incubation of $CD8^+$ T-cells with the antibody induces expression of activation markers such as CD69 and ribosomal proteins and elevate the metabolic and mitochondrial activity of $CD8^+$ T-cells (original Figure 4c-i)). Second, incubation of $CD8^+$ T-cells with anti-Ly6a antibody rescues T-cells from exhaustion, deactivates mitochondria (**new Figure 6g**), induces phosphorylation of Erk1/2 (**new Figure 6i**), and induces mobilization of Myc from the cytoplasm to the nucleus (**new Figure 6j**). These data are consistent with the function of the Erk-Myc axis in mitochondrial metabolism[14,15]. These new findings strengthen our hypothesis that the influence of anti-Ly6a antibody on $CD8^+$ T-cells does not depend on the presence of a ligand found on other cells of the immune system or on cancer cells. Yet, to avoid any confusion, we refer to this antibody as crosslinking throughout the revised version of the text.

Others:
1. In fig. 1c, the dosage of tumor cells for lung metastasis should be titrated. The negative result can be simply because that the dosage used is too higher to overwrite the potential difference.

To address this concern, we performed the experiment by intravenously injecting $5x10^5$ and $1x10^5$ melanoma cells (n=6-7 mice in each group). Bioluminescence quantification indicated that, in contrast to the enhanced tumor growth in the skin, in the UVB-irradiated group versus the control group, no differences in growth of lung metastases between the UVB-irradiated mice and the control group were observed at either dose (**new Figure 1c and new extended data Figure 1c**). This excludes the possibility that the negative results obtained in our original experiment were due to the high dose that was used.

2. Naïve OT-1 cells with primed are not good at killing tumor cells in 24 hours.

We apologize for an error in the description of the method for this experiment. In the experiment shown in Figure 1e, we performed a melanoma killing assay by co-culturing B16-OVA melanoma cells with OT1 $CD8^+$ T-cells isolated from mice that were UVB radiated for 8 weeks, 5 days a week. Therefore, the OT1 $CD8^+$ T-cells are not naïve. For example, under these conditions, expression of Ly6a is induced as we showed in Figure 2. The Methods section has been corrected in the revised manuscript.

3. The assessment of the Ly6a level in the TILs between B16 and B16-OVA tumors is a good indicator for determining TCR dependency. The authors should examine the Ly6a level between OVA-tetramer-positive and -negative TILs of B16-OVA.

This was a great suggestion. To test this possibility, we challenged C57Bl/6 and OT-I mice with B16-ova melanoma cells injected subcutaneously. Once tumors were palpable, we analyzed the infiltrating $CD8^+$ T-cells for their expression of Ly6a concomitantly with staining with H-2Kb:OVA (SIINFEKL) tetramer (MBL, cat# TB-5001-4). We found that approximately 20% of H-2Kb:OVA-reactive $CD8^+$ T-cells that infiltrate the tumors are $Ly6a^{high}$ (**new extended data 4d**). This is equivalent to the percentages of $Ly6a^+$ $CD8^+$ T-cells infiltrating tumor tumors in control mice (original Extended Data Figure 4b.) This indicates that the increase in Ly6a is not restricted by the TCR specificity, but probably results from the inflammatory environment of the tumor. This further supports our hypothesis that Ly6a is a marker of T-cells that have been exposed to an inflammatory or an immunosuppressive environment that could result from chronic UVB exposure or cancerous lesions.

Reviewer #3 (Remarks to the Author): with expertise in cancer immuno-metabolism

This manuscript shows that Ly6a mediates UV exposure-induced sDLN T cell suppression. Outside the context of UV exposure, Ly6a crosslinking enhances T cell anti-tumoral cytotoxic activity and reprograms their mitochondrial metabolism. Treatment with anti-Ly6a antibody reduces tumor resistance to anti-PD1 therapy, leading to new immunotherapy treatment. The following points need to be further addressed:

We thank the reviewer for the thoughtful insights and comments. Addressing the concerns raised has improved our manuscript.

Major:

1. Why did the authors specifically focus on Ly6a but not other upregulated molecules in T cells due to UVB exposure? What is the rationale? The analysis showed no marked difference in Ly6a expression induced by UVB exposure.

We apologize for not clearly describing our results. Among all relevant markers of CD4 and CD8 that were examined by mass cytometry, Ly6a was the most significantly changed marker upon UV exposure (original extended data Figure 2b). We validated the increase in Ly6a using flow cytometry (**new Figure 2g** and **extended data Figure 2k-o)** Further we conducted single-cell RNA-seq of $CD4^+$ and $CD8^+$ T-cells from skin-draining lymph nodes following UVB. We found a significant induction in Ly6a expression in CD4 and CD8 following UVB exposure (**new Figure 3a-e** and **extended data Figure 3a-b**).

2. Besides T cells, it is unclear whether Ly6a is expressed in CD11b+ cells, including tumor-associated macrophages (TAMs) or dendritic cells. If so, why did the authors specifically focus on Ly6a in T cells but ignore myeloid cells?

Consistent with previous reports[1,2], we found that expression of Ly6a is not limited to T-cells; it is expressed on other immune cells including B cells, DCs, and macrophages. However, both unsupervised and supervised analyses using CyTOF, accompanied by flow cytometry indicated that UVB induces an increase in Ly6a expression predominantly on T-cells (**new Figure 2g** and **new extended data Figure 2c**). In the revised version of our manuscript, we validated this by further characterizing the changes in Ly6a expression on a variety of immune cells across mice chronically exposed to UVB. Our discovery pipeline was based on immune suppression induced by chronic exposure to UVB. UVB likely induces a simpler response than the enormous complexity of immune suppression induced in cancer. We highlighted these results in **new Figure 2g** and added a paragraph to the Results section describing the logic behind focusing on T-cells in subsequent experiments.

3. Given that the authors have shown that skin DLN CD11b+ cells specifically induce Ly6a expression in T cells (Fig. 2n), the mechanism is unclear to me. Additionally, is UVB exposure-induced Ly6a expression in T cells dependent on CD11b+ cells or independent of them?

This is a very important issue, and we thank the reviewer for highlighting it. To better understand this mechanism, we isolated DCs from the skin, skin-draining lymph nodes, and spleens of mice chronically irradiated with UVB and incubated them with naïve splenic $CD8^+$ T-cells isolated from control mice. We found a significant increase of $Ly6a^{high}/CD8^+$ T-cell upon incubation with DCs isolated from the skin and skin-draining lymph nodes but not upon incubation with spleen DCs, from UVB-irradiated mice (**new Figure 3k-l** and **extended data Figure 3q-r**). It is noteworthy that the effect of skin DCs on Ly6a expression of $CD8^+$ T-cells was much higher than the effect of skin-draining lymph nodes.

To further dissect the mechanism by which DCs induce Ly6a expression on $CD8^+$ T-cells, we conducted single-cell RNA-seq of $CD4^+$ and $CD8^+$ T-cells from skin-draining lymph nodes following UVB. We found a significant increase in Ly6a expression in certain CD4 and CD8 clusters (**new Figure 3a-e** and **extended data Figure 3a-b**). Enrichment tests showed a notable and distinct impact of the type 1 IFN response, and, notably, IFNα and IFNβ were the only cytokines identified in this analysis (**new Figure 3b**). These findings indicate the central role of type 1 IFN in the activation of Ly6a *in vivo* and suggests that Ly6a identifies a subset of interferon exposed CD4 and CD8 T-cells that must also exist also out of UVB context.

To further explore this, we incubated naïve $CD8^+$ T-cells with DCs from skin and from skin-draining lymph nodes in the presence of blocking antibodies for IFNAR-1 (BioCell, catalog #BE024) or with control IgG. We observed a significant reduction in the ability of DCs to induce Ly6a expression on $CD8^+$ T-cells in the presence of anti-IFNAR-1 antibody (**new extended data Figure 3q-r**). Lastly, chronic exposure of naïve splenic $CD8^+$ T-cells to type I IFN induced Ly6a expression in the majority of cells. Taken jointly, our new data suggest that chronic exposure of $CD8^+$ T-cells to type I IFN predominantly secreted by UV-activated DCs induces Ly6a expression.

4. Since Ly6a was highly induced in TILs, it is necessary to investigate Ly6a function in T cells through genetic Ly6a knockdown in CD8+ T cells for tumor cell killing and Ly6a x Cd8a cKO mice for tumor control.

We apologize for not making it clearer in the initial version of the manuscript. We found that Ly6a induces T-cell activation through crosslinking and not through blocking. Therefore, we do not believe that genetic manipulation resulting in reduced Ly6a expression mimics the effects observed with anti-Ly6a treatment. In this revised version we further explored the molecular mechanism that governs Ly6a downstream signaling. Briefly, crosslinking of Ly6a activates the MAP kinase pathway resulting in phosphor-ERK induction and in nuclear localization of Myc (**new Figure 6i-j**). This results in rescue of T-cells from exhaustion and deactivates mitochondrial de-activation (**new Figure 6g**). We also now report *ex vivo* and *in vivo* data that show that the increase of Ly6a expression on T-cells is IFNα signaling dependent (**new Figure 3 a-e, new Figure 3k-l** and **new extended data Figure 3q-r**). Indeed, chronic activation of T-cells with IFNα induces Ly6a expression on almost 100 percent of the cells, thus endogenously, the system reaches 100% expression of Ly6a which is a benefit when ubiquitous phenotype is analyzed. We demonstrate that the impact of anti-Ly6a treatment on T-cell killing is increases when T-cells are concurrently treated with type I interferon (**new extended data Figure 5a**). Notably, treatment with type I interferon alone does not enhance the T-cells' killing ability (**new extended data Figure 5a)**. This suggests a strong correlation between the activity of the anti-Ly6a antibody and the expression level of Ly6a. Therefore, while we acknowledge that genetic manipulation could potentially contribute to a better understanding of Ly6a, we believe that our data concerning the effects of anti-Ly6a and the role of Ly6a as a marker for UVB-induced effects on T-cells hold independent significance.

5. Considering that chronic IFNa significantly increases Ly6a expression in CD8+ T cells, can IFNAR blockade phenocopy the results obtained with anti-Ly6a treatment?

This is a wonderful suggestion. To address that we demonstrated that blocking IFNAR completely abrogated the induction of Ly6a on T-cells induced by DCs from skin and skin-draining lymph nodes (**new extended data Figure 3q-r**). To assess its activity *in vivo*, we next challenged mice with Ret melanoma cells and blocked IFNAR-1 using a mouse monoclonal antibody (BioCell, Catalog #BE0241). Consistent with reduction of Ly6a on T-cells, anti-INFAR-1 blockade significantly inhibited tumor growth compared to control IgG injection. We added these results as a **new Figure 5f**.

6. Why did anti-Ly6a promote mitochondrial activity in T cells? Does Ly6a signaling control mitochondrial functions such as membrane potential, ROS generation, ATP production, and oxidative phosphorylation (OXPHOS)? If so, how?

To dissect the mechanism of Ly6a-mediated activation, we incubated naïve splenic $CD8^+$ T-cells with anti-Ly6a antibody alone or in combination with anti-CD3 antibody. T-cell proteins were extracted and analyzed by Prof. Tami Geiger at the Weizmann Institute, a proteomics expert. In addition to elevation in mitochondrial protein expression, we also found an increase in proteins associated with cMyc (original Figures 4c-h). In this version we performed subsequent that corroborated the increase in T-cells membrane potential following crosslinking of anti-Ly6a antibody (original Figures 4i). We have now demonstrated that incubation of $CD8^+$ T-cells with anti-Ly6a

antibody rescues T-cells from exhaustion, causes mitochondrial de-activation (**new Figure 6g**), induces phosphorylation of Erk1/2 (**new Figure 6i**), and results in mobilization of cMyc from the cytoplasm to the nucleus (**new Figure 6j**). These data are consistent with the established role of the Erk-cMyc axis as an inducer of mitochondrial metabolism[14,15].

7. In Fig. 4, does anti-Ly6a treatment affect dendritic cell migration to skin DLN? Does anti-Ly6a treatment reduce T cell exhaustion? Can anti-Ly6a treatment prolong the survival rate of tumor-bearing mice?

To address these important questions, we used a mouse melanoma model. We challenged mice with *Ret* melanoma cells and allowed tumors to reach a palpable size. Mice were treated with anti-Ly6a or control antibodies and tumor size and the phenotype of infiltrating T-cells were assessed (**new Figure 5c**). We did not observe DC migration following treatment, as their subsets and numbers in the draining lymph nodes were comparable in the two treatment groups (**extended data Figure 5d**). We did however detect a 2 to 3 fold induction in the percentages of $CD8^+$ T-cells (out of $CD45^+$ cells) infiltrating tumors following treatment with anti-Ly6a antibody (**new Figure 5d**). Strikingly, the percentages of $CD8^+$ T-cells that express GrB was significantly higher in tumors, but not in the draining lymph node, from anti-Ly6a antibody treated mice compared to the controls (**new Figure 5d**). Consistent with this, we observed a prolonged survival of tumor bearing mice upon treatment with anti-Ly6a antibodies (**new extended data Figure 5c**).

Minor:
In Fig. 2f, the label of row 3 should be L_CD8_3, not L_CD4_3. In Extended Fig. 1e and f, the figure legends do not match the figures."

We thank the reviewer for pointing out these errors. They have been corrected.

Reviewer #4 (Remarks to the Author): with expertise in cancer immunology

The authors have identified that UV exposure induces immune suppression and expression of Ly6A in T cells, which is also confirmed to be present in tumor infiltrating T cells. Anti-Ly6A treatment affects metabolism of T cells and inhibited tumor growth in mice resistant to anti-PD-1 therapy. Comprehensive approaches were used to validate the findings. However, overall the observations are rather preliminary due to the small number of mice used in the studies, and many observations are needed to be clarified, especially for the following:

We thank the reviewer for the thoughtful insights and comments. Addressing the concerns raised has improved our manuscript.

1) It is unclear if UV induced ly6A expression in T cells also depends on IFN-a, if so, are CD11b+ cells in lymph nodes the source of it?

To assess the mechanism though which Ly6a is upregulated on $CD8^+$ T-cells, we isolated DCs from the skin, skin-draining lymph nodes, and spleen of mice chronically

irradiated with UVB and incubated them with naïve splenic $CD8^+$ T-cells isolated from control mice. We found a significant increase of $Ly6a^{high}/CD8^+$ T-cells upon incubation with DCs isolated from the skin and skin-draining lymph nodes of UVB irradiated mice but upon incubation with DCs from spleen (**new Figure 3k-l**). Skin DCs had a greater effect on Ly6a expression than did skin-draining lymph node DCs. We have also conducted single-cell RNA-seq of $CD4^+$ and $CD8^+$ T-cells from skin-draining lymph nodes following UVB. We found a significant increase in Ly6a expression in certain clusters $CD4^+$ and $CD8^+$ T-cells (**new Figure 3a-e** and **extended data Figure 3a-b**). Further, an enrichment test for predicting the active cytokines within these clusters using Response Enrichment Analysis showed a notable and distinct impact of the type 1 interferon (IFN) response, un this clusters both in CD4 and in CD8. Notably, IFNα and IFNβ were the only cytokines identified in this analysis (**new Figure 3b**). These findings suggest that type 1 IFN is important for the activation of Ly6a *in vivo*, and it suggests that Ly6a identifies subset of IFN-exposed $CD4^+$ and $CD8^+$ T-cells that exist also out of UVB context.

To test this hypothesis, we incubated naïve $CD8^+$ T-cells with DCs from skin and skin-draining lymph nodes of UVB treated mice, in the presence of blocking antibodies for IFNAR-1 (BioCell, catalog #BE024) or with control IgG. There was a significant reduction in the ability of DCs to induce Ly6a expression on $CD8^+$ T-cells in the presence of anti-IFNAR-1 antibody (**new extended data Figure 3q-r**). Further, chronic exposure of naïve splenic $CD8^+$ T-cells to type I IFN induced Ly6a expression in the majority of cells. These new data suggest that chronic exposure of $CD8^+$ T-cells to type I IFN predominantly secreted by UV-activated DCs induces Ly6a expression.

Does anti-Ly6A antibody treatment reverse immune suppression induced by UV?

In response to this suggestion, we performed a study in mice. C57BL/6 mice were exposed to UVB radiation or were mock irradiated for 5 days/week over 8 weeks (50 mJ up to 200 mJ, a gradual increase similar to that used in phototherapy). Post radiation, $2x10^5$ Ret melanoma cells were injected subcutaneously. Once tumors reached a palpable size, mice were injected intraperitoneally twice a week with 100 µg anti-Ly6a antibody (clone E13 161-7) or with anti-IgG2a as a control (n=7 mice in each condition). Treatment with anti-Ly6a antibody significantly inhibited tumor growth in mice that were exposed to UVB and to those that were mock irradiated (**new Figure 5c**). The inhibitory impact of the anti-Ly6a antibody on tumor growth after UVB treatment was associated with an elevation in activated CD8 cells within the tumor microenvironment (**new Figure 5d**). Further, this is in sharp contrast to the effect of anti-PD1 antibody, which did not reduce tumor growth following chronic exposure to UVB (original Figure 1g, h and **new Figure 1h** and **new extended data Figure 5e**).

In summary, our results demonstrate that anti-Ly6a treatment hinders the accelerating effect of UVB-induced immunosuppression on tumor growth.

2) Although the authors show Ly6A can be induced by IFNa in vitro, it does not necessarily mean Ly6A is induced by IFNa in TME. Other factors such as IL-27 can also induce Ly6A. Antibody blocking experiments are needed to confirm it.

This is an important point. Our discovery pipeline was based on the immune suppression induced by chronic exposure to UVB. To further dissect the mechanism that triggers Ly6a signaling in $CD8^+$ T-cells *in vivo* we conducted single-cell RNA-seq of $CD4^+$ and $CD8^+$ T-cells from skin-draining lymph nodes following UVB. There was a significant increase in Ly6a expression in certain clusters of $CD4^+$ and $CD8^+$ cells (**new Figure 3a-e** and **new extended data Figure 3a-b**). Enrichment analysis showed a significant enrichment of the type 1 interferon (IFN) response in these clusters (**new Figure 3b**). Notably, IFNα and IFNβ were the only cytokines identified in this analysis (**new Figure 3b**). These findings indicate that type 1 INF is important for the activation of Ly6a *in vivo*, and it suggests that Ly6a identifies a subset of interferon exposed $CD4^+$ and $CD8^+$ T-cells that exist even in the absence of UVB irradiation.

We also performed an experiment to block signaling through IFNAR-1. We incubated naïve $CD8^+$ T-cells with DCs from skin and from skin-draining lymph nodes in the presence of blocking antibodies for IFNAR-1 (BioXcell, catalog #BE024) or with control IgG. There was a significant reduction in the ability of DCs to induce Ly6a expression on $CD8^+$ T-cells in the presence of anti IFNAR-1 (**new extended data Figure 3q-r**). Moreover, chronic exposure of naïve splenic $CD8^+$ T-cells to type I IFN induced Ly6a expression in the majority of cells. Taken jointly, our new data suggests that chronic exposure of $CD8^+$ T-cells to type I IFN predominantly secreted by UV-activated DCs induces Ly6a expression.

With that being said, the immune suppression induced by the tumor microenvironment is much more complicated than reflected in this experiment. Blocking IFNAR-1 *in vivo* did not completely recapitulate the effect of anti-Ly6a antibodies, suggesting other factors are important. DeLong and colleagues have shed important insights in the mechanistic process through which T-cell express Ly6a. They demonstrated that mediators such as IFNγ and IL-27 modulate the expression of Ly6a on T-cells in a model of parasitic protozoan[7]. Beyond that, their results challenge the notion that induction of Ly6a expression is limited to stem cell or antigen-experienced T-cells, but rather indicates exposure to specific inflammatory factors.

Earlier studies have suggested that crosslinking Ly6a on T-cells induces IL-2 secretion but that this crosslinking suppresses IL-2 secretion when combined with anti-CD3 antibodies[12,16]. These however were based on transfected T-cell lines, and the relevance to the role of Ly6a following activation may be complicated. In contrast, other publications have shown that crosslinking of Ly6a induces intracellular calcium influx and IL-2 production[10,11]. One potential explanation for these discrepancies may be that different antibody clones were used. In the present study we observed significant differences in the effects of different anti-Ly6a antibody clones. Other evidence for a suppressive role of Ly6a came from genetically modified mice. Stanford et al. demonstrated that T-cells from Ly6a-deficient mice have lower proliferation rates following stimulation with anti-CD3 antibody than do T-cells from wild-type mice[8]. Interestingly, T-cells from the mutant mice were equally responsive to other stimuli such as LPS, ConA, PMA, and the mixed lymphocyte reaction[8]. We have added a paragraph describing the limitations of our findings and discussing the elusive role of Ly6a on T-cells to the Discussion.

3) The mode of action of anti-Ly6A antibody is intriguing. How the authors conclude that these antibodies are doing cross-linking rather than blocking or depleting Ly6A+ cells in vivo?

We thank the reviewer for highlighting this important point. Since the endogenous ligand of Ly6a is not known, it is difficult to address this issue. Nonetheless, several lines of evidence strongly suggest that anti-Ly6a antibody affects the biological activity of T-cells through crosslinking of Ly6a rather than by blocking its interactions with ligand. First, incubation of $CD8^+$ T-cells with anti-Ly6a antibody induces activation markers such as CD69 and ribosomal proteins and elevates the metabolic and mitochondrial activity of $CD8^+$ T-cells (original Figure 4c-i)). Second, we demonstrated that incubation of $CD8^+$ T-cells with anti-Ly6a antibody rescues T-cell from exhaustion-induces mitochondrial de-activation (**new Figure 6g**), induces phosphorylation of Erk1/2 (**new Figure 6i**), and causes cMyc to move from the cytoplasm to the nucleus (**new Figure 6j**). These data are in agreement with the established role of the Erk-cMyc axis as a major inducer of mitochondrial metabolism[14,15]. These findings strengthen our hypothesis that the effects of anti-Ly6a antibody on $CD8^+$ T-cells do not depend on the presence of a ligand found in other cells of the immune system or in cancer cells. Yet, to avoid any confusion, we refer to this antibody as crosslinking throughout the revised version of the text.

4) The data are not rigorous. Very low numbers of mice (n=3-4) were used for essentially all in vivo experiments. It is also unclear how many times the experiments were repeated.

We have repeated all *in vivo* experiments to enhance the statistical power:
New Figure 1b. New experiment n=6 per treatment for a total of n=10 per group.
New Figure 1c. New experiment n=6 per treatment for a total of n=11 per group.
New Figure 1h. New experiment n=6 per treatment for a total of n=10 per group.
New Figure 5c. n=7 per group.
New Figure 5h. New experiment n=7 per treatment for a total of n=10-11 per group.

5) The human Ly6E appears to have very broad expression, it does not seem very relevant to Ly6A expression in mouse models.

We have removed the human Ly6E data from the manuscript.

Minor: There are quite some messed up labeling of Figures and figure legends. e.g., Figure 3 i, j, k, and extended data Figure 1.

We have carefully corrected labeling in the figures.

Reviewer #5 (Remarks to the Author): with expertise in melanoma, UVB

This is a largely well-written manuscript that describes a novel finding that Ly6a-high T-cells in UV irradiated skin have reduced target cell killing activity and maybe a mechanism of UV-induced immunosuppression and resistance to checkpoint blockade immunotherapy. Similar Ly6a-high T-cells were found in melanoma tumor

microenvironment, which seem to induce Ly6a expression in response to IFN-alpha. Treatment of tumor-bearing mice with anti-Ly6a antibodies inhibited tumor growth, suggesting that this could be a viable immunotherapeutic strategy. These are interesting and novel results that would be of interest to the readers of Nature Communications.

We thank the reviewer for the thoughtful insights and comments. Addressing the concerns raised has improved our manuscript.

However, there are several concerns that should be addressed to bring this manuscript up to par for publication:

Fig.1: It is not clear what Ret melanoma cell line was used, as no name has been mentioned. Please provide the name of the cell line, the source, and reference(s) for this cell line (Ref. 20 doesn't seem to be appropriate as it does not describe any Ret cell lines). The authors have not provided a rationale for why this cell line was preferred over some of the other readily available cell lines, e.g., the YUMM series of cell lines. Further details of the characteristics of this cell line should be included in the materials & methods section.

The Ret melanoma line was established in 1992 by 10 back crosses of line 304 in C57BL/6 mice[17,18]. This line is widely used in melanoma models and induces spontaneous melanoma progression that resembles human melanoma in its progression, metastases potential, melanotic level, and immunogenicity. It results in activation of MAPK and cJun signaling, which are downstream of the Ret oncogene[17–19]. Most importantly, like the YUMM melanoma model, tumors induced by Ret melanoma cells are responsive to checkpoint blockade[20]. We have added the rationale for use of Ret melanoma cells to the methods section.

Fig. 1b: Only 3 mice per group have been used for this experiment, which is insufficient to draw any conclusions.

We have repeated this experiment with 6 additional mice in each treatment group for a total of n=10 per group.

It is not clear what the authors mean by the following statement on page 3, in the context of a subcutaneous model: "Because the primary tumor volumes were significantly greater in UVB-treated mice, we reasoned that the number of circulating melanoma cells were higher and thus the number of cells available for metastasis formation were higher in the UVB-treated group compared to the control group."

Thank you for your comment, and we apologize for the lack of clarity in this paragraph. We deleted the unclear text and replaced with a simple description “To further reveal the effect of UVB exposure on metastases development, we performed an intravenous injection of melanoma using melanoma metastases model”[21].

Fig 1c: Only 4 mice have been used for this experiment, which is insufficient. Why is there a different number of mice shown for the same experiment in Suppl. Fig. 1c? The authors have failed to mention the data for liver mets shown in Suppl. Fig. 1c, where there seems to be a significant reduction in UVB-irradiated mice.

The experiment shown in Figure 1C included 5 mice in the original version. To address the reviewer's concern we performed this experiment again with 6 mice per group. Lung metastases were quantified using an IVIS bioluminescence apparatus. We found that, in contrast to the enhanced tumor burden observed in the skin of UVB-irradiated mice, there was no significant change in the growth rate of melanoma in the lungs (**new Figure 1c**). Supplementary Figure 1c has been removed from the revised version of the manuscript.

Overall, **all** of these and related in vivo experiments **throughout** the manuscript have insufficient number of mice (as low as 2) included and robust conclusions cannot be made from these data.

We have repeated all *in vivo* experiments to enhance the statistical power:
New Figure 1b. New experiment n=6 per treatment for a total of n=10 per group.
New Figure 1c. New experiment n=6 per treatment for a total of n=11 per group.
New Figure 1h. New experiment n=6 per treatment for a total of n=10 per group.
New Figure 5c. n=7 per group.
New Figure 5h. New experiment n=7 per treatment for a total of n=10-11 per group.

The spleen and sDLN CD8+ cells have not been characterized for the expression of effector molecules such as IFN-gamma, granzyme B, perforin, etc. This is important to conclude that UVB reduces CD8+ T cell activity.

We have now characterized expression of the effector molecule GrB on $CD8^+$ T-cells from skin-draining lymph nodes and spleens following UVB exposure. We found a significant reduction in GrB expression following UVB exposure on $CD8^+$ T-cells from skin-draining lymph nodes compared to mock-irradiated animals (**new Figure 1g**). No significant change was observed in GrB expression on $CD8^+$ T-cells isolated from the spleen and no change was detected in GrB expression on $CD4^+$ T-cells in any of the conditions.

There seems to be a disconnect between UVB-induced skin immunosuppression and IFN-alpha in an inflammatory tumor microenvironment, both leading to the induction of Ly6a expression. The authors have to address and explain these seemingly contrasting concepts.

In the revised version, we present data that, in both cases, IFNα is the major modulator of Ly6a on T-cells. To identify what mediator induces Ly6a following chronic exposure to UVB, we conducted single-cell RNA-seq of $CD4^+$ and $CD8^+$ T-cells from skin-draining lymph nodes of mice treated with UVB. We found a significant increase in Ly6a expression in certain clusters of $CD4^+$ and $CD8^+$ T-cells (**new Figure 3a-e** and **extended data Figure 3a-b**). Enrichment analysis showed a notable and distinct impact of the type 1 IFN response in both clusters. Notably, IFNα and IFNβ were the only cytokines identified in this analysis (**new Figure 3b**). These findings indicate the central role of type 1 IFN in the induction of Ly6a expression *in vivo* and suggest that Ly6a identifies a subset of interferon-exposed T-cells that exist also out of UVB context.

To delineate the process through which Ly6a is upregulated, we isolated naïve splenic T-cells and incubated them with DCs from skin and skin-draining lymph nodes from UVB and mock-irradiated mice. Only DCs from irradiated mice induced Ly6a

expression on naïve T-cells (**new Figure 3**k-l). Blocking the type I IFN receptor completely abrogated Ly6a expression (**new extended data Figure 3q-r**). Collectively, our data indicate that in conditions of chronic UVB exposure and within the tumor microenvironment, IFNα is the major modulator of Ly6a expression on T-cells. We added a paragraph in the Discussion that explains the two concepts of UVB immunosuppression and tumor microenvironment inflammation, that both, independently, induce Ly6a expression via type 1 interferon signaling.

The manuscript needs some editing to remove several typographical errors.

The manuscript has been proofread by a professional scientific editor.

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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The additional experiment in new Figure 5C has greatly strengthened the manuscript as it is now clear that anti-Ly6a improve UVB induced tumour suppression in vivo. The data in new Figure 5D and 1G are unclear. There is no y-axis label, and if the authors are using %granzyme B positive as a marker of activation this is not appropriate for CD4+ T cells. CD44+/CD62L- would be more appropriate. The new data on crosslinking of Ly6a activating the MAP kinase pathway are convincing; however, the data on the nuclear localisation of Myc (new Figure 6J) are unclear. Why have the authors shown a single CD8+ cell in the control group and how was this quantified in the right-hand panel? How many cells and samples were analysed here? The effect of the skin DCs versus the lymph DCs on Ly6a expression is interesting. The schematic in Figure 3K does not match the groups in Figure 3I. Given the previous issues with the flow data it would be good to show representative flow data for Figure 3I, like Figure 4B. The authors should be consistent in showing either Ly6a high in terms of % or MFI across the manuscript. The title of Figure 6 should be more specific. I acknowledge that if my comments here are addressed the findings in this manuscript are significant in the absence of further genetic validation.

Reviewer #2 (Remarks to the Author):

In the revised manuscript, the authors had performed additional experiments and provided with detailed interpretations. Many questions raised previously have been addressed adequately. However, it is still unclear to me what the natural role of Ly6a on CD8+ T cell is. And the effect of ly6a antibody in vivo can be complicated by other ly6a-expressing immune cells, even though the ly6a antibody assumes to be agonistic. Given the lack of knowledge of endogenous ligands for ly6c and relevant data from ly6c-knockout mice, it is not convincing to me that ly6a acts an immunosuppressor for CD8 T cells in UVB-induced tumor suppression.

Reviewer #3 (Remarks to the Author):

Authors have addressed my concerns.

Reviewer #4 (Remarks to the Author):

The authors have adequately responded to my previous critiques. I have no further comments.

Reviewer #5 (Remarks to the Author):

The authors have done a thorough and satisfactory job of responding to my prior critique by including new experimental data and other appropriate revisions of text and figures. I have no further comments.

Point by Point:

Reviewer #1 (Remarks to the Author):

The additional experiment in new Figure 5C has greatly strengthened the manuscript as it is now clear that anti-Ly6a improve UVB induced tumour suppression in vivo.

The data in new Figure 5D and 1G are unclear. There is no y-axis label, and if the authors are using %granzyme B positive as a marker of activation this is not appropriate for CD4+ T cells. CD44+/CD62L- would be more appropriate.

In response to this comment, we have added titles for the Y axis and have removed the $CD4^+$ T cell data from Figure 1G. We agree with the reviewer that additional markers should be analyzed, and in the mass cytometry analysis shown in Figure 2 we used additional markers of $CD4^+$ cells, including CD44 and CD62L. We observed no significant difference in $CD44^+$ or $CD62L^-$ $CD4^+$ cells but a notable increase in $Ly6a^+$ $CD4^+$ cells. We now emphasize this observation in the results section. In Figure 5d, we refer to the total percentage of $CD4^+$ cells within the tumor, whereas the expression of granzyme B was measured only in the $CD8^+$ cells. We added a title to the Y axis and changed the graph to present the analysis more clearly.

The new data on crosslinking of Ly6a activating the MAP kinase pathway are convincing; however, the data on the nuclear localisation of Myc (new Figure 6J) are unclear. Why have the authors shown a single CD8+ cell in the control group and how was this quantified in the right-hand panel? How many cells and samples were analysed here?

These experiments were performed in three independent biological replicates. In the control group, seven fields were tested, and in the anti-Ly6a treated group, eight fields were tested. In each of the fields there were four to nine $CD8^+$ cells, and the graph plots the percentage of $CD8^+$ cells in which there was nuclear staining of cMYC. We have now added this clarification to the methods.

The effect of the skin DCs versus the lymph DCs on Ly6a expression is interesting. The schematic in Figure 3K does not match the groups in Figure 3I.

We apologize for this error and have fixed the schematic in Figure 3K.

Given the previous issues with the flow data it would be good to show representative flow data for Figure 3I, like Figure 4B.

We added a representative image to **new Figure 3I**.

The authors should be consistent in showing either Ly6a high in terms of % or MFI across the manuscript.

For consistency, we removed the MFI graph from Figure 4B, and in all other figures we plot the percent of Ly6a^{high} cells.

The title of Figure 6 should be more specific.

We have revised the Figure 6 title. It is now “Anti-Ly6a antibody prevents CD8^{+} T cell loss of mitochondrial function via cMYC signaling”.

I acknowledge that if my comments here are addressed the findings in this manuscript are significant in the absence of further genetic validation.

We greatly thank the reviewer for these thoughtful comments as addressing them improved our manuscript.

Reviewer #2 (Remarks to the Author):

In the revised manuscript, the authors had performed additional experiments and provided with detailed interpretations. Many questions raised previously have been addressed adequately.

We greatly thank the reviewer for the thoughtful comments as addressing them improved our manuscript.

However, it is still unclear to me what the natural role of Ly6a on CD8+ T cell is. And the effect of ly6a antibody in vivo can be complicated by other ly6a-expressing immune cells, even though the ly6a antibody assumes to be agonistic.

We thank the reviewer for this comment. Our in-vitro and ex-vivo data provide the mechanism by which anti-Ly6a enhances CD8 cytotoxicity and reduces tumor growth. This identified mechanism can explain the significant tumor growth repression in-vivo. We agree with the reviewer that we cannot exclude the effect of the anti-Ly6a antibody on other immune cells. However, the effect on CD8 T-cells is sufficient to explain the in-vivo results.

Given the lack of knowledge of endogenous ligands for ly6c and relevant data from ly6c-knockout mice, it is not convincing to me that ly6a acts an immunosuppressor for CD8 T cells in UVB-induced tumor suppression.
We agree with the reviewer that we have not addressed all questions related to Ly6a's biological role. However, the novelty of our paper lies in our ability to overcome resistance to immunotherapy by targeting a molecule whose expression increases following a state of immunosuppression, such as that induced by UVB exposure.

Reviewer #3 (Remarks to the Author):

Authors have addressed my concerns.
We greatly thank the reviewer for the thoughtful comments as addressing them improved our manuscript.

Reviewer #4 (Remarks to the Author):

The authors have adequately responded to my previous critiques. I have no further comments.
We greatly thank the reviewer for these thoughtful comments as addressing them improved our manuscript.

Reviewer #5 (Remarks to the Author):

The authors have done a thorough and satisfactory job of responding to my prior critique by including new experimental data and other appropriate revisions of text and figures. I have no further comments.
We greatly thank the reviewer for these thoughtful comments as addressing them improved our manuscript.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

I would like to follow up on my previous comment on figure 6J. The authors clarify that:

These experiments were performed in three independent biological replicates. In the control group, seven fields were tested, and in the anti-Ly6a treated group, eight fields were tested. In each of the fields there were four to nine CD8+ cells, and the graph plots the percentage of CD8+ cells in which there was nuclear staining of cMYC. We have now added this clarification to the methods.

In the representative images, the control condition shows a single CD8 T cell without Myc nuclear localisation. If you have 7 fields tested, each with four to nine CD8 T cells, surely there is a more representative image you can use here with multiple CD8 T cells as in the antiLy6a condition. I think it is misleading that you show 3 cells without nuclear staining, but only 1 is CD8. What are these other cells as in the main text you state that the experiment was done on CD8+ T cells?

My other comments have been addressed.

Point by point

REVIEWERS' COMMENTS

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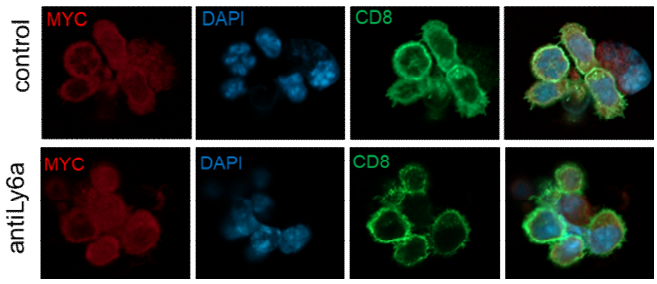
We thank the reviewer for this comment and agree that the visualization in the representative figure might be misleading due to technical reasons. CD8 cells are non-adherent, making Immunostaining analysis challenging because each cell adheres to the slide at a different angle, and different zoom layers reflect different cells. Therefore, an image that focuses on a single layer might misrepresent the data. In our experiment, we used a pure CD8 population (as we detailed in the results section), thus there is no doubt about the cells that were stained. In response to the reviewer's important comment, we have now submitted a new figure for the control treatment, where most cells are at the same zoom level. The **new Figure 6j** clearly represents our analysis of Myc nuclear transportation in CD8 cells following anti Ly6a antibody treatment.



My other comments have been addressed.