

Reviewer 1:**Open Review**

(x) I would not like to sign my review report

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English language and style

() Extensive editing of English language and style required

() Moderate English changes required

(x) English language and style are fine/minor spell check required

() I don't feel qualified to judge about the English language and style

	Yes	Can be improved	Must be improved	Not applicable
Does the introduction provide sufficient background and include all relevant references?	(x)	()	()	()
Is the research design appropriate?	()	(x)	()	()
Are the methods adequately described?	()	(x)	()	()
Are the results clearly presented?	()	(x)	()	()
Are the conclusions supported by the results?	()	(x)	()	()

Comments and Suggestions for Authors

1. Answer to comment (2): **"In the figure legends, "n" refers to the number of independent experiments, i.e. experiments performed at different time points."** It is still unclear how many donors were processed for an experiment at any given timepoint. Pooling data obtained at different timepoints is very tricky as the authors know. So when the revised figure legends now state "n" independent experiments, does that mean n repeat timepoints, with cells from all the 9 donors used per timepoint?

NB: Nowadays, separate datapoints are often overlayed on bar graphs to provide a clearer picture of data replicates

Answer:

Indeed, "n" refers to the repeated timepoints of every independent experiment. Per timepoint one donor was used.

2. Figure 3a: for relative MFI, the ratio rather than arithmetic subtraction of MFIs should be used, and MFI of the FMO, not the isotype control should be used as the denominator. It is correct that isotype control can be used to ascertain the effectivity of your Fc blocking protocol, but FMO should be used as background signal for further analysis as it allows to determine the extent of spectral spread between channels. There is a lot of not-so-very-recent methodological literature on this.

Answer:

We changed figure 3a and 3b by showing the relative MFI as the ratio of the MFI of the antigen-specific mAb compared to the MFI of the isotype control mAb.

We apologize by not using FMO to determine background signals. However, we are convinced we were able to correctly distinguish positive populations from negative populations as shown in Figure 2a and 2b, where we define the gating strategy that was used.

3. Figure 3c: huge error bar, no statistical significance, and data presumably not sufficient to claim "strong increase in expression". I don't think this graph is of much use. Why not show the PD-1 expression pre/post by flow in figure 3b?

Answer:

We agree with the reviewer that this is insufficient to make the following statement: "strong increase in expression". Since we only have data obtained with qPCR and not with flow cytometry evaluating PD-1 expression levels after co-culture we decided to not show this data. We deleted the following statement out of the manuscript:

“Quantitative PCR analysis showed a strong increase in PD-1 expression on CD8^{pos} T cells upon co-culture with DC-MEL when compared to CD8^{pos} T cells that were not stimulated [Figure 3c].”

4. Figure 3f: missing short description of proliferation assay used

Answer:

The proliferation assay was described in the materials and methods section as follows:

2.6 Proliferation assay

PBMCs depleted from CD14^{pos} cells from healthy donor were labeled with 0.5μM Cell Trace Violet (Invitrogen). These cells (10⁵) were co-cultured for 6 days with or without DC-MEL (4.8μg Melan-A mRNA) at a effector:stimulator ratio of 10:1 in 200μL IMDM containing 1% heat-inactivated human AB serum, PS, L-Glu and non-essential amino acids. T-cell proliferation was measured in flow cytometry as the dilution of the CellTrace Violet dye in the CD8^{pos} T-cell population. Proliferation observed in cultures without DC-MEL was considered as background.

We have adapted figure 3 in the revised manuscript as follows:

As suggested by reviewer 3, we now present the percentage of proliferating cells instead of the fold increase in CD8^{pos} T cell proliferation (figure 3e).

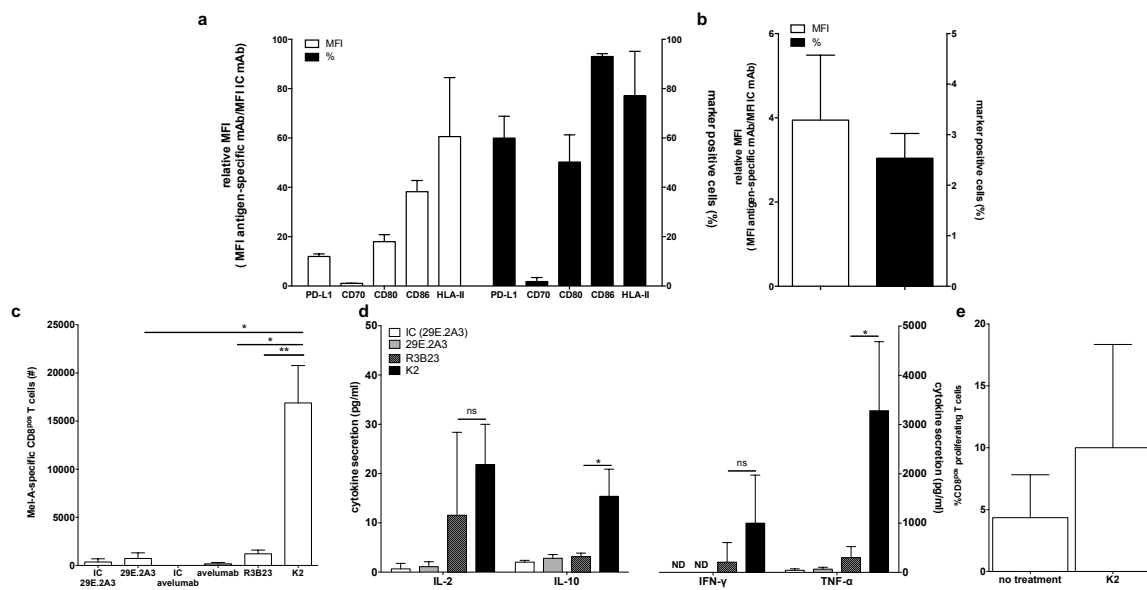


Figure 3: Activation of Melan-A-specific CD8^{pos} T cells by DC-MEL is enhanced in the presence of sdAb K2. [a] Graph showing the percentage of marker positive cells as well as the relative MFI (MFI antigen-specific mAb/MFI IC mAb) for the expression of PD-L1, CD70, CD80, CD86, and HLA-II on DC-MEL. The graph summarizes the results of three independent experiments as mean ± SEM. [b] Graph showing the percentage of PD-1^{pos} cells as well as the relative MFI (MFI antigen-specific mAb/MFI IC mAb) for the expression of PD-1 on T cells. The graph summarizes the results of three independent experiments as mean ± SEM (left). [c] Graph showing the number Melan-A-specific T cells after co-culture with DC-MEL in the presence of IC mAbs [IC], anti-PD-L1 mAbs [29E.2A3 and avelumab], sdAb R3B23 [R3B23] or sdAb K2 [K2]. The graph summarizes the results of three independent experiments as mean ± SEM. [d] Graph showing the production of IL-2, IL-10, IFN-γ and TNF-α by Melan-A-specific CD8^{pos} T cells co-cultured with DC-MEL in the presence of sdAb K2 compared to sdAb R3B23. The graph summarizes the results of three independent experiments as mean ± SEM. [e] Graph showing the percentage of CD8^{pos} proliferating T cells co-cultured with DC-MEL in the presence of sdAb K2. The graph summarizes the results of two independent experiments as mean ± SEM.