

Dendritic cell progenitors engineered to express extracellular-vesicle–internalizing receptors enhance cancer immunotherapy in mouse models

Corresponding Author: Professor Michele De Palma

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Ghasemi and colleagues describe the effect of engineering DC precursor (DCP) cells with an extracellular receptor (EVIR) specific to a surface tumor antigen, which internalizes tumor-derived EVs and promotes antigen presentation to boost anti-tumor activity of endogenous T cells. Outfitting murine DCPs with an EVIR enhances tumor control and the efficacy of PD-1 blockade in a couple of tumor models. Further, the authors demonstrate feasibility of generating human DCPs transduced with a lentiviral vector encoding an EV and show activation of CD8+ T cells specific to a peptide artificially presented on tumor cells. Overall, the study is well done and build further on their prior report describing the concept and mechanism of EVIRs in antigen-presenting cells.

Addressing a few questions would help understand the mechanism and potential application of this approach.

1. In Figure 2, the effect of exogenous DCP on TILs is unclear. The authors show an overall increase in CD45+ cells and a modest increase in PD-1+ CD8+ T cells within those hematopoietic cells in some animals. However, an increase in effector (IFN γ +, TNF α +) TILs is not statistically significant, so the conclusions in the text are not supported. Further, there is no difference in frequency of those cells in mice receiving EVIR DCP with and without PD-1 blockade. These data need to be rectified or removed. Further, if PD1+ CD8+ T cells are only a few percent of total CD45+ cells (Fig 2c), what are the other major populations modulated by the DCP EVIRs?

2. Comparison of DCP and moDC in Fig. 2d-g is interesting. Is the limited effect of moDC ascribed to their poor trafficking/infiltration into tumors? What is the phenotype and differentiation status of DCP vs moDC in tumors?

3. Can DCP EVIRs cross-present tumor antigens and invoke CD4+ T cell responses? The authors did some studies in the previous paper but it is unclear whether DCP (either human or mouse) can be used to stimulate both subsets of T cells.

3.1. For the clinical translation, do the authors envision using allogeneic (off-the-shelf) EVIR-DCP or autologous? If the latter, it is unclear whether cross-dressing shown in Fig. 5 would contribute significantly to the intended function of DCPs. If the former, would allo-immunogenicity of these DCP be a problem or the authors envision HLA editing?

Minor point

1. In Fig. 5d, statistical tests with n=2 is discouraged.

Reviewer #2

(Remarks to the Author)

Ali Ghasemi et al., explore the use of engineered dendritic cell progenitors (DCPs) expressing a CAR construct known as EVIR for cancer therapy, specifically targeting solid tumors like melanoma. By utilizing murine models, the efficacy of these engineered DCPs in activating immune responses, facilitating antigen transfer, and their interactions with antigen-specific T lymphocytes was analyzed. The study also explores the combined effect of CAR-expressing DCPs with PD-1 blockade. The

findings highlight the potential of CAR EVIR-engineered DCPs to enhance antitumor immune responses, offering new avenues for therapeutic strategies in melanoma treatment.

Overall, the manuscript is well written and the results are well presented and discussed. Here are some suggestions for the manuscript revision:

- 1) The number of experiments herein presented is high. To enhance clarity, consider providing a summary table of key results.
- 2) In some experiments the number of subjects is low and highly variable, is there an explanation for this? (i.e Fig1c and Ext Fig 1b); Could this may have an impact in the statistical power of the analyses, please explain this.
- 3) Please, begin each results section with a concise and clear description of the tumor model (e.g., melanoma, HER2+ colon cancer) and the treatment used (DCP-IL-12/EVIR or other variants) to help clarify the distinctions between experiments. Moreover, figures and extended data (extended figures) are sometimes separated within different results sections, please consider reorganising the figure panels and additional figures to make the results easier to read.
- 4) Please emphasise the added benefits of combining EVIR strategy with IL-12, both for antigen presentation and immune response. Summarise the key advantages of this combination at the end of the results to highlight its superiority.
- 5) The implication of this promising approach on CD8+ T cells functionality, could also be declined then in terms of an increase in the number of clonal profiles, or in clonal diversity? Do the authors have any observations on this?
- 6) Improve the discussion with the importance of the EVIR approach in managing tumor heterogeneity. Emphasise that, unlike ex vivo loading, EVIR allows the recognition of multiple tumor antigens in vivo, reducing the risk of immune escape.
- 7) What could be the clinical scenario of this novel approach? Can the author briefly comment on this also in the manuscript discussion, highlighting both potential impact and possible limitations?

Reviewer #3

(Remarks to the Author)

This paper is of great significance in the field of tumor immunotherapy as it presents novel concepts and approaches for dendritic cell progenitor (DCP)-based immunotherapy. It pioneers a cell therapy platform based on DCPs co-expressing IL-12 and EVIR. In contrast to traditional monocyte-derived dendritic cell (moDC) therapies, DCPs can more effectively differentiate into cDC1 within tumors and exhibit enhanced antigen uptake and presentation capabilities, thereby providing a novel solution to the limitations of traditional moDC therapies. The design of EVIR is highly innovative. It can specifically bind to antigens (such as GD2 and HER2) on the surface of tumor cells and their membrane particles, facilitating tumor antigen uptake and cross-presentation. This approach of using chimeric receptors to enhance antigen uptake has demonstrated favorable results in diverse tumor models.

Suggestions for Improvement.

1. In the study, the long-term safety and potential immune-related adverse events of engineered DCPs in vivo have not been fully evaluated. It is recommended to conduct long-term animal experiments to observe the impact of engineered DCPs on the overall health of animals, the function of important organs, and the homeostasis of the immune system, providing more comprehensive safety data for future clinical applications.
2. The interaction between other cellular components in the tumor microenvironment (such as macrophages, myeloid-derived suppressor cells, etc.) and engineered DCPs has not been deeply studied. Future research can further explore the roles and mechanisms of these cells in the immune response mediated by engineered DCPs and their impact on treatment efficacy, which is helpful to more comprehensively understand the complexity of the tumor immune microenvironment and the mechanism of action of engineered DCPs. Single-cell sequencing and other technologies can be used to deeply analyze the gene expression changes and cell-cell communication networks of different immune cells during the treatment process.

Minor points:

1. Has the image e in Figure 4 undergone any special processing? It looks quite different from conventional immunofluorescence images.
2. For different antigens targeted by EVIR, whether there are some antigen-specific influencing factors or mechanisms that have not been fully explored, such as the influence of the regulatory mechanism of antigen expression in different tumor types on the treatment effect.

Overall, this is a high-quality research paper with important academic value and clinical application prospects. Through rigorous experimental design, it provides new strategies and theoretical bases for the field of tumor immunotherapy. Although there are some deficiencies, through further research and improvement, it is expected to promote the clinical application of engineered DCPs in cancer treatment.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have addressed my queries and re-organized/amended the manuscript

Reviewer #2

(Remarks to the Author)

The authors answered all the questions reported in the previous report and further implemented the work with additional experiments and evidence. I am satisfied with the review and congratulate them on excellent research in the field of new cell therapies.

Reviewer #3

(Remarks to the Author)

Sufficient additional experiment and consideration has been given to the revise we requested, and we have no further requests.

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POINT BY POINT RESPONSE TO REVIEWERS

Response to all reviewers

We thank the reviewers for their expert analysis of our paper, which we have revised according to their suggestions.

Briefly, we have provided additional data and made text revisions in these areas:

- 1) As requested, we have reorganized the manuscript to improve its clarity and readability, namely, by focusing Figure 2 on DCPs and Figure 3 on their comparison with moDCs (previously, both DCPs and moDCs were shown in Figures 2 and 3).
- 2) We have added explanations and new data illustrating the effects of engineered DCPs on cells of the tumor microenvironment other than T cells (namely, macrophages) ([new Figure 2i-j](#)).
- 3) We have studied the antigen-presentation modality of DCP-EVIR. Briefly, our results indicate that DCP-EVIR largely function via cross-dressing of pre-formed, MHCI-loaded antigens ([new Extended Data Figure 4](#)).
- 4) We have performed a safety study to evaluate the impact of the engineered DCPs on commonly evaluated biomarkers of organ function. Overall, the data suggest that systemic administration of DCP-IL-12/EVIR only induces mild and transient liver toxicity without significant renal or long-term tissue damage, supporting the safety profile of this platform for future clinical translation ([new Figure 5](#)).
- 5) We have added additional data with human DCs, showing transduction of both human DCPs and moDCs and using two different donors to show EVIR-mediated internalization of EVs in both DCPs and moDCs ([new Figure 6b-c](#) and [6e-f](#)).

We have made additional changes to the text, as requested, and have detailed all revisions in the response letter to the individual referees, shown below.

Reviewer #1 (Remarks to the Author):

Ghasemi and colleagues describe the effect of engineering DC precursor (DCP) cells with an extracellular receptor (EVIR) specific to a surface tumor antigen, which internalizes tumor-derived EVs and promotes antigen presentation to boost anti-tumor activity of endogenous T cells. Outfitting murine DCPs with an EVIR enhances tumor control and the efficacy of PD-1 blockade in a couple of tumor models. Further, the authors demonstrate feasibility of generating human DCPs transduced with a lentiviral vector encoding an EV and show activation of CD8⁺ T cells specific to a peptide artificially presented on tumor cells. Overall, the study is well done and build further on their prior report describing the concept and mechanism of EVIRs in antigen-presenting cells.

Response: We thank the reviewer for the careful analysis of our paper and for their positive assessment of our work.

Addressing a few questions would help understand the mechanism and potential application of this approach.

1. In Figure 2, the effect of exogenous DCP on TILs is unclear. The authors show an overall increase in CD45⁺ cells and a modest increase in PD-1⁺ CD8⁺ T cells within those hematopoietic cells in some animals. However, an increase in effector (IFN γ ⁺, TNF α ⁺) TILs is not statistically significant, so the conclusions in the text are not supported. Further, there is no difference in frequency of those cells in mice receiving EVIR DCP with and without PD-1 blockade. These data need to be rectified or removed. Further, if PD1⁺ CD8⁺ T cells are only a few percent of total CD45⁺ cells (Fig 2c), what are the other major populations modulated by the DCP EVIRs?

Response: We thank the reviewer for this comment. In response to this criticism and that of another reviewer, who suggested the paper be reorganized for improving clarity, we have now re-worked Figures 2 and 3 by focusing Figure 2 on DCPs and Figure 3 on moDCs and their comparison with DCPs.

In the revised Figure 2, we now present three independent DCP experiments, all involving cells expressing IL-12 with or without the EVIR. (These experiments were present in the original version of the manuscript, but we have added further analyses, as suggested.)

In all experiments, the addition of the EVIR to IL-12 provided clear additive therapeutic benefits (in terms of tumor growth delay), demonstrating the role of the EVIR in the anti-tumoral response. In our view, this is significant if one considers that IL-12 is, per se, a potent anti-tumoral cytokine.

In the first experiment (Figure 2a-c), flow cytometry analysis of the tumors revealed IL-12-dependent promotion of CD8⁺ T cell infiltration and EVIR-dependent enhancement of their activation, which may have cooperatively contributed to the improved response (**Figure 2c**).

In the second experiment (now shown in **Figure 2d-e**), we observed that the EVIR-dependent enhancement of CD8⁺ T cell activation was variable and clear only in a fraction of the tumors (**Extended Data Figure 2a-b**), as rightfully noted by the reviewer. We now comment in the Results section that this less significant effect of the EVIR on T cell activation may be due to the longer time interval between the last DCP infusion and tumor analysis in this second experiment compared to the first one (14 versus 8 days). For transparency, we prefer to show these data, which are now presented in Extended Data Figure 2a-b.

Overall, the results of these two independent experiments show EVIR-dependent contribution to tumor growth inhibition, which was associated with enhanced CD8⁺ T cell activation in at least a fraction of the tumors. It should be stressed that IL-12 is a major promoter of T cell responses (see Figure 2a-c) and the effect of the EVIR in Extended Data Figure 2a-b is shown in combination with IL-12. We hope that clarifications have addressed the concern of the reviewer.

The reviewer then asked “what are the other major populations modulated by the DCP EVIRs?”. We have now added additional analyses to address this important question, now shown in Figure 2f-j, which illustrates a third, independent DCP experiment (CD8 T cell depletion study, already present in the original version of the manuscript).

In agreement with the study shown in Figure 2a-c, immune profiling of the tumors indicated that DCP treatment increased the relative proportions of CD8⁺ T cells in the tumors. In addition, the DCPs greatly increased M1-like (inflammatory) macrophages while decreasing M2-like (pro-tumoral) macrophages, compared to tumors of vehicle-treated mice (**new data in Figure 2i-j**). These effects on macrophage polarization illustrate the reprogramming of the tumor microenvironment induced by the engineered DCPs. Interestingly, CD8⁺ T cell depletion did not reverse DCP-induced M1-like macrophage programming, potentially explaining why tumor growth was only partially rescued (see Figure 2f-h). Overall, these data suggest that, beyond promoting CD8⁺ T cell infiltration and activation, DCP-IL-12/EVIR plus α PD-1 skews macrophages toward an anti-tumoral, M1-like phenotype, likely in a mechanism that is dependent on IL-12.

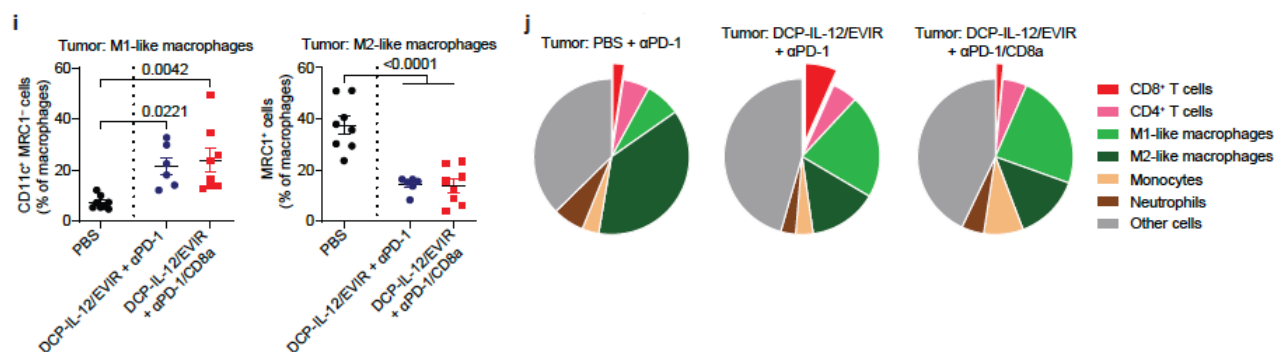


Figure 2i-j. Effects of DCPs on the tumor microenvironment. i, Frequency of the indicated macrophage states in the tumors (mean $\pm$ s.e.m.; DCP-IL-12-P2A-EVIR + α PD-1, n=6 mice; other groups, n=8 mice). Statistical analysis by one-way ANOVA with Tukey's multiple comparison test. j, Pie charts showing the hematopoietic cell composition of the tumors. The area of each cell population corresponds to the mean percentage value in the total CD45⁺ cells (DCP-IL-12-P2A-EVIR + α PD-1, n=6 mice; other groups, n=8 mice).

2. Comparison of DCP and moDC in Fig. 2d-g is interesting. Is the limited effect of moDC ascribed to their poor trafficking/infiltration into tumors? What is the phenotype and differentiation status of DCP vs moDC in tumors?

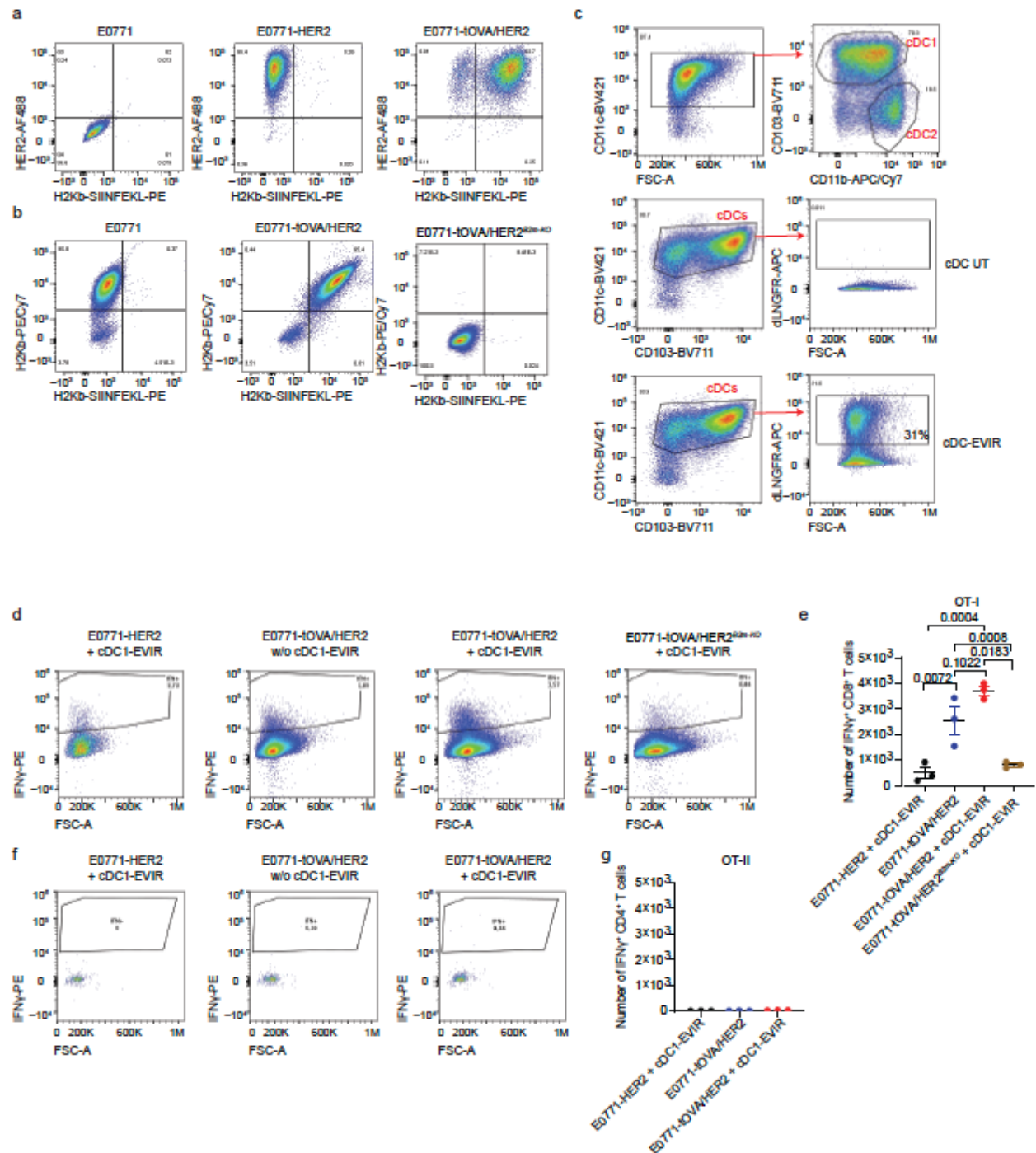
Response: The reviewer is correct. We have extensively studied the trafficking and fate of both DCPs and moDCs in our previous paper (Ghasemi et al., Nat Cancer 2024) and showed that, after systemic injection in the general circulation, DCPs efficiently engraft in mice to generate cDCs, whereas moDCs engraft poorly and retain their moDC phenotype. We have now clarified this point in the Discussion: *"In our study, moDCs engineered to co-express the EVIR and IL-12 showed limited efficacy and failed to sensitize tumors to PD-1 blockade. [...] we have previously shown that moDCs exhibit lower engraftment compared to DCPs²⁴, which may limit both the duration of antigen uptake and presentation and the magnitude and temporal window of local IL-12 release. In contrast, DCPs efficiently engraft in tumor and lymph nodes and mainly differentiate into cDC1, a cDC subset involved in priming or boosting tumor-reactive CD8⁺ T cells⁴⁴⁻⁴⁶. Notably, cDC1-derived IL-12 is biologically relevant, as it more efficiently engages T and NK cells in the anti-tumor response compared to IL-12 secreted by other myeloid cells^{42,47}. Overall, our data suggest that DCPs may offer advantages over moDCs in terms of both antigen presentation and IL-12-mediated T cell activation, which are paramount for robust anti-tumor immunity."*

3. Can DCP EVIRs cross-present tumor antigens and invoke CD4⁺ T cell responses? The authors did some studies in the previous paper but it is unclear whether DCP (either human or mouse) can be used to stimulate both subsets of T cells.

Response: Motivated by this question, we have studied the antigen-presentation modality of DCP-EVIR. An entire new figure has been added to the manuscript (**new Extended Data Figure 4a-g**) that reports data obtained with both OT-I cells (to study MHCI-restricted presentation to CD8⁺ T cells) and OT-II cells (to study MHCII-restricted presentation to CD4⁺ T cells). This study involved the generation of genetically-modified cancer cells suited to distinguish cross-dressing from cross-presentation or direct presentation of a model antigen.

Briefly, our results indicate that DCP-EVIR largely function via cross-dressing of pre-formed, MHCI-loaded antigens (as also shown for human DCPs in Figure 6) and lack the ability to directly present antigens to CD4⁺ T cells. This is likely because the cancer cells do not express MHCII and, therefore, cannot cross-dress the DCs with preformed MHCII-peptide complexes.

While we could positively verify the occurrence of MHC I cross-dressing but not MHC II antigen presentation *in vitro*, it should be stressed that our cell therapy platform also involves co-expression of IL-12 and the ability of the DCP to differentiate into both cDC1 and cDC2 *in vivo*. These features may broaden the mode of action of the engineered DCPs in tumor-bearing mice.



Extended Data Figure 4. DCP-EVIR induce CD8 $^{+}$ T cell responses via antigen cross-dressing but do not activate CD4 $^{+}$ T cells

a, Flow cytometry analysis of human HER2 and H-2Kb–SIINFEKL expression in engineered E0771 cells. **b**, Flow cytometry analysis of H-2Kb and H-2Kb–SIINFEKL expression in E0771-OVA/HER^{B2m-KO} cells confirming genetic ablation of MHC-I. **c**, Flow cytometry analysis of cDC1 (CD11c⁺CD103⁺) and cDC2 (CD11c⁺CD11b⁺CD103⁻) markers and EVIR expression (measured by dLNGFR staining) in transduced cDCs. UT, untransduced. **d**, Representative flow cytometry analysis of intracellular IFN γ in OT-I CD8⁺ T cells after co-culture with cDC1-EVIR in the presence of the indicated cancer cell lines. **e**, Quantification of IFN γ ⁺ OT-I cells from d (mean $\pm$ s.e.m.; n = 3 wells per condition). Statistical analysis by one-way Anova with Tukey's multiple comparison test. **f**, Representative flow cytometry dot plots of intracellular IFN γ in OT-II CD4⁺ T cells after co-culture with cDC1-EVIR in the presence of the indicated cancer cell lines. **g**, Quantification of IFN γ ⁺ OT-II cells from f (mean $\pm$ s.e.m.; n = 3 wells per condition).

Each data point represents an independent co-culture well.

We have addressed the limitations in our current understanding of the mode of action of the DCP-EVIR in the Discussion, as follows: *“Although the precise mechanisms underlying the anti-tumor response induced by DCPs expressing the EVIR and IL-12 remain to be fully identified, our results provide several insights. We show that EVIR expression by DCPs contributes to eliciting T cell responses in combination with IL-12. This is illustrated, for example, by the increased numbers of activated, intra-tumoral CD8⁺ T cells, which may mediate the tumor response to PD-1 blockade 48. Indeed, depletion of CD8⁺ T cells partially rescued tumor growth in DCP-treated mice, implicating CD8⁺ T cells in the tumor response. In contrast, depleting CD8⁺ T cells in mice treated with DCPs engineered to co-express FLT3L and IL-12 – but not the EVIR – did not impair the anti-tumoral response 24, suggesting a specific role for the EVIR in the cross-priming of CD8⁺ T cells. Additionally, DCP-derived IL-12 reprograms the tumor microenvironment by activating NK cells, enhancing IFN γ production, and polarizing macrophages toward an anti-tumoral, M1-like phenotype 24. Given the roles of various immune cells and their bioactive mediators in orchestrating anti-tumor immunity, further studies will be required to identify the cellular and molecular underpinnings of the tumor response mediated by EVIR-engineered DCPs. While DCP-IL-12/EVIR elicited OVA-specific T cells in the spleen of treated mice without the need for ex vivo OVA protein pulsing, it remains to be seen whether the engineered DCPs alter the number and composition of T cell clonotypes in the periphery and tumors of treated mice.”.*

3.1. For the clinical translation, do the authors envision using allogeneic (off-the-shelf) EVIR-DCP or autologous? If the latter, it is unclear whether cross-dressing shown in Fig. 5 would contribute significantly to the intended function of DCPs. If the former, would allo-immunogenicity of these DCP be a problem or the authors envision HLA editing?

Response: Thanks for these sensible comments. We currently envision an autologous approach based on engineered DCPs, most likely in combination with chemo-immunotherapy to improve the response. In addition, we are also developing a platform of iPSC-derived DCs with disrupted endogenous HLA, in which the EVIR will be used to facilitate DC cross-dressing with patient-derived HLA-peptides. These considerations have now been included in the Discussion, as follows: *“The EV internalization capacity of EVIR-engineered human DCPs supports the clinical potential of our platform. Our preclinical studies suggest mild and transient toxicity of DCP-IL-12/EVIR in mice, which is consistent with the short-lived engraftment of the engineered cells 24. To further mitigate potential safety*

concerns associated with constitutive IL-12 expression, non-integrating gene delivery approaches such as mRNA electroporation or lipid nanoparticle (LNP)-mediated mRNA delivery ⁵⁸ may allow for transient cytokine expression. Human DCP-IL-12/EVIR may be conveniently and efficiently produced from mobilized peripheral blood for autologous cell therapy applications. Alternatively, to facilitate scalable manufacturing of engineered DCPs, induced pluripotent stem cell (iPSC)-derived DCPs could be used for standardized, large-scale production, while the development of allogeneic "off-the-shelf" DCPs combined with genome editing strategies, such as HLA knockout or suppression ⁵⁹, may further broaden clinical applicability. Notably, expression of the EVIR, which facilitates DC cross-dressing with tumor-derived p-MHCI complexes ¹⁹, may surrogate for the absence of endogenous MHCI molecules in the DCs. Together, these approaches position DCP-EVIR as a promising platform for the development of next-generation DC-based immunotherapies for cancer”.

Minor point

1. In Fig. 5d, statistical tests with n=2 is discouraged.

Response: We have now added new data to this figure (now Fig. 6). In particular, we now show transduction of both human DCPs and moDCs (**new Fig. 6b-c**) and used two different donors to show EVIR-mediated internalization of EVs in both DCP progeny cells and moDCs (**new Fig. 6e-f**). The latter experiments show that the EVIR promotes GD2-dependent internalization of EVs in two different donors and two different DC populations, thus confirming the robustness of the finding.

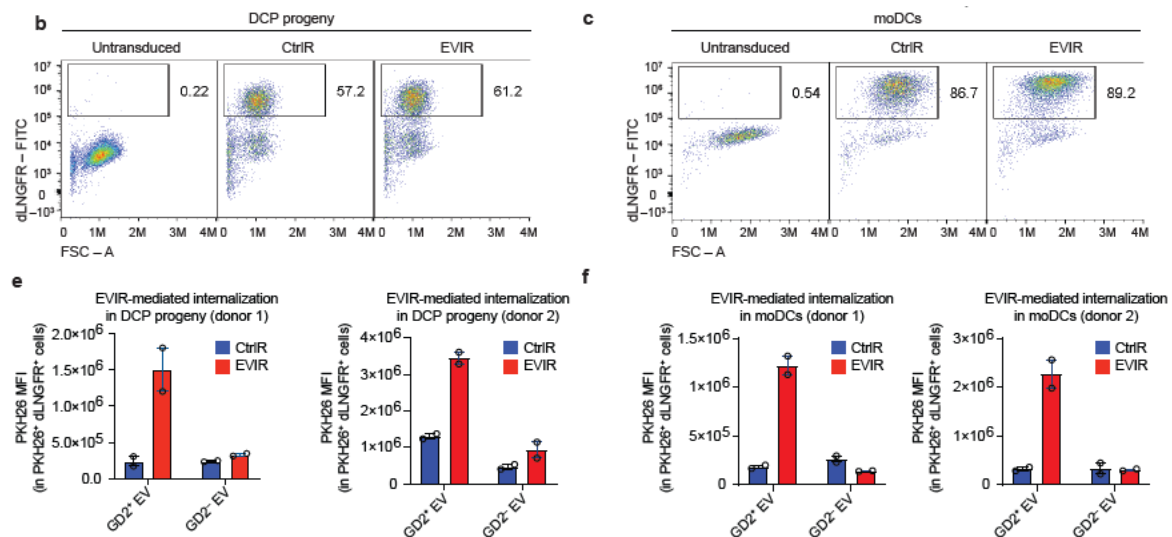


Figure 6. αGD2 EVIR promotes antigen internalization and presentation by human DCPs

b-c, Representative dot plots showing the expression of CtrlR and EVIR in human DCP progeny and moDCs. **e-f**, PKH26 MFI in transduced human DCP progeny (**e**) or human moDCs (**f**) measured by flow cytometry from two independent donors and two biological replicates for each (mean ± s.e.m.).

With respect to former Fig. 5d (now Fig. 6h-i), we agree that statistical tests with $n=2$ may be discouraged (some conditions in this experiment have $n=3$). However, upon consultation with a biostatistician, we concluded that two-way Anova tests with post-hoc correction for multiple comparisons may be conducted even if some groups have $n=2-3$ (provided that groups sizes are similar, which is the case here). So, in this specific case, we would prefer to retain the statistical analysis. However, we have carefully reassessed the text pertaining to this experiment to make sure that data were not overinterpreted, as follows: *“Flow cytometry analysis of IFN γ in CD8⁺ T cells indicated that co-expression of the EVIR and hIL-12 by the DCP progeny – and, to a lesser extent, moDCs – increased the magnitude of the T cell response in a GD2 and HLA-A2–dependent manner, demonstrating efficient cross-dressing conducive to T cell stimulation (Figure 6h-i). Collectively, these results demonstrate that dual EVIR and hIL-12 expression enhances antigen internalization and presentation by human DCs.”*

Reviewer #2 (Remarks to the Author):

Ali Ghasemi et al., explore the use of engineered dendritic cell progenitors (DCPs) expressing a CAR construct known as EVIR for cancer therapy, specifically targeting solid tumors like melanoma. By utilizing murine models, the efficacy of these engineered DCPs in activating immune responses, facilitating antigen transfer, and their interactions with antigen-specific T lymphocytes was analyzed. The study also explores the combined effect of CAR-expressing DCPs with PD-1 blockade. The findings highlight the potential of CAR EVIR-engineered DCPs to enhance antitumor immune responses, offering new avenues for therapeutic strategies in melanoma treatment.

Overall, the manuscript is well written and the results are well presented and discussed.

Response: We thank the reviewer for the careful analysis of our paper and for their positive assessment of our work.

Here are some suggestions for the manuscript revision:

1) The number of experiments herein presented is high. To enhance clarity, consider providing a summary table of key results.

Response: We thank the reviewer for their advice. We have now reorganized the manuscript to improve its clarity, namely, by focusing Figure 2 on DCPs and Figure 3 on their comparison with moDCs (previously, both DCPs and moDCs were shown in Figures 2 and 3). We have also revised the text to improve clarity. As per editorial guidelines, it is not possible to provide a summary table of the results. We hope, however, that the reviewer will find the revised paper better organized and easy to read.

2) In some experiments the number of subjects is low and highly variable, is there an explanation for this? (i.e Fig1c and Ext Fig 1b); Could this may have an impact in the statistical power of the analyses, please explain this.

Response: The reviewer here refers to a sample that was used as a negative control for mCherry staining (MC38-GD2 cancer cells). In principle, that sample could have been omitted, as it is not part of the experimental groups (being a fluorescence control only used to set a threshold for negative mCherry fluorescence). In fact, it was not used for statistical analysis. Yet, it is included for reference. In general, all experimental groups shown in the manuscript involve several independent mice, ranging from 3 to 10 in different experiments (typically 7-10 per group).

3) Please, begin each results section with a concise and clear description of the tumor model (e.g., melanoma, HER2+ colon cancer) and the treatment used (DCP-IL-12/EVIR or other variants) to help clarify the distinctions between experiments. Moreover, figures and extended data (extended figures) are sometimes separated within different results

sections, please consider reorganising the figure panels and additional figures to make the results easier to read.

Response: Thanks for these comments. We have included brief but precise descriptions of the various experiments before the description of the results. We also show a schematic of the experimental outline for each experiment, which helps the reader with the experimental design. Also, as stated in response to point 1 above, we have now reorganized the manuscript to improve its clarity, namely, by focusing Figure 2 on DCPs and Figure 3 on their comparison with moDCs.

4) Please emphasise the added benefits of combining EVIR strategy with IL-12, both for antigen presentation and immune response. Summarise the key advantages of this combination at the end of the results to highlight its superiority.

Response: We have included summary sentences at the end of each section of the Results, and broadened the Discussion section to emphasize the key results of our study.

5) The implication of this promising approach on CD8+ T cells functionality, could also be declined then in terms of an increase in the number of clonal profiles, or in clonal diversity? Do the authors have any observations on this?

Response: Thanks for these comments. We currently lack data on T cell clonality, although the data in Figure 4c-d (left panels) show that the engineered DCPs plus anti-PD1 increase OVA-specific T cells, compared to vehicle/anti-PD1, without ex vivo OVA loading. While this result is indicative of the elicitation of tumor-specific T cells by the engineered DCPs, we have now stressed in the Discussion that further studies should address the clonal composition of the T cell repertoire in the tumors, as follows: *“Given the roles of various immune cells and their bioactive mediators in orchestrating anti-tumor immunity, further studies will be required to identify the cellular and molecular underpinnings of the tumor response mediated by EVIR-engineered DCPs. While DCP-IL-12/EVIR elicited OVA-specific T cells in the spleen of treated mice without the need for ex vivo OVA protein pulsing, it remains to be seen whether the engineered DCPs alter the number and composition of T cell clonotypes in the periphery and tumors of treated mice.”*

6) Improve the discussion with the importance of the EVIR approach in managing tumor heterogeneity. Emphasise that, unlike ex vivo loading, EVIR allows the recognition of multiple tumor antigens in vivo, reducing the risk of immune escape.

Response: Thanks for the suggestion, this has been emphasized in the Discussion as follows: *“In contrast, our strategy uses engineered DCPs to enforce tumor antigen acquisition in vivo and amplify potentially broad and diverse immune effector mechanisms. This approach relies on the bait molecule targeted by the EVIR (e.g., GD2) to facilitate tumor EV/particle uptake, but not to specifically elicit direct cancer cell killing or phagocytosis. This strategy is less likely to induce immune escape through downregulation of the bait molecule*

or clonal selection of bait molecule-negative cells, and may result in the destruction of both bait molecule-positive and -negative cancer cells.”.

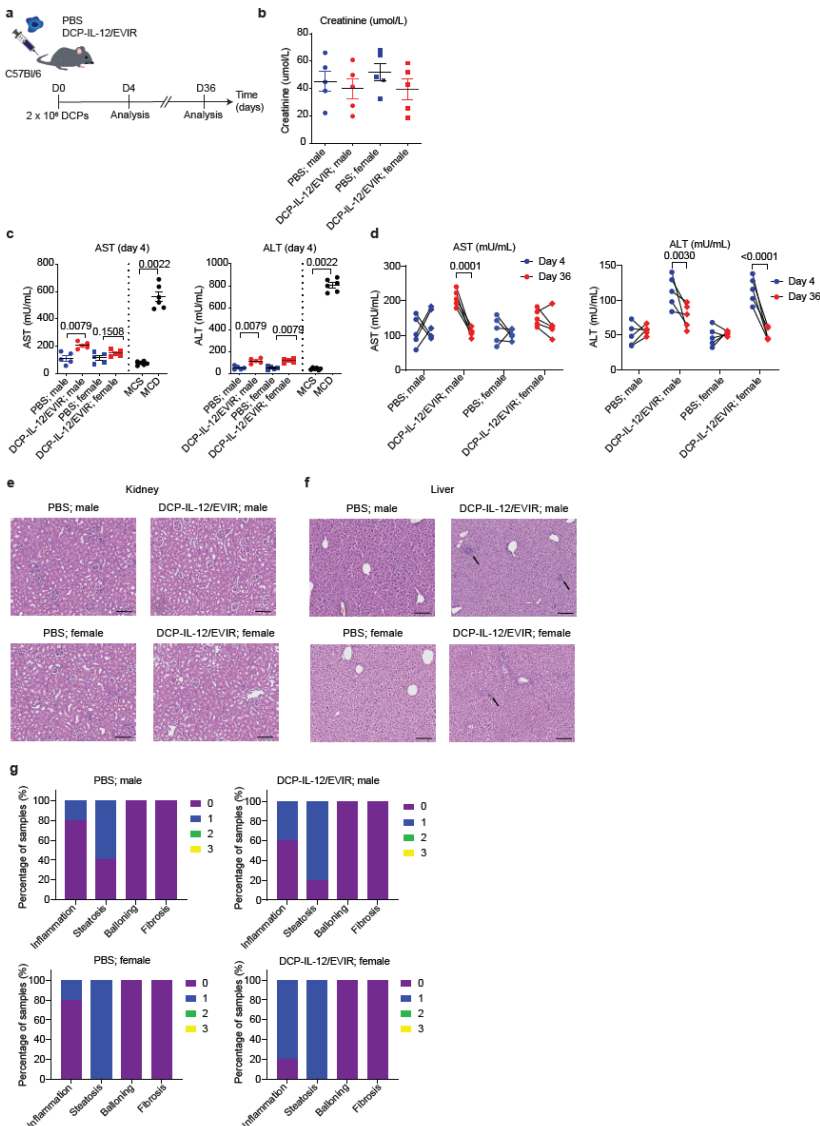
7) What could be the clinical scenario of this novel approach? Can the author briefly comment on this also in the manuscript discussion, highlighting both potential impact and possible limitations?

Response: We currently envision an autologous approach based on engineered DCPs, most likely in combination with chemo-immunotherapy to improve the response. In addition, we are also developing a platform of iPSC-derived DCs with disrupted endogenous HLA, in which the EVIR will be used to facilitate DC cross-dressing with patient-derived HLA-peptides. These considerations have now been included in the Discussion, as follows: *“The EV internalization capacity of EVIR-engineered human DCPs supports the clinical potential of our platform. Our preclinical studies suggest mild and transient toxicity of DCP-IL-12/EVIR in mice, which is consistent with the short-lived engraftment of the engineered cells ²⁴. To further mitigate potential safety concerns associated with constitutive IL-12 expression, non-integrating gene delivery approaches such as mRNA electroporation or lipid nanoparticle (LNP)-mediated mRNA delivery ⁵⁸ may allow for transient cytokine expression. Human DCP-IL-12/EVIR may be conveniently and efficiently produced from mobilized peripheral blood for autologous cell therapy applications. Alternatively, to facilitate scalable manufacturing of engineered DCPs, induced pluripotent stem cell (iPSC)-derived DCPs could be used for standardized, large-scale production, while the development of allogeneic “off-the-shelf” DCPs combined with genome editing strategies, such as HLA knockout or suppression ⁵⁹, may further broaden clinical applicability. Notably, expression of the EVIR, which facilitates DC cross-dressing with tumor-derived p-MHCI complexes ¹⁹, may surrogate for the absence of endogenous MHCI molecules in the DCs. Together, these approaches position DCP-EVIR as a promising platform for the development of next-generation DC-based immunotherapies for cancer.”.*

Reviewer #3 (Remarks to the Author):

This paper is of great significance in the field of tumor immunotherapy as it presents novel concepts and approaches for dendritic cell progenitor (DCP)-based immunotherapy. It pioneers a cell therapy platform based on DCPs co-expressing IL-12 and EVIR. In contrast to traditional monocyte-derived dendritic cell (moDC) therapies, DCPs can more effectively differentiate into cDC1 within tumors and exhibit enhanced antigen uptake and presentation capabilities, thereby providing a novel solution to the limitations of traditional moDC therapies. The design of EVIR is highly innovative. It can specifically bind to antigens (such as GD2 and HER2) on the surface of tumor cells and their membrane particles, facilitating tumor antigen uptake and cross-presentation. This approach of using chimeric receptors to enhance antigen uptake has demonstrated favorable results in diverse tumor models.

Response: We thank the reviewer for the careful analysis of our paper and for their positive assessment of our work.



Suggestions for Improvement.

1. In the study, the long-term safety and potential immune-related adverse events of engineered DCPs in vivo have not been fully evaluated. It is recommended to conduct long-term animal experiments to observe the impact of engineered DCPs on the overall health of animals, the function of important organs, and the homeostasis of the immune system, providing more comprehensive safety data for future clinical applications.

Response: We thank the reviewer for this important suggestion. We have now performed a safety study to evaluate the impact of the engineered DCPs on

commonly evaluated biomarkers of organ function. Briefly, the mice were intravenously injected with either PBS or DCP-IL-12/EVIR and monitored over a 36-day period. Liver function was evaluated by measuring serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT), whereas kidney function was evaluated by measuring creatinine levels. These parameters were assessed both at day 4, to capture early effects, and day 36, the study endpoint. We also performed pathology analysis of liver and kidney at day 36. Overall, the data suggest that systemic administration of DCP-IL-12/EVIR only induces mild and transient liver toxicity without significant renal or long-term tissue damage, supporting the safety profile of this platform for future clinical translation. The data are shown in **new Figure 5**.

Figure 5 (shown above). αGD2 DCP-IL-12/EVIR do not induce liver or kidney toxicity in mice

a, Procedure to study liver and kidney toxicity following systemic DCP-IL-12/EVIR inoculation in tumor-free mice. **c**, Serum creatinine at day 4 post-DCP injection (mean ± s.e.m.; $n = 5$ mice). **b**, Serum AST and ALT at day 4 post-DCP injection (mean ± s.e.m.; $n = 5$ mice, except for MCS and MCD, $n = 6$ male mice). The mice in the MCS and MCD groups originated from an independent experiment. Statistical analysis by two-tailed Mann-Whitney test comparing mice with same sex. **d**, Serum AST and ALT at day 4 and 36 post-injection (mean ± s.e.m.; $n = 5$ mice). Statistical analysis by two-tailed Mann-Whitney test comparing mice with same sex. **e**, Representative H&E-stained kidney sections of mice from the indicated treatment group. **f**, Representative H&E-stained liver sections of mice from the indicated treatment group. Arrows indicate areas of mild inflammatory infiltration in liver. **g**, Frequency of mice exhibiting histological features of inflammation, steatosis, ballooning, or fibrosis in liver sections ($n = 5$ mice).

Each data point in all panels represents one sample from an independent mouse except in **g**, in which values represent the percentage of mice per group showing the indicated pathology grade.

2.The interaction between other cellular components in the tumor microenvironment (such as macrophages, myeloid-derived suppressor cells, etc.) and engineered DCPs has not been deeply studied. Future research can further explore the roles and mechanisms of these cells in the immune response mediated by engineered DCPs and their impact on treatment efficacy, which is helpful to more comprehensively understand the complexity of the tumor immune microenvironment and the mechanism of action of engineered DCPs. Single-cell sequencing and other technologies can be used to deeply analyze the gene expression changes and cell-cell communication networks of different immune cells during the treatment process.

Response: We agree that future studies should better address the immunological effects of the DCPs. For now, we have included additional data obtained by flow cytometry analysis of the tumors from the experiment in which we studied the effects of CD8⁺ T cell depletion (now shown in Figure 2f-h). Immune profiling of the B16F10 melanomas indicated that DCP treatment increased the relative proportions of CD8⁺ T cells in the tumors (as also shown in other experiment). In addition, we now show that the DCPs greatly increased M1-like (inflammatory) macrophages while decreasing M2-like (pro-tumoral) macrophages, compared to tumors of vehicle-treated mice (**new data in Figure 2i-j**). These effects on macrophage polarization illustrate the reprogramming of the tumor microenvironment induced by the engineered DCPs. Interestingly, CD8⁺ T cell depletion did not reverse DCP-induced M1-like macrophage programming, potentially explaining why tumor growth was

only partially rescued (see Figure 2h). Overall, these data suggest that, beyond promoting CD8⁺ T cell infiltration and activation, DCP-IL-12/EVIR plus αPD-1 skews macrophages toward a an anti-tumoral, M1-like phenotype, likely in a mechanism that is dependent on IL-12.

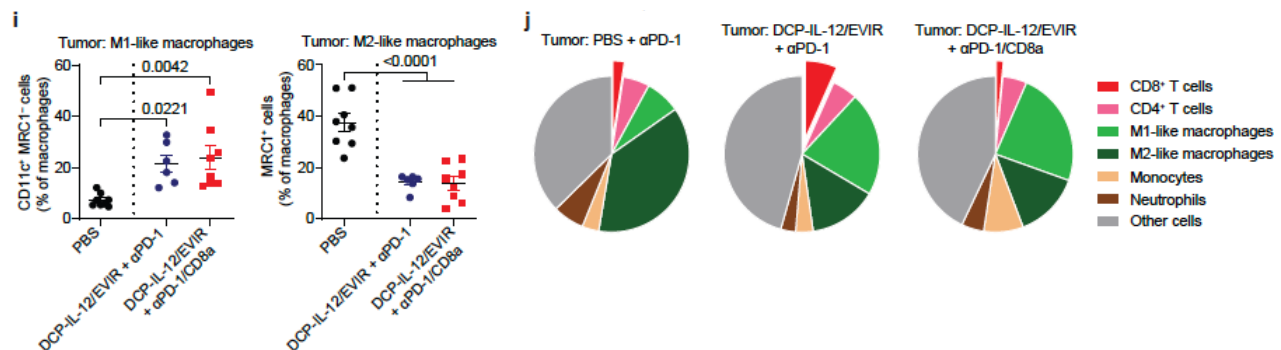


Figure 2i-j. Effects of DCPs on the tumor microenvironment. **i**, Frequency of the indicated macrophage states in the tumors (mean ± s.e.m.; DCP-IL-12-P2A-EVIR + αPD-1, n=6 mice; other groups, n=8 mice). Statistical analysis by one-way ANOVA with Tukey's multiple comparison test. **j**, Pie charts showing the hematopoietic cell composition of the tumors. The area of each cell population corresponds to the mean percentage value in the total CD45⁺ cells (DCP-IL-12-P2A-EVIR + αPD-1, n=6 mice; other groups, n=8 mice).

Minor points:

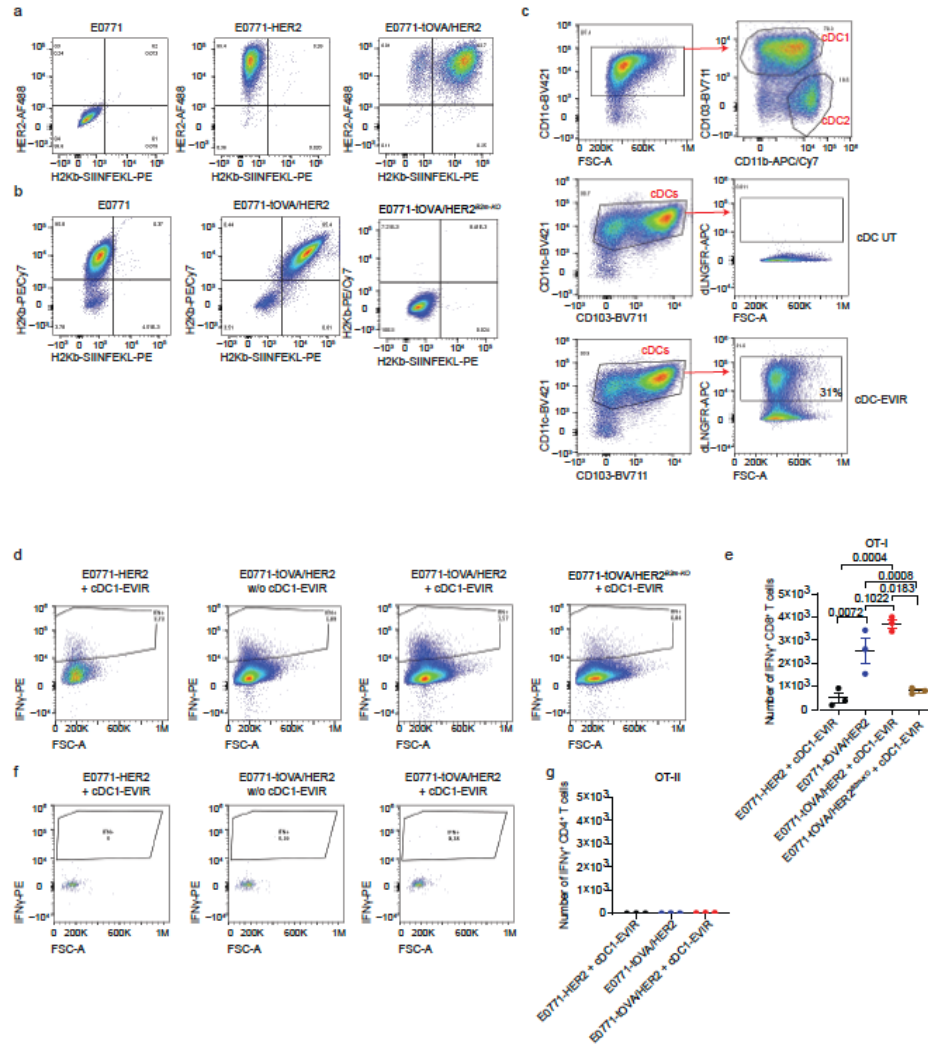
1.Has the image e in Figure 4 undergone any special processing? It looks quite different from conventional immunofluorescence images.

Response: We thank the reviewer for this comment. We have now improved the resolution of the images, which show GD2 staining together with nuclear staining in size-matched tumors. The appearance of GD2 staining, which is largely membrane-associated, is consistent with other reports in the literature.

2.For different antigens targeted by EVIR, whether there are some antigen-specific influencing factors or mechanisms that have not been fully explored, such as the influence of the regulatory mechanism of antigen expression in different tumor types on the treatment effect.

Response: We thank the reviewer for requesting additional information on the mode of action of the EVIR. We have now added additional data that investigate the mode of antigen presentation by the DCP-EVIR.

An entire new figure has been added to the manuscript (**new Extended Data Figure 4a-g**) that reports data obtained with both OT-I cells (to study MHCI-restricted presentation to CD8⁺ T cells) and OT-II cells (to study MHCII-restricted presentation to CD4⁺ T cells). This



study involved the generation of genetically-modified cancer cells suited to distinguish cross-dressing from cross-presentation or direct presentation of a model antigen.

Briefly, our results indicate that DCP-EVIR largely function via cross-dressing of pre-formed, MHC-I-loaded antigens (as also shown for human DCPs in Figure 6) and lack the ability to directly present antigens to CD4⁺ T cells. This is likely because the cancer cells do not express MHCII and, therefore, cannot cross-dress the DCs

with preformed MHCII-peptide complexes.

While we could positively verify the occurrence of MHC-I cross-dressing but not MHCII antigen presentation in vitro, it should be stressed that our cell therapy platform also involves co-expression of IL-12 and the ability of the DCP to differentiate into both cDC1 and cDC2 in vivo. These features may broaden the mode of action of the engineered DCPs in tumor-bearing mice.

Extended Data Figure 4. DCP-EVIR induce CD8⁺ T cell responses via antigen cross-dressing but do not activate CD4⁺ T cells

a, Flow cytometry analysis of human HER2 and H-2Kb–SIINFEKL expression in engineered E0771 cells. **b**, Flow cytometry analysis of H-2Kb and H-2Kb–SIINFEKL expression in E0771-OVA/HER^{B2m-KO} cells confirming genetic ablation of MHC-I. **c**, Flow cytometry analysis of cDC1 (CD11c⁺CD103⁺) and cDC2 (CD11c⁺CD11b⁺CD103⁺) markers and EVIR expression (measured by dLNFR staining) in transduced cDCs. UT, untransduced. **d**, Representative flow cytometry analysis of intracellular IFN γ in OT-I CD8⁺ T cells after co-culture with cDC1-EVIR in the presence of the indicated cancer cell lines. **e**, Quantification of IFN γ ⁺ OT-I cells from d (mean $\pm$ s.e.m.; n = 3 wells per condition). Statistical analysis by one-way Anova with Tukey's multiple comparison test. **f**, Representative flow cytometry dot plots of intracellular IFN γ in OT-II CD4⁺ T cells after co-culture with

cDC1-EVIR in the presence of the indicated cancer cell lines. **g**, Quantification of IFN γ ⁺ OT-II cells from **f** (mean $\pm$ s.e.m.; n = 3 wells per condition). Each data point represents an independent co-culture well.

Overall, this is a high-quality research paper with important academic value and clinical application prospects. Through rigorous experimental design, it provides new strategies and theoretical bases for the field of tumor immunotherapy. Although there are some deficiencies, through further research and improvement, it is expected to promote the clinical application of engineered DCPs in cancer treatment.

Response: We thank the reviewer for these very encouraging comments.

POINT BY POINT RESPONSE TO REVIEWERS

Reviewer #1 (Remarks to the Author):

The authors have addressed my queries and re-organized/amended the manuscript

We thank the reviewer for their expert advice and suggestions, which have improved our manuscript.

Reviewer #2 (Remarks to the Author):

The authors answered all the questions reported in the previous report and further implemented the work with additional experiments and evidence. I am satisfied with the review and congratulate them on excellent research in the field of new cell therapies.

We thank the reviewer for their expert advice and suggestions, which have improved our manuscript.

Reviewer #3 (Remarks to the Author):

Sufficient additional experiment and consideration has been given to the revise we requested, and we have no further requests.

We thank the reviewer for their expert advice and suggestions, which have improved our manuscript. While carefully checking the raw data and corresponding figures, we discovered one error in Fig. 5c-d (AST and ALT values at day 4 and 36). The error was due to the incorrect transfer of some raw numerical data from the spreadsheet generated by the plate reader to the final data table used to generate the graphs (with the identified treatment groups). The error has now been corrected. While the correction slightly changed several numerical values in the charts, it did not alter any of the biological effects (the changes are minimal). The observation that DCPs cause only mild and transient elevation of AST and ALT remains valid, so we haven't modified the results and conclusions of that experiment.

