EMBO reports

Pericyte stem cells induce Ly6G+ cell accumulation and immunotherapy resistance in pancreatic cancer

Zhichong Wu, Kevin Thierry, Sophie Bachy, Xinyi Zhang, Pia Gamradt, Hector Hernandez-Vargas, Ivan Mikaelian, Laurie Tonnon, Roxanne Pommier, Yajie Zhao, Philippe Bertolino, and Ana Hennino
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Corresponding author(s): Ana Hennino (ana.hennino@inserm.fr)

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1st Editorial Decision **22nd Nov 2022**

Dear Dr. Hennino,

Thank you for transferring your manuscript to EMBO reports. I now went through your manuscript and the referee (advisor) report from The EMBO Journal (attached below). The referee acknowledges that the revised manuscript has improved, but explanations for several of the concerns mentioned by reviewers have not been provided.

EMBO reports emphasizes novel functional over detailed mechanistic insight, but asks for strong in vivo relevance of the findings, and clear experimental support of the major conclusions. Thus, we will not require addressing points regarding more mechanistic details experimentally. However, it will be necessary that in a further revised manuscript you address all points questioning the main conclusions of the study, and all technical concerns, or points regarding the experimental designs, model systems used, or data presentation.

I thus invite you to further revise your manuscript with the understanding that all remaining concerns of the advisor must be addressed in the revised manuscript or in a detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of another (final) round of review.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

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3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14693178/authorguide). Please insert page numbers in the checklist to indicate where the requested information can be found in the manuscript. The completed author checklist will also be part of the RPF.

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5) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq and array data) are deposited in an appropriate public database. This is now mandatory (like the COI statement). If no primary datasets have been deposited in any database, please state this in this section (e.g. 'No primary datasets have been generated and deposited').

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The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Methods) that follows the model below. Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

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Moreover, I have these editorial requests:

6) We now request the publication of original source data with the aim of making primary data more accessible and transparent to the reader. Our source data coordinator will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

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If n<5, please show single datapoints for diagrams.

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11) We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and add a statement declaring your competing interests. Please name that section 'Disclosure and Competing Interests Statement' and add it after the author contributions section.

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Title page - Abstract - Introduction - Results - Discussion - Materials and Methods - Data availability section (DAS) - Acknowledgements - Disclosure and Competing Interests Statement - References - Figure legends - Expanded View Figure legends

13) Please make sure that all the funding information is also entered into the online submission system and is complete and similar to the one in the manuscript text file (acknowledgements).

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15) Please upload the 'key resources table' as 'Reagents and Tools table'. I have attached templates for that in word or excel format. Please upload the filled in table to the manuscript tracking system as a 'Reagent Table' file. The example below shows how the table will display in the published article and includes examples of the type of information that should be provided for the different categories of reagents and tools. Please list your reagents/tools using the categories provided in the template and do not add additional subheadings to the table. Reagents/tools that do not fit in any of the specific categories can be listed under "Other":
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Kind regards,

Achim

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

This submission was previously reviewed and revised for a journal outside of EMBO press. The authors investigated the CD106+ pericyte stem cell (PeSC) subtype that contribute to the accumulation of Ly6G+ cells in pancreatic cancer. The authors demonstrate the early arrival of CD106+ CD24+ CD44+ population during pancreatitis and PanIN initiation. They provide a pointwise response to the reviewer's comments from the previous journal. The work is interesting; however, there are several major issues that remain unresolved or have not been addressed.

1) There appears to be two conceptual inconsistencies in this manuscript. First, the authors demonstrate in-vitro that PeSCs promote cancer cell growth and proliferation (Fig.4). Next, the authors utilize Rag2KO mice that are T and B cell deficient to implicate Ly6G+ Gr-MDSCs to mediate the tumor promoting effects of PeSCs (Fig. 5 and 6). Further, depletion of Ly6G+ Gr-MDSCs results in tumor suppression and negates the tumor promoting effects of PeSCs. The in-vitro and in-vivo experiments thus far seem to be contradictory. The in-vitro experiments seem to suggest that PeSCs have direct impact on tumor proliferation, whereas the subsequent in-vivo experiments and Ly-6G depletion indicates that the tumor promoting effects of PeSCs are secondary due to their influence on Gr-MDSCs. Second, in Fig.7, the authors show that PeSCs suppress anti-tumor CD4+ and CD8+ T cell response. Earlier in Fig. 5 and 6, the authors demonstrate that the effects PeSCs and Ly6G+ MDSCs on tumor proliferation are T cell independent. These two findings appear contradictory. Please clarify.
2) Figure 1A shows the colocalization between CD24 and CD44 in a proportion of cells in KC or KPC pancreata. However, the colocalization between CD106 and CD24 (or CD44) was not shown.
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4) In Fig. 1C, there is no staining with viability dye to identify live cells and is not mentioned in the methods section too. This is a potential technical issue as dead cells would bind to antibodies/ fluorophores non-specifically.
5) Images in Fig. 1D is poor quality. Please include better quality images.
6) Please include H&E histology and % PanIN lesions vs. normal pancreas in KC and KPC mice tissues in Figure 1.
7) Figure 2 was a key figure to show the PeSC in single-cell RNA-seq data. However, this result was only superficially presented without in-depth analyses or adequate data presentation to support the main conclusion or other results of this study. As mentioned by Reviewer 2 Q1 in Fig. 2A, the surface markers used for PanIN-enriched population (containing CD106+ PeSCs) sorting are DAPI-CD45-CD31-Lectin PNA-EpCAM+. It is understandable that after EpCAM sorting the total cell mixture can still have EpCAM-low clusters such as the Cluster7. However, it is still confusing whether the cells in

Figure 2B refer to only the DAPI-CD45-CD31-Lectin PNA-EpCAM+ (the lower panel of Figure 2A, PanIN cell enriched fraction), or from a combination of both fibroblast-enriched fraction (the upper panel of Figure 2A) and PanIN cell enriched fraction. Figure 2C heatmap is just the top genes from the cell clusters automatically defined by algorithm, without showing the key genes such as epithelial cell genes (EpCAM, Krt8, Krt18, Krt19), fibroblast genes (Pdgfra, Col1a1, Dcn, Pdpn), pericyte genes (Cspg4, Rgs5, Pdgfrb), or stem cell genes (Cd24a, Cd44). Only a few of these genes were shown in supplementary figure 4 as UMAP format but not shown in heatmap or violin plot format.

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9) The author mentioned that the single-cell RNA-seq was done on 5 KC mice with a total cell number of 2000. The total cell number is too low to support any conclusion on the identification of Cluster 7 PeSC (which has perhaps only 100 cells or so). Such low number of cells for Cluster 7 (as well as all other cell types combined) will cause the issue of inaccuracy in cell clustering. Based on in supplementary figure 4, the potential PeSC cluster (Cluster 7) has very low expression of Cspg4 and Rgs5, with certain expression of Pdgfrb, and with robust expression of Col1a1/2, Col3a1, Cd34, Cxcl12. It seems that the Cluster 7 is more fibroblast-like, than pericyte-like.

10) The analysis on human PDAC single-cell RNA-seq data does not appear to support the conclusion regarding the presence of PeSC cluster. The usage of PeSC score has no specific relevance to the so-called PeSC, but rather reflects a combination of mesenchymal genes associated with all fibroblasts and pericytes combined. This is likely why Figure 3H right panel showed the high PeSC score in the entire fibroblast cluster. The analysis of this human PDAC single-cell RNA-seq data did not show supporting data for the cell clustering or identification (only superficially shown in supplementary figure 5). Together with the point raised above, the identification of the PeSC in PDAC is not robustly validated, and appears disconnected from the remaining functional studies using isolated cells, the identiy of which remain obscure.

11) With respect to Figure 5, the legend appears to be incomplete. In addition, murine macrophages are frequently known to also express CD11c and contaminate DC populations. Therefore, it could be the case that a decrease in F4/80+ macrophages (Fig. 5 E) is reflected in the CD11c+ population (in Fig. 5F and G). DCs could be identified as CD45+ F4/80- CD11c+ population. Please include gating strategy for Figure 5.

12) In Figure 5 and 6, the authors show that in Rag2KO mice, PeSCs induce Ly6G+ CD11b+ Gr-MDSCs infiltration and depletion of Gr-MDSCs result in tumor inhibition independent of T cells. In Figure 7, the authors demonstrate that in C57BL6 mice, PeSCs induce a T cell suppression response. However, there is a similar increase in tumor weights induced by PeSCs in Rag2KO mice (Figure 5b) and C57BL6 mice (Figure 7b). In this case, which effector cell populations are the PeSC and MDSCs suppressing?

13) MDSCs play an important role in suppressing T cell response, resulting in unchecked tumor proliferation. In Figure 6, the authors demonstrate tumor suppression after Ly-6G depletion in the absence of T cells. It is unclear as to how the Ly-6G+ MDSCs affect tumor progression in the absence of T cells.

14) It is important to note in Figure 5, 6 and 7 that the immune profiling is performed on subcutaneous tumors and not in pancreatic orthotopic tumors. The authors could explain why they chose the subcutaneous tumor implantation over pancreatic orthotopic tumors.

Centre de Recherche en Cancérologie de Lyon
UMR Inserm 1052 - CNRS 5286 - UCBL - CLB

Ana Hennino, PhD
INSERM U 1052
28, rue Laennec, F-69008 Lyon, France
Phone: +33-469 166 669
e-mail: ana.hennino@inserm.fr

Dear Dr. Achim Breiling

Please find here bellow the point-by-point response to the reviewers requests on our manuscript entitled "Identification of a $CD106^{+}$ pericyte stem cell leading to $Ly6G^{+}$ cell accumulation responsible for resistance to immunotherapy in pancreatic cancer".

We addressed point-by-point the requests and proposed several results and/or edits that would be incorporated into the revised manuscript.

Furthermore we have included (in addition to the initially described human tumor microarray) new data in Figure 1, demonstrating the presence of CD106 population associated with the tumor adjacent stroma rather than in the tumor core in human pancreatic cancer samples.

These data further validate the identification of CD106 cell population in human and mouse context.

We hope that you will find our work as exciting as we do and consider it for publication in EMBO Reports.

Referee #1:

This submission was previously reviewed and revised for a journal outside of EMBO press. The authors investigated the CD106+ pericyte stem cell (PeSC) subtype that contribute to the accumulation of Ly6G+ cells in pancreatic cancer. The authors demonstrate the early arrival of CD106+ CD24+ CD44+ population during pancreatitis and PanIN initiation. They provide a pointwise response to the reviewer's comments from the previous journal. The work is interesting; however, there are several major issues that remain unresolved or have not been addressed.

1) There appears to be two conceptual inconsistencies in this manuscript. First, the authors demonstrate in-vitro that PeSCs promote cancer cell growth and proliferation (Fig.4). Next, the authors utilize Rag2KO mice that are T and B cell deficient to implicate Ly6G+ Gr-MDSCs to mediate the tumor promoting effects of PeSCs (Fig. 5 and 6). Further, depletion of Ly6G+ Gr-MDSCs results in tumor suppression and negates the tumor promoting effects of PeSCs. The in-vitro and in-vivo experiments thus far seem to be contradictory. The in-vitro experiments seem to suggest that PeSCs have direct impact on tumor proliferation, whereas the subsequent in-vivo experiments and Ly-6G depletion indicates that the tumor promoting effects of PeSCs are secondary due to their influence on Gr-MDSCs. Second, in Fig.7, the authors show that PeSCs suppress anti-tumor CD4+ and CD8+ T cell response. Earlier in Fig. 5 and 6, the authors demonstrate that the effects PeSCs and Ly6G+ MDSCs on tumor proliferation are T cell independent. These two findings appear contradictory. Please clarify.

We thank the reviewer for the comment and wish to clarify.

We show in vitro that PeSC promote directly cancer growth and proliferation in Figure 4D by assessing the proliferation rate by counting GFP accumulation (Incucyte) or by staining Ki67 (FACS) (Figure EV4A-4B). *In vivo*, we did not detect any impact of the proliferation of tumor cell by analyzing the percentage of Ki67 or GFP^+.
We do agree that the impact of PeSC on tumor cell proliferation is not the same *in vitro* and *in vivo* setup. However, in our perspective, we don't consider these results as "contradictory". Pancreatic cancer microenvironment contains several cell populations which include tumor cells, fibroblasts as well as immune cells that interact together. Therefore, in an attempt to understand the interactions between the different partners we designed our experiments in a stepwise manner, starting from 1) *in vitro* co-culture of PeSC with tumor cells to understand the direct impact of PeSC on tumor cells, 2) *in vivo s.c.* injection in Rag2KO in order to take into consideration the innate immunity and further 3) *in vivo* s.c. injection in C57BL6 mice in order to take into consideration the innate and adaptive immunity. Therefore, our conclusion based on our stepwise experiments are : 1) PeSC impact tumor cell proliferation in absence of the immune cells *in vitro.* 2) However, the PeSC effect on tumor proliferation is minor in the presence of innate immune cells *in vivo*. Our *in vivo* results show that in the presence of PeSC there is a significant MDSC

accumulation in Rag2KO mice. This accumulation is also observed in C57Bl6 mice and further diminish $CD8^+$ and $CD4^+$ T cells activation.
Therefore, we believe that in the context of the tumor microenvironment, the interaction between PeSC and tumor cells favors the accumulation of MDSC that drives diminished $CD8^+$ and $CD4^+$ T cell activation. We have attempted to understand how this interaction drives the MDSC accumulation. We show that extended analysis the cytokine/chemokine production by LegendPlex assay pointed out an increased production of CCL2 and CCL5 but also CCL20, CXCL1, CXCL5 and CXCL10 (Appendix Figure S5A-S5H). These results suggest that a complex interplay between these soluble factors might be important for the emergence of MDSC population.

2) Figure 1A shows the colocalization between CD24 and CD44 in a proportion of cells in KC or KPC pancreata. However, the colocalization between CD106 and CD24 (or CD44) was not shown.

We could not performed co-staining for CD106 and CD44, because our antibodies are both of rabbit origin (CD106 Abcam Cat# ab134047 and CD44 Abcam Cat# ab157107).

In order to bypass this technical issue, we performed FACS staining for the fresh PanIN tissue from KC mice (Figure 1C) and PeSC cell line (Appendix Figure S3A). We show that $CD106^+$ cells are co-expressing CD24 and CD44.

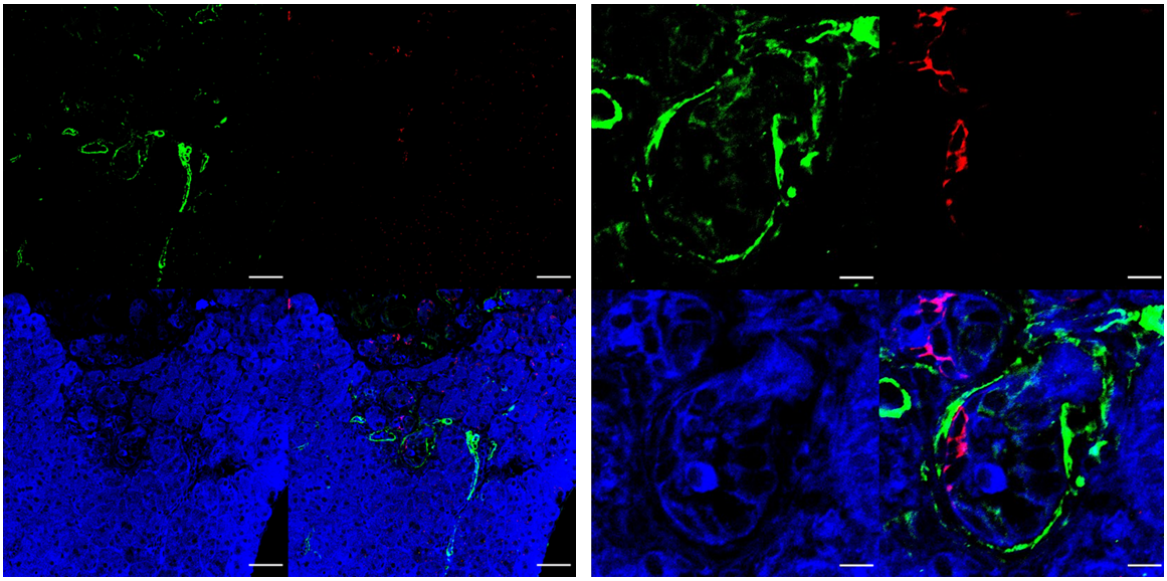
3) Figure 1B showed aSMA-CD106 colocalization and CK19-CD106 colocalization separately. A co-staining of aSMA-CD106-CK19 is necessary. Figure 1B left panel showed aSMA-CD106 colocalization in the ADM area, but the right panel selected an area away from the ADM areas to show aSMA-CD106 colocalization. Figure 1B left panel also showed large areas with many positive CD106 staining (upper right corner areas) without aSMA-staining positivity. It is unclear what is the identity of those cells.

The aim of Figure 2B is to demonstrate that "$CD106^+$ population was in close contact with αSMA^+ cells (Figure 1B left) and absent in the $CK19^+$ nascent duct region (Figure 1B right)". This phrase has been re-modified with detailed Figure numbers in the manuscript.

With regard to the reviewer's concern in the question "the right panel selected an area away from the ADM areas to show aSMA-CD106 colocalization".
In our perspective, the right panel is to show that $CD106^+$ cells are not of tumor origin since they do not express CK19 (Figure 1B) (***It is not to show "aSMA-CD106 colocalization"***). **Similar localization was found in PanIN lesions and tumor adjacent regions from human surgery samples (Modified in New Figure 1A, New Figure EV1A-EV1B, New Appendix Figure S1) where the fibroblast-like $CD106^+$ cells were in close contact with the transforming acinar cells suggesting a role of those cells in sustaining the metaplasia and transformation.**

With regard to another question from the reviewer: "Figure 1B left panel also showed large areas with many positive CD106 staining (upper right corner areas) without aSMA-staining positivity. It is unclear what is the identity of those cells".

We go back to our original IF pictures from confocal. We re-adjust the signal in CD106 channel and find that those CD106^{+} area in the upper right corner in Figure 1B left can be considered as backgrounds in the acinar cells. We do find that CD106 can un-specifically bind to acinar cells or islet cells for two reasons: 1. CD106 Ab dilution for IF is high. 2. The fluorescence of Cy3 is more likely to go extinction under excitation. Therefore, the acquisition of Cy3 is comparably weak and contains more background. "Figure 1B left" has been replaced by the re-adjusted IF pictures. Please also refer to the split channels below. The original IF image has been uploaded to the respective Figure Folder for editor to review. We also include the original IF of the 5x magnification.



4) In Fig. 1C, there is no staining with viability dye to identify live cells and is not mentioned in the methods section too. This is a potential technical issue as dead cells would bind to antibodies/ fluorophores non-specifically.

We do agree with the reviewer that we did not include viability dye in our staining due to the high number of Abs in our matrices. Nevertheless, we have tested in pilot experiments the percentage of viable cells using the viability dye and show 90 to 95% of the cells were viable.

5) Images in Fig. 1D is poor quality. Please include better quality images.

We have included higher resolution images in the manuscript. **(modified in New Figure EV1C)**

6) Please include H&E histology and % PanIN lesions vs. normal pancreas in KC and KPC mice tissues in Figure 1.
We do not understand the purpose of the request.

7) Figure 2 was a key figure to show the PeSC in single-cell RNA-seq data. However, this result was only superficially presented without in-depth analyses or adequate data presentation to support the main conclusion or other results of this study. As mentioned by Reviewer 2 Q1 in Fig. 2A, the surface markers used for PanIN-enriched population (containing CD106+ PeSCs) sorting are DAPI-CD45-CD31-Lectin PNA-EpCAM+. It is understandable that after EpCAM sorting the total cell mixture can still have EpCAM-low clusters such as the Cluster7. However, it is still confusing whether the cells in Figure 2B refer to only the DAPI-CD45-CD31-Lectin PNA-EpCAM+ (the lower panel of Figure 2A, PanIN cell enriched fraction), or from a combination of both fibroblast-enriched fraction (the upper panel of Figure 2A) and PanIN cell enriched fraction. Figure 2C heatmap is just the top genes from the cell clusters automatically defined by algorithm, without showing the key genes such as epithelial cell genes (EpCAM, Krt8, Krt18, Krt19), fibroblast genes (Pdgfra, Col1a1, Dcn, Pdpn), pericyte genes (Cspg4, Rgs5, Pdgfrb), or stem cell genes (Cd24a, Cd44). Only a few of these genes were shown in supplementary figure 4 as UMAP format but not shown in heatmap or violin plot format.

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(only superficially shown in supplementary figure 5). Together with the point raised above, the identification of the PeSC in PDAC is not robustly validated, and appears disconnected from the remaining functional studies using isolated cells, the identiy of which remain obscure.

We addressed the question 7-10 concerning the RNA single cell data altogether here below.
Reviewer's comments 7 to 10 concerned the single cell transcriptome analyses. They asked for more details, a deeper analyses, and pointed at limitations due to the number of cells and markers used for the identification of PeSCs.
In this new version of the analyses we approach these issues by:
1. adding detailed information about the characteristics of the dataset and the methods used to identify the relevant clusters.
2. producing additional visualizations (heatmaps, dotplots and violin plots) with relevant genes, including those suggested by the reviewers.
3. introducing several independently published pericyte genelists to better score each cell across different datasets.
including an additional large human PDAC dataset to account for limitations in the number of cells and provide stronger evidence of PeSC identification.

Although we acknowledge the few number of cells in our Ductal single cell analyses, the cluster we identified seems to diverge drastically from the other cell subpopulations. Moreover, we show now that, in addition to our PeSC score, it is enriched in pericyte scores independently obtained from the literature (ovarian and brain cancer scores, Figure EV2A-EV2B).
Our new data also points to the presence of mesenchymal cells in human PDAC enriched in our PeSC score.

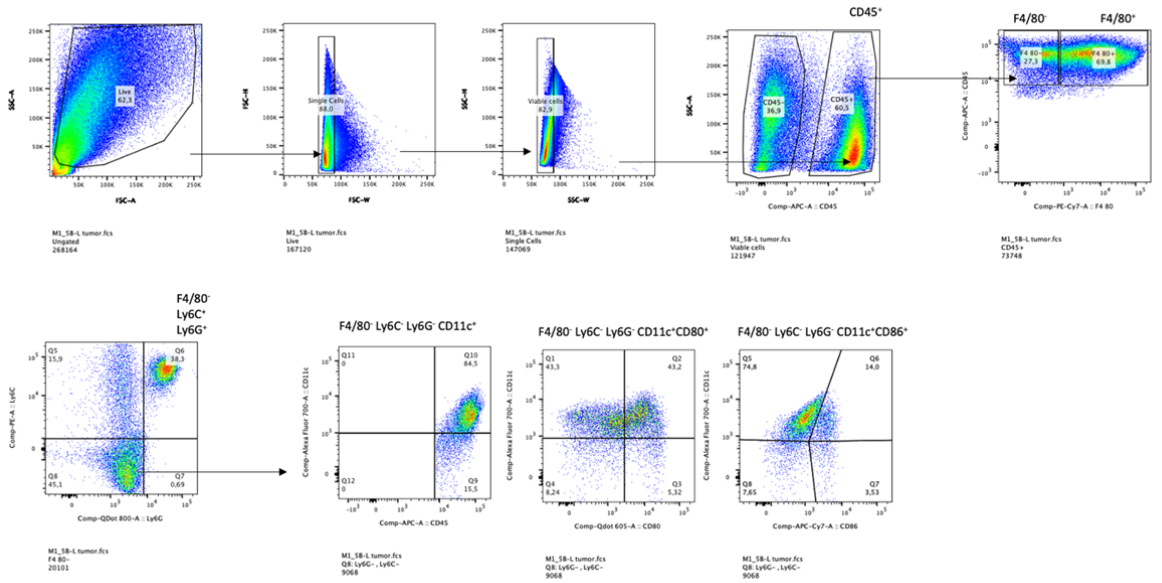
11) With respect to Figure 5, the legend appears to be incomplete. In addition, murine macrophages are frequently known to also express CD11c and contaminate DC populations. Therefore, it could be the case that a decrease in F4/80+ macrophages (Fig. 5 E) is reflected in the CD11c+ population (in Fig. 5F and G). DCs could be identified as CD45+ F4/80- CD11c+ population. Please include gating strategy for Figure 5.

We re-edited Figure 5 with more detailed arrows and dotted-lines to clarify our points in the manuscript. The respective figure legend has been modified as follow:

Figure 5. ***In vivo*** **injection of PeSCs in the context of epithelial tumors induce Ly6G$^+$ MDSCs in the microenvironment.** (A) Experimental setting. (B) Tumor weight. FACS analysis of the percentages of CD45$^+$ cells (C), Ly6G$^+$CD45$^+$ MDSCs (D), F4/80$^+$CD45$^+$ Macrophages (E), CD11c$^+$CD45$^+$ DCs (F), and CD11c$^+$CD80$^+$ / CD11c$^+$CD86$^+$ DCs (G). The results shown are cumulative from three independent experiments (each dot represents one mouse, 12-15 mice per group). Representative immunofluorescence staining of implanted tumors in Rag2KO mice for CK19, mCherry, GFP and DAPI (H) and E-cadherin, ZO-1 and GFP (I). Solid white arrows

indicated the representative cells which expressed E-cadherin and ZO-1 in the cell junctions. Hollow white arrows indicated the representative cells whose E-cadherin or ZO-1 expression were shifting to the cytoplasm. The white dotted line indicated two representative area where E-cadherin and ZO-1 were reversely expressed. Scale bar, 50 mm. *P < 0.05, **P < 0.01 and *** P < 0.001, **** P < 0.0001.

Here below the gating strategy for identifying the macrophage, dendritic cell and MDSC populations.



12) In Figure 5 and 6, the authors show that in Rag2KO mice, PeSCs induce Ly6G+ CD11b+ Gr-MDSCs infiltration and depletion of Gr-MDSCs result in tumor inhibition independent of T cells. In Figure 7, the authors demonstrate that in C57BL6 mice, PeSCs induce a T cell suppression response. However, there is a similar increase in tumor weights induced by PeSCs in Rag2KO mice (Figure 5b) and C57BL6 mice (Figure 7b). In this case, which effector cell populations are the PeSC and MDSCs suppressing?

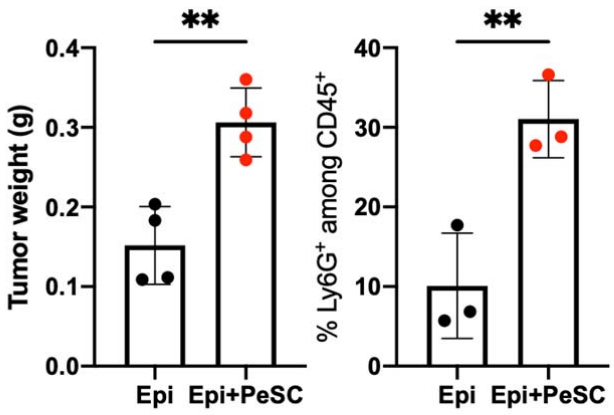
We show that co-injection of PeSC and tumor cells leads to accumulation of MDSC as well as reduction of F4/80 macrophages and CD11c dendritic cells in Rag2KO mice. Elimination of tumor cells by F4/80 macrophage population is therefore reduced in the PeSC+Epi condition compared to Epi alone one, leading to increased tumor weight. In absence of T cell, F4/80 macrophages population represent the major effector cells (about 60% of $CD45^+$ cells, Figure 5E) that is reduced to 40% of $CD45^+$ cells in PeSC+Epi condition. Depletion of Gr-MDSCs restores the F4/80 macrophages population back to 60% of $CD45^+$ cells allowing the reduction of the tumor weight.
In C57BL6 mice, the F4/80 macrophage population is also reduced but it represents only 20% of the $CD45^+$ cells (Figure 7E). In this case the majority of effector cells are T cells that are sensitive to the suppression exerted by MDSC.

13) MDSCs play an important role in suppressing T cell response, resulting in unchecked tumor proliferation. In Figure 6, the authors demonstrate tumor suppression after Ly-6G depletion in the absence of T cells. It is unclear as to how the Ly-6G+ MDSCs affect tumor progression in the absence of T cells.

See response in 12)

14) It is important to note in Figure 5, 6 and 7 that the immune profiling is performed on subcutaneous tumors and not in pancreatic orthotopic tumors. The authors could explain why they chose the subcutaneous tumor implantation over pancreatic orthotopic tumors.

Given to the fact that we used 8-10 mice per group in Figure 5 and two more PD-1 treated group in Figure 7. It was very difficult to perform all the experiments orthotopically. Nevertheless, we did perform the orthotopic tumor implantation to check the microenvironment in the two conditions. We demonstrate that the tumor weight increase as well as Ly6G+ MDSC enrichment is a key event in PeSC+Epi condition compared to Epi condition, which is consistent to the result in s.c. Inj condition.



Dear Dr. Hennino,

Thank you for the submission of your revised manuscript to our editorial offices. I have now received the report from the referee/arbitrator that I asked to re-evaluate your study, you will find below. As you will see, s/he now fully support the publication of your study in EMBO reports.

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- Please provide the abstract with not more than 175 words and written in present tense throughout.

- Please add up to 5 keywords below the abstract.

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- The 'Data Availability' section (DAS) should inform if large datasets have been submitted to a public database. If no datasets have been submitted, please just state there 'No large primary datasets have been generated and deposited'. Please remove all the other information presently part of the DAS.

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- Please add scale bars of similar style and thickness to the microscopic images (main, EV and Appendix figures), using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend. Presently, some scale bars show text nearby, or they are too thin.

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- two to four short bullet points highlighting the key findings of your study (two lines each).
- a schematic summary figure that provides a sketch of the major findings (not a data image) in jpeg or tiff format (with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

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Senior Editor
EMBO Reports

Referee #1:

Histopathology information in appendix informs sufficiently on the sample studied. The response to this reviewer' comments were addressed satisfactorily for EMBO Reports.

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100 characters (including spaces).

Pericyte stem cells induce Ly6G^{+} cell accumulation and immunotherapy resistance in pancreatic cancer

- Please provide the abstract with not more than 175 words and written in present tense throughout.

175 words

We report the identification of a cell population that shares pericyte, stromal and stemness features, does not harbour the *Kras*^{G12D} mutation and drives tumoral growth *in vitro* and *in vivo*. We term these cells pericyte stem cells (PeSCs) and define them as CD45^{-}EPCAM^{-} CD29^{+}CD106^{+}CD24^{+}CD44^{+} cells. We perform studies with *p48*-Cre;*Kras*^{G12D} (KC), *pdx1*-Cre;*Kras*^{G12D};*Ink4a/Arf*$^{fl/fl}$ (KIC) and *pdx1*-Cre;*Kras*^{G12D};*p53*^{R172H} (KPC) and tumor tissues from PDAC and chronic pancreatitis patients. We also perform single cell RNAseq analysis and reveal a unique signature of PeSC. Under steady-state conditions, PeSCs are barely detectable in the pancreas but present in the neoplastic microenvironment in both humans and mice. The co-injection of PeSCs and tumor epithelial cells leads to increased tumor growth, differentiation of Ly6G^{+} myeloid-derived suppressor cells and a decreased amount of F4/80^{+} macrophages and CD11c^{+} dendritic cells. This population induces resistance to anti-PD-1 immunotherapy when coinjected with epithelial tumor cells. Our data reveals the existence of a cell population that instruct immunosuppressive myeloid cell responses to bypass PD-1 targeting and thus suggest potential new approaches for overcoming resistance to immunotherapy in clinical settings.

- Please add up to 5 keywords below the abstract.

pancreatic cancer, pericytes, stem cells, myeloid-derived suppressor cells, PD-1 therapy

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OK

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OK

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number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective Appendix figure legends. Please avoid phrases like 'independent experiment', but clearly state if these were biological or technical replicates. Please provide all this information in a section called 'Data information' for each legend (as it has been done for the main figure legends). Finally, please remove the Appendix legends from the main manuscript text file.
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- Please make sure that all figure panels are called out separately and sequentially (main, EV and Appendix figures). Presently, Figure 6F is called out after Figure 7N OK, the callout for 6J is before 6H, OK callouts for Figure 6I, OK 7O OK, Appendix Figure S6D are missing OK; the callout for EV1B is before EV1A OK and the callout for Appendix Figure S3C is after Appendix Figure S3F OK. Please check, rewrite the text or change the order of the panels in the figure. OK

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OK

In addition, I would need from you:
- a short, two-sentence summary of the manuscript (not more than 35 words).

We report the identification of a cell population that shares pericyte, stromal and stemness features and drives tumoral growth in pancreatic cancer. This study deciphers the role of this cell within the tumor microenvironment.

- two to four short bullet points highlighting the key findings of your study (two lines each).

-Under steady-state conditions, PeSCs were barely detectable in the pancreas.
-PeSC were present in the neoplastic microenvironment in pancreatic cancer in both humans and mice.

-The co-injection of PeSCs and tumor epithelial cells led to increased differentiation of $Ly6G^{+}$ myeloid-derived suppressor cells and induced resistance to anti-PD-1 immunotherapy

- a schematic summary figure that provides a sketch of the major findings (not a data image) in jpeg or tiff format (with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

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Dr. Ana Hennino
Cancer Research Centre of Lyon (CRCL), INSERM U1052, CNRS UMR5286, Claude Bernard University, Lyon, France
France

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Abridged guidelines for figures

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The data shown in figures should satisfy the following conditions:

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- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

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Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
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Select "Not Applicable" only when the requested information is not relevant for your study.

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Include a statement about **blinding** even if no blinding was done.	Not Applicable	
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For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Materias and Methods

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Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Materials and Methods
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

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Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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