THE EMBO JOURNAL

SPICE-Met: Profiling and imaging energy metabolism at the single-cell level using a fluorescent reporter mouse

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision **25th May 2022**

Dear Philippe,

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see, the referees find the analysis interesting and support publication here. They raise a number of different concerns that I would like to ask you to address in a revised version.

I think it is helpful to discuss the revisions further either via email or a video call. Let me know what works best for you.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

Thank you for the opportunity to consider your work for publication. I look forward to discussing your revisions further with you.

Yours sincerely,

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

I have attached a PDF with helpful tips on how to prepare the revised version.

Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

We realise that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (23rd Aug 2022). As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed.

Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

Use the link below to submit your revision:

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #1:

The authors here present a new way to profile immune cell metabolism at single-cell resolution using a fluorescent reporter mouse that measures ADP/ATP ratios. This new tool would be of great value for a broad research community. I really like the idea and enjoyed reading the paper.

I have no major concerns but rather suggestions to further improve the paper and its use by the community.

- It is not clear why the authors moved to 2P imaging for macrophages. This should be better explained and those data should be supplemented with flow cytometry data.
- As written by the authors, a main advantage is the combination with immune activation/surface/phenotype markers but the authors missed the chance to do this for the macrophages. It would be strong added value to include macrophage activation markers and extend the study to LPS/IFNg and IL-4-induced cells to align potential heterogeneity in metabolism to phenotypic and functional differences. Especially combining with intracellular iNOS would be important since NO suppresses OXPHOS and could aid to explain observed differences in mito vs glycolytic dependency.
- Another important advantage of the technique is the ability to assess changes over time. Performing a kinetics to follow-up LPS/IFNg-induced metabolic changes in real-time would be a nice way to showcase the power of this technique.
- In general the discussion should be extended (e.g. 3 metabolic profiles of macrophages) and limitation/darwbacks of the

technique should be highlighted along with its pros.
- Validation against Seahorse is nice and well appriacited. For the complex samples, it would be worth comparing to SCENITH. Both techniques can be probably combined.

Great effort from the authors to push the immunometabolism forward and I personally look forward to apply it in our own research also.

Referee #2:

In the manuscript by Russo et al, the authors have generated a novel transgenic mouse model expressing PercevalHR, a fluorescent reporter, allowing single cell evaluations of cellular ATP:ADP ratios. Using this model, they monitor cellular metabolism by SPICE-Met, a new method for evaluating ex vivo and in vivo metabolism. ATP, but not ADP, results in a shift in the fluorescence excitation spectrum of PercevalHR. This is an innovative and exciting technology that has significant potential for advancing our understanding of both ex vivo and in vivo cell metabolism. The experiments are very well performed and presented and will add significantly to the literature in this field.

A few points are raised below:

-For the vast majority of experiments, the authors report data as ATP/ADP ratios but there is only little information on total ATP or ADP levels. For example, in figure 2, how do total ATP and ADP levels differ in naïve, EM, and CM T cells in draining and non-draining LN? How do levels change between tetramer- and tetramer+ T cells? In figure 3B, it appears that the level of PercevalHR staining in B cells is higher than in other cell types (as monitored by MFI ratio). Do these cells harbor higher ATP levels than other immune cell types?

-Given the lower MFI of PercevalHR (excitation at 405nm detection with a 525 band-pass filter) in CD4 than CD8 T cells, it appears that CD4 T cells harbor a lower number of ADP molecules than CD8 T cells but the MFI ratio of ATP/ADP is higher in CD4 T cells. Therefore, what information does SPICE-Met provide as regards the relative energetic state of CD8 vs CD4 T cells?

-Differences in the responsiveness of ex-vivo BMDM and in-vivo activated peritoneal macrophages to oligomycin is very impressive (Figure 4). Is the transient loss of response of in vivo-activated macrophages following oligomycin treatment maintained if oligomycin is added in glucose-free media?

-The 3 profiles of macrophage metabolism shown in Figure 5 is of much interest. While likely outside the scope of this study, have the authors evaluated differences in the phenotypes of the macrophages that fall into these 3 separate categories?

Minor
-The authors cite SCENITH technology (i.e. protein synthesis as a function of puromycilation) but do not compare their methodologies. It would therefore be important to compare with other technologies that evaluate energy status, i.e. NADPH metabolism (see for example- https://doi.org/10.1038/nmeth.4306).

Referee #3:

In this manuscript Erica Russo et al. describe a transgenic reporter mouse model that in combination with a series of metabolic inhibitors allows to infer metabolic dependencies in living cells, ex-vivo and in-vivo. They show the way the PercevalHR transgenic mice report for changes in intracellular ATP/ADP ratio in response to metabolic inhibitors. Authors demonstrate that the results obtained with their mouse model correlates with Seahorse measurements. They use their Vav-iCre x PercevalHR mice to analyze the metabolic profile of resting or activated macrophages, in vitro and ex-vivo and identify heterogenous profiles.

The article presents a resource that is valuable for future immunometabolic studies. Their mice represent an important resource for the community, especially if they would allow for profiling metabolism in-vivo by imaging.

However, authors do not mention limitations of the study and the results shown are mainly ex-vivo or in-vitro; showing only an incremental advantage compared to transfecting with the PercevalHR reporter. Also, authors mention developing a new method; while they are using the same inhibitors and the same reporter as previously published to reveal the metabolic profile.

In order to be acceptable for publication, some major corrections need to be addressed:

Practical points:

A. Authors show that metabolic changes occur in terms of seconds/minutes of treatment; and they show ex-vivo data. As the

ATP/ADP ration can change rapidly; authors show address:
o Kinetics of ATP/ADP ratio of cells, when cells are extracted from their natural microenvironment? Impact of tissue dissociation? Does cell culture media bias in the measured metabolic dependencies?
o If a treatment is done in-vivo, how long can we keep the cells in FACS buffer of PBS before the ATP/ADP ratio changes? In other words how stable is the readout?
o Their resource could be interesting for studies in stem cells and other cells that are difficult to transfect with the PercevalHR reporter plasmid/lentivirus. Authors should exploit this advantage of their mice and mention about the sensitivity and expression level of their reporter in quiescent cells. Can you easily measure the metabolic profile of quiescent cells? Stem cells? Does the level of expression of the reporter in different cell types impact on the results?
o It has been shown that pH can impact on PercevalHR fluorescence. Does the glycolytic state and low pH impact on the metabolic profile measured in activated macrophages? Or activated CD8 T cells?
B. Is the emission spectra of PercevalHR suitable for in-vivo imaging of metabolism?

Conceptual points:

C. Authors quantify the changes in ATP/ADP ratio in response to an inhibitor of pathway X and label the parameter as the "% contribution of pathway X" to ATP/ADP ratio. However this is not entirely correct, when pathway X is inhibited, cells can very rapidly respond by upregulating the use of compensatory pathways (as ussually observed in the ECAR upon Oligomycin by Seahorse). If a decrease in the ATP/ADP ratio is observed, it means that cells cannot compensate more by using other pathways. For this reason, instead of % contribution; authors should use "% dependency on pathway X" is more accurate.

Minor points:
D. In Figure 1, the FACS histograms X axis shows ATP or ADP and that is incorrect; label the fluorescent channel and label accordingly FL-X (PercevalHR-ATP).
E. Figure 1F, 1G, 1H are identical to Figure S1B, S1C and S1D.
F. In the introduction, authors mention protein translation; the correct term is mRNA translation or protein synthesis.

Point-by point

Referee #1:

The authors here present a new way to profile immune cell metabolism at single-cell resolution using a fluorescent reporter mouse that measures ADP/ATP ratios. This new tool would be of great value for a broad research community. I really like the idea and enjoyed reading the paper.

I have no major concerns but rather suggestions to further improve the paper and its use by the community.

We thank the referee for the nice comments on our manuscript and suggestions to further improve it.

- It is not clear why the authors moved to 2P imaging for macrophages. This should be better explained and those data should be supplemented with flow cytometry data.

Following referee suggestion, we have now added flow cytometry analyses corresponding to our macrophages imaging experiments.

-> Flow cytometry-based SPICE-Met profiling of macrophages is shown in **Figure S4**.

- As written by the authors, a main advantage is the combination with immune activation/surface/phenotype markers but the authors missed the chance to do this for the macrophages. It would be strong added value to include macrophage activation markers and extend the study to LPS/IFNg and IL-4-induced cells to align potential heterogeneity in metabolism to phenotypic and functional differences. Especially combining with intracellular iNOS would be important since NO suppresses OXPHOS and could aid to explain observed differences in mito vs glycolytic dependency.

We thank the referee for these suggestions. We have now added IL-4-treated macrophages as an additional condition analyzed by SPICE-Met both by flow cytometry and imaging. Intracellular iNOS (and cell permeabilization and fixation) is unfortunately not compatible with our fluorescent probe. Instead, we tested the role of NO using a pharmacological inhibitor of iNOS. With these experiments, we confirmed the impact on NO in blocking OXPHOS in activated macrophages.

-> Flow cytometry and imaging based SPICE-Met profiling of alternatively activated macrophages is shown in **Figure S4** and **Figure S3**, respectively.

-> Role of iNOS in macrophage metabolic profiling is shown in **Figure S5C-E**.

- Another important advantage of the technique is the ability to assess changes over time. Performing a kinetics to follow-up LPS/IFNg-induced metabolic changes in real-time would be a nice way to showcase the power of this technique.

We agree with the referee that SPICE-Met facilitates kinetic studies. As suggested, we report the kinetics of metabolic switch in macrophages upon LPS/IFN-g stimulation.

-> Kinetics of metabolic switch during classical macrophage activation is shown in **Figure S5A-B**.

- In general the discussion should be extended (e.g. 3 metabolic profiles of macrophages) and limitation/darwbacks of the technique should be highlighted along with its pros.

We now discussed in more details advantages and limitation of the technique (results and discussion section) and in particular the role of culture media used during the assay.

- Validation against Seahorse is nice and well appreciated. For the complex samples, it would be worth comparing to SCENITH. Both techniques can be probably combined.

As mentioned before, cell permeabilization/fixation is not compatible with our biosensor. It is therefore not possible to combine directly with SCENITH. Of note, most of our findings (T cells, monocytes) are very consistent with the results already obtained by Arguello et al. using the SCENITH approach.

Great effort from the authors to push the immunometabolism forward and I personally look forward to apply it in our own research also.

Referee #2:

In the manuscript by Russo et al, the authors have generated a novel transgenic mouse model expressing PercevalHR, a fluorescent reporter, allowing single cell evaluations of cellular ATP:ADP ratios. Using this model, they monitor cellular metabolism by SPICE-Met, a new method for evaluating ex vivo and in vivo metabolism. ATP, but not ADP, results in a shift in the fluorescence excitation spectrum of PercevalHR. This is an innovative and exciting technology that has significant potential for advancing our understanding of both ex vivo and in vivo cell metabolism. The experiments are very well performed and presented and will add significantly to the literature in this field.

We are grateful to the reviewers for the very positive remarks.

A few points are raised below:

-For the vast majority of experiments, the authors report data as ATP/ADP ratios but there is only little information on total ATP or ADP levels. For example, in figure 2, how do total ATP and ADP levels differ in naïve, EM, and CM T cells in draining and non-draining LN? How do levels change between tetramer- and tetramer+ T cells? In figure 3B, it appears that the level of PercevalHR staining in B cells is higher than in other cell types (as monitored by MFI ratio). Do these cells harbor higher ATP levels than other immune cell types?

Following referee suggestion, we now report separately the ATP and ADP levels in T cells subjected to metabolic inhibitors. The results confirmed that drop in ATP:ADP ratio corresponded to a decrease in ATP and an increase in ADP.

-> Levels of ATP and ADP during oligomycin treatment is shown in **Figure S1B**.

While the ratio is expected to be independent of the level of expression of the probe, it is not the case for ATP or ADP signals only. Therefore, Perceval probes is best used when comparing ATP:ADP ratios (and cannot be used to compare ATP only).

-Given the lower MFI of PercevalHR (excitation at 405nm detection with a 525 band-pass filter) in CD4 than CD8 T cells, it appears that CD4 T cells harbor a lower number of ADP molecules than CD8 T cells but the MFI ratio of ATP/ADP is higher in CD4 T cells. Therefore, what information does SPICE-Met provide as regards the relative energetic state of CD8 vs CD4 T cells?

As noted by the referee, the ATP:ADP ratio is higher in $CD4^+$ T cells as compared to $CD8^+$ T cells. While the biological consequences of this difference are unclear, $CD4^+$ and $CD8^+$ T cells have similar energic profiles when subjected to SPICE-Met.

-Differences in the responsiveness of ex-vivo BMDM and in-vivo activated peritoneal macrophages to oligomycin is

very impressive (Figure 4). Is the transient loss of response of in vivo-activated macrophages following oligomycin treatment maintained if oligomycin is added in glucose-free media?

We thank the reviewer for this suggestion and indeed now report that glucose is key for the reversal of metabolism upon oligomycin.

-> The role of glucose in the reversal of peritoneal macrophage metabolism after oligomycin is shown in **Figure S6**.

-The 3 profiles of macrophage metabolism shown in Figure 5 is of much interest. While likely outside the scope of this study, have the authors evaluated differences in the phenotypes of the macrophages that fall into these 3 separate categories?

We thank the referee for his/her interest in the 3 metabolic profiles identified. We agree that this is of much interest and should be the focus of further studies.

Minor
-The authors cite SCENITH technology (i.e. protein synthesis as a function of puromycilation) but do not compare their methodologies. It would therefore be important to compare with other technologies that evaluate energy status, i.e. NADPH metabolism (see for example- https://doi.org/10.1038/nmeth.4306).

We now discuss the similarity of the energy metabolic profiling in T cells performed by SPICE-Met and SCENITH (in addition to the SeaHorse data already included).

Referee #3:

In this manuscript Erica Russo et al. describe a transgenic reporter mouse model that in combination with a series of metabolic inhibitors allows to infer metabolic dependencies in living cells, ex-vivo and in-vivo. They show the way the PercevalHR transgenic mice report for changes in intracellular ATP/ADP ratio in response to metabolic inhibitors. Authors demonstrate that the results obtained with their mouse model correlates with Seahorse measurements. They use their Vav-iCre x PercevalHR mice to analyze the metabolic profile of resting or activated macrophages, in vitro and ex-vivo and identify heterogenous profiles.

The article presents a resource that is valuable for future immunometabolic studies. Their mice represent an important resource for the community, especially if they would allow for profiling metabolism in-vivo by imaging.

However, authors do not mention limitations of the study and the results shown are mainly ex-vivo or in-vitro; showing only an incremental advantage compared to transfecting with the PercevalHR reporter. Also, authors mention developing a new method; while they are using the same inhibitors and the same reporter as previously published to reveal the metabolic profile. In order to be acceptable for publication, some major corrections need to be addressed:

We thank the reviewer for finding our methodology valuable and an important resource for the community. We now discussed in more details advantages and limitation of the technique (results and discussion section) and in particular the role of culture media used during the assay.

Practical points:

A. Authors show that metabolic changes occur in terms of seconds/minutes of treatment; and they show ex-vivo data. As the ATP/ADP ration can change rapidly; authors show address:
Kinetics of ATP/ADP ratio of cells, when cells are extracted from their natural microenvironment?
Impact of tissue dissociation? Does cell culture media bias in the measured metabolic dependencies?
If a treatment is done in-vivo, how long can we keep the cells in FACS buffer of PBS before the ATP/ADP ratio changes? In other words how stable is the readout?

We agree that the points raised by the referee regarding stability/role of culture media in the assay are important. We now have performed several experiments and discuss these points in the revised version.

In our assay, we choose to use complete media to maximize energic resources and to reveal how a given cell generate energy in the absence of constraints. By modifying the media (i.e removing the glucose), we can reveal plasticity in energy metabolism. For example, we now show that in the absence of glucose, activated T cells are able to reverse to OXPHOS. We also show that in the absence of glucose, macrophages are not able to restore their ATP levels after oligomycin treatment.

-> The profiling of energy metabolism in T cells in the presence or absence of glucose is shown in **Figure S7**.

->Effect of the medium composition on peritoneal macrophage response to oligomycin is shown in **Figure S6**.

We also performed experiments to test the stability of the read-out ex vivo. We compared the measurements made immediately after extraction or after 90 min at 37°C. Similar results were obtained suggesting that the measurements could be performed within this time window at least (what we always do).

-> The comparison of SPICE-Met profiling performed directly ex vivo or after 90 min incubation at 37°C is shown in **Figure S2**.

o Their resource could be interesting for studies in stem cells and other cells that are difficult to transfect with the PercevalHR reporter plasmid/lentivirus. Authors should exploit this advantage of their mice and mention about the sensitivity and expression level of their reporter in quiescent cells. Can you easily measure the metabolic profile of quiescent cells? Stem cells? Does the level of expression of the reporter in different cell types impact on the results?

We now explain that the actual ATP:ADP ratio can vary mildly between cell types at steady state (as shown in Figure 3C), although the biological relevance remains to be established.
Because it is a ratiometric probe, the level of expression of the probe is not expected to influence the measurements. Future studies that would use different Cre-expressing mice (to analyze different cell type) would need to confirm that the level of expression is sufficient but so far, we have seen sufficient expression of the probe in all the cells tested (including quiescent cells and HSCs).

o It has been shown that pH can impact on PercevalHR fluorescence. Does the glycolytic state and low pH impact on the metabolic profile measured in activated macrophages? Or activated CD8 T cells? B. Is the emission spectra of PercevalHR suitable for in-vivo imaging of metabolism?

We have performed experiments to show that metabolic inhibitor treatment (i.e oligomycin) has little impact on pH during Perceval measurements. To this end, we have mixed B cells transduced with pHRed probe (pH sensitive genetically-encoded probe) or Perceval and treated them with oligomycin. As seen in the figure below, the drastic change in ATP:ADP ratio upon oligomycin is not associated with a change of pH during the time of the assay.

The emission spectra of PercevalHR is indeed suitable for in vivo imaging. The use of the probe directly in vivo faces several challenges (delivery of the inhibitor, level of expression) that we (or others) will address in future studies. (Data shown to referees, but removed from Review Process File)

Conceptual points:

C. Authors quantify the changes in ATP/ADP ratio in response to an inhibitor of pathway X and label the parameter as the "% contribution of pathway X" to ATP/ADP ratio. However this is not entirely correct, when pathway X is inhibited, cells can very rapidly respond by upregulating the use of compensatory pathways (as ussually observed in the ECAR upon Oligomycin by Seahorse). If a decrease in the ATP/ADP ratio is observed, it means that cells cannot compensate more by using other pathways. For this reason, instead of % contribution; authors should use "% dependency on pathway X" is more accurate.

We understand the referee point and both terms have advantages and drawbacks. Since adaptation can take minutes or hours, we think that the term contribution may better reflect the pathway used by the cell at the time of the assay.

Minor points:

D. In Figure 1, the FACS histograms X axis shows ATP or ADP and that is incorrect; label the fluorescent channel and label accordingly FL-X (PercevalHR-ATP).

We thank the referee for this remark. This has been corrected.

E. Figure 1F, 1G, 1H are identical to Figure S1B, S1C and S1D.

We are very grateful to the referee for noticing this mistake. Data for CD4+ T cell and CD8+ T cell profiling are very similar and we had inadvertently duplicated the CD8+ T cell graph in the supplementary Figure S1B (now FigureS1C). We have corrected this mistake and inserted the correct graph (CD4+ T cells) in Figure S1C. Figure S1D is not concerned by this inversion (although the graph looks quite similar to that of CD8+ T cells)

F. In the introduction, authors mention protein translation; the correct term is mRNA translation or protein synthesis.

The referee is absolutely right. We thank the referee for noticing the inaccurate term that we have now corrected.

Dear Philippe,

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been seen by the two referees and their comments are provided below.

As you can see both referees appreciate the added revisions. Referee #3 has a few remaining concerns that can be sorted out with text changes. Please take a careful look at the comments regarding "the naming of the metabolic parameter". In the end it it is your study but give it a thought and see if it makes sense to change as the referee suggests.

Once we get the revised version back in then I will formally accept the MS

Will you also look at the grant information if it is complete enough. I am just asking as there are no grant numbers mentioned.

That should be all. You can use the link below to upload the final version.

Congratulations on a nice study

Best Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

Use the link below to submit your revision:

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #1:

The authors answered my concerns and I have no further questions. Nice work!

Referee #3:

- general summary and opinion about the principle significance of the study, its questions and findings

I still believe the article is interesting and an interesting resource for the community. Authors added interesting results in response to our concerns. I would like to congratulate the authors for their work.

- specific major concerns essential to be addressed to support the conclusions

Only one point/discussion needs to be addressed and closed before, in my opinion, accepting the manuscript.

I raised a concern and shared my arguments to change the naming of the metabolic parameter that is calculated throughout this manuscript. I proposed to change:
"% contribution of pathway x" to
"% dependence of pathway x" or "X pathway dependence (%)"

Authors answered:

" We understand the referee point and both terms have advantages and drawbacks. Since adaptation can take minutes or hours, we think that the term contribution may better reflect the pathway used by the cell at the time of the assay."

I would like to further discuss the arguments (not clear) of the authors to keep this nomenclature that will impact all future studies with this method.

Authors are confusing adaptation to metabolic switching or rapid compensation. The calculations in their experiments are done in cells after 40 minutes of treatment. In the timeframe of the experiments of Seahorse, that are the same as for SCENITH and SPICE-met, hundreds of laboratories have shown that the switch to glycolysis (increase of ECAR) takes place seconds (within 360 sec) after addition of oligomycin. In other words, cells that cannot compensate by switching to glycolysis in response to oligomycin will reveal their dependency on OXPHOS. For this reason, I hope we agree that SPICE-met does not measure the metabolic profile at basal conditions (other than the ATP/ADP basal ratio) and thus it cannot reflect the "contribution of pathway X" to cellular metabolism. Instead SPICE-met measure responses of cells to inhibitors, and thus it can only reveal the " Dependencies on pathway X" and/or the maximal capacities of the cells to exploit alternative pathways. For example, in response to oligomycin cells will rapidly reveal their maximal glycolitic capacity, and this will differ; as observed with ECAR and seahorse with the "% contribution of glycolysis" at steady state. Again, the mean Ratio MFI used for the calculations in this manuscript was obtained at 40 minutes after treatment, leaving plenty of time for the cells to switch and reveal their maximal metabolic capacities.

I hope that if we agree in this definitions, authors change that definition of the metabolic parameter in the manuscript.

- minor concerns that should be addressed

A)
In figure 1.I, it is not clear in the legend that they are showing the ratio of OCR/ECR; and is not clear why they choose to graph the ratio instead of showing the Raw ECAR and Raw OCR profiles, that is the usual way to represent Seahorse data. Please clarify the legend, or show the raw ECAR and raw OCR profiles.

B)
I raised the point about how to isolate, stain and pass the cells in the FACS while keeping the cells with a physiolocial ATP/ADP ratio. The authors performed experiments that show similar profiles in cells kept in the cell culture media for 0 vs 90 minutes. It would be good to show in the graph if the differences still statistically significant.
An issue that I did not raise before is that most fluorescent monoclonal antibodies, contain sodium azide an inhibitor of mitochondrial respiration. Did authors observed any impact of some antibodies, depending on their diluition? In material methods authors do not mention any washing steps after the addition of the staining antibodies during 10 minutes, can authors clarify this.

- any additional non-essential suggestions for improving the study (which will be at the author's/editor's discretion)

No.

In response to the final comments of reviewer 3

• We agree with the referee and decided to change the terminology from contribution to dependence.

• We have indicated in the figure legends that OCR:ECAR ratio is represented

• Abs dilution is at least 1/200 and we have not detected any problem to the small concentration of azide present in the assay.

2nd Revision - Editorial Decision **3rd Aug 2022**

Dear Philippe,

Thanks for submitting your revised manuscript. I have now looked at everything and all looks good. I am therefore very pleased to accept the manuscript for publication here.

Congratulations on a nice study

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. If you do NOT want this, you will need to inform the Editorial Office via email immediately. More information is available here: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

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Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs 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 and any statistical test employed should be iustified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

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;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Typically 3 mice were used per condition and per experiments.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Sample size estimate was performed according to our ethical protocol
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No animals were excluded from the analysis
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Treated and untreated mice were age-mateched and sex-matched animails.
For animal studies, include a statement about randomization even if no randomization was used.	No randomization was used.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No blinded analysis was performed
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was done
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes
Is there an estimate of variation within each group of data?	Yes

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	included in the manuscript
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	N/A

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Done
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Done
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	OK

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
12. 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.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Done
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	OK
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	N/A