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Fetal monocytes possess increased metabolic capacity and replace primitive macrophages in tissue macrophage development

Fengqi Li, Katarzyna Maria Okreglicka, Lea Maria Pohlmeier, Christoph Schneider, Manfred Kopf

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Transaction Report:

(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. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 1st Oct 2019

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

While both referees appreciate the technical aspects of the study, referee #2 also finds that the advance and insight over Guilliams et al. paper not sufficient to consider publication here. Referee #1 is more positive regarding this aspect, but also requests further in imaging analysis to test the capacity of primitive macrophages vs monocytes to colonize the lung. I have discussed the issue of novelty further with referee #1 and while we agree that the Guilliams et al. paper takes a bit of the novelty away we also find that the present analysis is valuable and provides new insight.

Therefore, should you be able to extend the analysis along the lines suggested by referee #1 then I would be able to consider a revised version. I should add that it is EMBO Journal policy to allow only a single round of revision, and that it is therefore important to resolve the major concerns at this stage.

REFEREE REPORTS:

Referee #1:

This is very well designed, interesting and concise study that will be of interest for the macrophage community. Below are some comments to improve the study.

One major issue is that the authors completely dismissed the hypothesis of a migration/motility defect of primitive macrophages vs monocytes in their capabilities to colonize the tissue niches in the developing lung. The authors should provide some imaging data/analysis to assess the localization of the transferred cells in the lung.

The statement " Myb-dependent hematopoietic stem cells (HSC) emerge in the aorta-gonad-mesonephros (AGM) around E10.5 and seed the fetal liver between E11.5-E12.5, where they differentiate to monocyte precursors that can contribute to the MΦTR pool (Hoeffel et al, 2015; Hoeffel & Ginhoux, 2015; McGrath et al, 2015a; McGrath et al, 2015b)." needs to be modulated, stating that the exact contribution of HSC-derived monocytes in such process remains not very well characterized and precisely quantified if existing.

On " Apart from the brain, which is populated by pMΦ-derived microglia, fetal liver monocytes seed the large majority of tissues starting from E13.5, where they expand and differentiate to MΦTR, thereby replacing pMΦ (Gomez Perdiguero et al, 2015; Hoeffel et al, 2015).", the authors should not cite Gomez Perdiguero et al, 2015 as this study and successive studies from this group are not in favor of such working model.

On " To assess the potential of YS-derived late EMP to differentiate into AM, we sorted viable CD45loC-kit+F4/80-CD11bloMHCII-CD11c- EMP", are these cells not considered to be pre-Mac by Mass et al?

In "The presence of pMΦ-derived AM delayed the crash in body temperature (Fig 4B) and death only by 2 days without amelioration of O2 saturation (Fig 4C,D).", the use of "crash" might not ideal. What about reduction?

About "For an unbiased comparison of the phenotype of pMΦ and fetal monocytes, we performed gene set enrichment analysis (GSEA) on Gene Ontology (GO) for Cellular Component (CC) of their transcriptomes.", the authors should have provided transcriptomes of grafted cells early and late after transfer. In addition, the transcriptomes of Feli mono derived AM vs Primitive mac AM is already available in Hoefel et al 2015 study.

Referee #2:

Schneider et al studies here the development of alveolar macrophages (AM) from the competitive transplantation of different AM precursors from YS, fetal liver, and lung into neonatal AM-deficient mice. Fetal monocytes, promoted by Myb, outcompeted primitive MΦ (pMΦ) to develop into mature AM. AMs derived from pMΦ failed to clear alveolar proteinosis and protect from fatal lung failure following influenza virus infection. The next claim a ditinct mitochondrial respiratory and glycolytic capacity and repression of the transcription factors c-Maf and MafB. The report is well written and constructed and the data are solid.
There are a few shortcomings however which need to be addressed:
1) The biggest weakness is the lack of novelty. The Guilliams paper in Immunity (2016) reported on pretty much everything that is in here. There is a clear lack of conceptual advance and lack of novelty.
2) They claim differences in metabolism to be responsible for the district behavior of the AM precursors studied here. While the data suggest this to be the underlying mechanism as to why monocytes are 'better', it is equally possible, that because monocytes are better this is the phenotype they show. It's a cause and effect question and as such the finding is purely descriptive.
3) The Csf2ra data in Fig 4 are very interesting. However, to state that CSF2 sensitivity is 'likely' not involved is an overstatement. This reviewer would suggest that they measure PStat5 in response to limiting dilutions of GM-CSF.

1st Revision - authors' response 6th Nov 2019

Please see next page.

Point-by-point response to referees - EMBOJ-2019-103205. 'Fetal monocytes possess increased metabolic capacity and replace primitive macrophages in tissue macrophage development'

****** Referee #1 ******

This is very well designed, interesting and concise study that will be of interest for the macrophage community. Below are some comments to improve the study.

Authors: Thank you for supporting our findings. We are grateful for your insightful comments.

1) One major issue is that the authors completely dismissed the hypothesis of a migration/motility defect of primitive macrophages vs monocytes in their capabilities to colonize the tissue niches in the developing lung. The authors should provide some imaging data/analysis to assess the localization of the transferred cells in the lung.

Authors: Thank you for pointing out this important possibility. We agree with the referee that we did not consider a potential difference in migration/localization between primitive macrophages and fetal monocytes, in particular when it comes to migration from the liver to the other organs/tissues (in our case the lung). In the revised version, we have discussed this possibility (please see underlined text in the discussion part).
However, since we perform the transfer by intranasal instillation, we provide the cells directly to the airways. Of course, there is a theoretical chance that fewer primitive macrophages reach the alveoli because they get stuck in the trachea, bronchi, and bronchioles. If this were the case, it can be assumed that they would not differentiate to alveolar macrophages (AM). However, when we harvest the cells from the airways 7-8 weeks after transfer, the transferred cells (derived from either primitive macrophages or fetal monocytes) have differentiated to CD11c^{+} SiglecF^{hi}CD11b^{lo} AM, indicating that they have found the right niche for differentiation.

Since the AM niche has not been identified yet, it is impossible to visualize proper niche localization of the transferred cells. There is another technical hurdle for visualization. Transfer of more than 80'000 -100'000 cells to neonates results in death (probably due to lung failure and/or asphyxiation). Further, we have demonstrated that around 10% of transferred cells can be harvested from the lung after transfer indicating that maximal 10'000 cells make it to the lower airways (Fig EV1). Visualizing and quantitating such a low number in an organ containing around 5x10^{9} cells would be very challenging.

2) The statement "Myb-dependent hematopoietic stem cells (HSC) emerge in the aorta-gonad-mesonephros (AGM) around E10.5 and seed the fetal liver between E11.5-E12.5, where they differentiate to monocyte precursors that can contribute to the MΦ^{TR} pool (Hoeffel et al, 2015; Hoeffel & Ginhoux, 2015; McGrath et al, 2015a; McGrath et al, 2015b)." needs to be modulated, stating that the exact contribution of HSC-derived monocytes in such process remains not very well characterized and precisely quantified if existing.

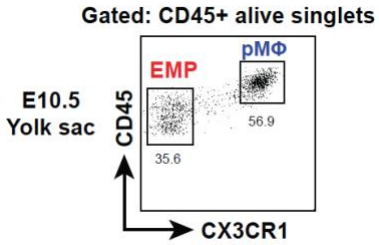
Authors: We thank referee #1 for the advice. We have changed the text accordingly (please see underlined text in the introduction part), i.e. "Myb dependent hematopoietic stem cells (HSC) emerge in the aorta-gonad-mesonephros (AGM) around E10.5 and seed the fetal liver between E11.5-E12.5. It is possible that HSC also could give rise to monocytes in the fetal liver then contribute to the MΦ^{TR} pool".

3) On "Apart from the brain, which is populated by pMΦ-derived microglia, fetal liver monocytes seed the large majority of tissues starting from E13.5, where they expand and differentiate to MΦ^{TR}, thereby replacing pMΦ (Gomez Perdiguero et al, 2015; Hoeffel et al, 2015).", the authors should not cite Gomez Perdiguero et al, 2015 as this study and successive studies from this group are not in favor of such working model.

Authors: Apologies for the sloppiness. We have removed the citation "Gomez Perdiguero et al, 2015" in the revised manuscript (please see underlined citation in the introduction part).

4) On "To assess the potential of YS-derived late EMP to differentiate into AM, we sorted viable CD45^{lo}C-kit^{+}F4/80^{-}CD11b^{lo}MHCII^{-}CD11c^{-} EMP", are these cells not considered to be pre-Mac by Mass et al?

Authors: We sorted EMP and primitive macrophages from E10.5 yolk sac based on markers CD45, C-kit, F4/80 and CD11b etc (Fig EV2A). There were clearly two CD45^{+} populations in yolk sac on E10.5, the CD45^{lo}C-kit^{+}F4/80^{-}CD11b^{lo} are EMP population, and CD45^{hi}C-kit^{-}F4/80^{+}CD11b^{hi} are primitive macrophages (Fig EV2A). Mass et al (Science. 2016) revealed that EMPs give rise to a population of premacrophages (Pre-Mac) that colonize the whole embryo from E9.5. We refer to this population as "primitive macrophages (pMΦ)" in our study. It has been shown that CX3CR1 is upregulated in pre-Macs and is important for embryo colonization. We also performed CX3CR1 staining in E10.5 yolk sac samples, which confirmed a clear separation of EMP (CD45^{lo}CX3CR1^{-}) and pMΦ (CD45^{hi} CX3CR1^{+}) (please see figure below, which was also added as Fig EV2B in the revised manuscript). Therefore, we are quite confident that we sorted the correct YS EMP population in our study.



5) In "The presence of pMΦ-derived AM delayed the crash in body temperature (Fig 4B) and death only by 2 days without amelioration of O2 saturation (Fig 4C, D).", the use of "crash" might not ideal. What about reduction?

Authors: We are sorry for the inappropriate slang. As suggested, we corrected it (please see underlined text in the result part).

6) About "For an unbiased comparison of the phenotype of pMΦ and fetal monocytes, we performed gene set enrichment analysis (GSEA) on Gene Ontology (GO) for Cellular Component (CC) of their transcriptomes.", the authors should have provided transcriptomes of grafted cells early and late after transfer. In addition, the transcriptomes of Feli mono derived AM vs Primitive mac AM is already available in Hoefel et al 2015 study.

Authors: We have added transcriptome data of AM harvested 6 weeks after transfer of the different precursors (please see Fig 5A-F). The data basically confirm results published previously by Guilliams and colleagues (van der Laar et al, Immunity 2016). Moreover, we have appropriately cited Hoeffel et al 2015.

****** Referee #2 ******

Schneider et al studies here the development of alveolar macrophages (AM) from the competitive transplantation of different AM precursors from YS, fetal liver, and lung into neonatal AM-deficient mice. Fetal monocytes, promoted by Myb, outcompeted primitive MΦ (pMΦ) to develop into mature AM. AMs derived from pMΦ failed to clear alveolar proteinosis and protect from fatal lung failure following influenza virus infection. The next claim a distinct mitochondrial respiratory and glycolytic

capacity and repression of the transcription factors c-Maf and MafB. The report is well written and constructed and the data are solid.

Authors: We thank the referee for the assessment of our manuscript.

There are a few shortcomings however which need to be addressed:

1) The biggest weakness is the lack of novelty. The Guilliams paper in Immunity (2016) reported on pretty much everything that is in here. There is a clear lack of conceptual advance and lack of novelty.

Authors: Unfortunately, the manuscript published by Guilliams and colleagues in 2016 scooped us. When their manuscript appeared online, we already had drafted a manuscript that contained about 80% of the data shown in the paper by van de Laar et al. Each paper contained about 10% additional results not shown in the manuscript of the others. We decided to continue on this story and better address the functional quality of alveolar macrophages derived from either primitive macrophages (pMΦ) or fetal monocytes (Mo).
In fact, in the present version of our manuscript, we deleted the large majority of overlapping results and present novel data, that extend the conclusions by the Guilliams group substantially.

Below a summary of the novelties:

(i) While van de Laar/Guillams and colleagues concluded that the function (i.e. clearance of surfactant) of AM derived from fetal Mo and pMΦ is comparable, we found a fundamental difference during influenza infection, when the mice with AM derived from pMΦ die in contrast to mice containing AM derived from fetal Mo.

(ii) Although the data of van de Laar/Guillams show that the development of pMΦ is somewhat impaired compared to fetal Mo, throughout the manuscript the authors focused on and highlighted that the two pMΦ and fetal Mo are very similar. We have compared the developmental capacities of pMΦ and fetal Mo from different locations (i.e. yolk sac, fetal liver and lung) and at different days of gestation in competitive transfer experiments. Our data demonstrate quantitatively the poor developmental capacity of pMΦ really, and that this actually declines over time of gestation.

(iii) Notably, based on a fate mapping approach, Hoeffel et al (Immunity 2015) proposed that pMΦ are replaced by fetal Mo in the tissues before birth. Conclusions from our elaborate competitive transfer experiments support this hypothesis.

(iv) In addition, we have tested the role of c-Myb by transfer of yolk sac and fetal liver Myb$^{-/-}$ precursors and conclude that c-Myb promotes the developmental capacity of EMP. Again, this was not shown by van de Laar et al.

(v) Importantly, we have addressed the underlying mechanisms that may (at least partially) explain the increased developmental capacities of fetal Mo and show that they have a superior metabolic fitness (both in glycolysis and mitochondrial respiration).

2) They claim differences in metabolism to be responsible for the district behavior of the AM precursors studied here. While the data suggest this to be the underlying mechanism as to why monocytes are 'better', it is equally possible, that because monocytes are better this is the phenotype they show. It's a cause and effect question and as such the finding is purely descriptive.

Authors: Thank you for asking this point. We basically agree with the referee on the cause and effect question. However, we think that this is a fundamental problem of the entire field of immunometabolism research rather than a weakness of our study. The large majority of publications in (immune) metabolism (including the high impact ones) are descriptively applying the cause and effect argument.

For example, is the switch from oxidative phosphorylation to glycolysis the cause or consequence of T cell proliferation. We think that this is sort of the hen and egg problem.

3) The Csf2ra data in Fig 4 are very interesting. However, to state that CSF2 sensitivity is 'likely' not involved is an overstatement. This reviewer would suggest that they measure pStat5 in response to limiting dilutions of GM-CSF.

Authors: Thank you for pointing this out. We previously showed reduced expression of Csf2ra in pMΦ compared to fetal Mo in the E17.5 lung (Schneider et al 2014). Moreover, van de Laar et al showed low surface expression of Csf2ra on yolk sac pMΦ, in contrast to fetal lung monocytes. Consistently, they showed reduced pSTAT5 signalling in yolk sac pMΦ in response to titrating amounts of GM-CSF. We think that there is no reason to repeat this experiment, because we will very likely get the same result. However, we argue that the reduced expression of Csf2ra on pMΦ does probably not explain their inability to fully reconstitute AM development. We found that Csf2ra surface expression is comparable 3-4 weeks after transfer of pMΦ and fetal monocytes. Despite, transfer of pMΦ failed to completely restore AM development within one year (50% reduction), while transfer fetal Mo resulted in 100% restorage of the AM population within 9-10 weeks after transfer. Thus, even though pMΦ may be disadvantaged compared to fetal Mo due to reduced expression of Csf2ra in the first 1-2 weeks after transfer, they should be able to fully reconstitute with a delay of 1-3 weeks compared to fetal Mo (i.e. 10-13 weeks after transfer).

Importantly, the difference in metabolism of pMΦ and fetal Mo was measured in the E14.5 fetal liver, where GM-CSF is not expressed and Csf2ra expression levels only slightly reduced on pMΦ compared to fetal Mo. This is the main argument, why we believe that Csf2ra expression levels are not responsible for the differences in developmental capacity. But we are happy to tone down this statement by saying that "… the difference in Csf2ra expression levels may contribute to but is certainly not the only reason for poor developmental capacity of pMΦ" (please see underlined text in the result and discussion part).

2nd Editorial Decision 16th Nov 2019

Thank you for submitting your revised manuscript. I have now had a chance to take a look at it and I happy with the revisions. I am therefore very pleased to accept the manuscript for publication here.

Before I can send you the formal accept letter there are just a few editorial points to be sorted out. You can use the link below to re-submit the manuscript.

2nd Revision - authors' response 20th Nov 2019

The authors performed the requested editorial changes.

3rd Editorial Decision 26th Nov 2019

Thanks for sending us your final version. I have now had a chance to take a look at it and all looks good.

I am therefore very pleased to accept the manuscript for publication here.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Manfred Kopf
Journal Submitted to: EMBOJ
Manuscript Number: EMBOJ-2019-103205

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 justified
- 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 $\chi 2$ 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?	At least 3 mice was used for each experiment. The number of mice and statistical evaluation of the data are described in each figure legend.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	The biological replicates and statistical methods is stated in the figure legend for each experiment.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No mice were excluded.
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.	Yes. To compare the proliferation capacity of primitive macrophages and fetal monocytes, we transferrd them in same neonatal mice to minimize the influences of different developmental environments.
For animal studies, include a statement about randomization even if no randomization was used.	Mice were randomly chosen to use as untreated or treated group.
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.	Yes. Both male and female mice are included in untreated and treated groups to minimize the gender influences.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Blinding was not used in this study.
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. The test used for each experiment is indicated in figure legend.
Is there an estimate of variation within each group of data?	Yes. The variation of each group of data was estimated calculating its standard error of the mean (SEM) as indicated in the figure legends.
Is the variance similar between the groups that are being statistically compared?	Yes

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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).	All antibodies (clone number) were used for this study were described in material and methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* 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.	C57BL/6 CD45.2 and congenic CD45.1 mice were originally from the Jackson Laboratory. Csf2ra-/- mice were recently established in our laboratory (Schneider et al, 2017). Csf2rb-/- mice (Stanley et al, 1994) were originally provided by A. Dunn, Ludwig Institute for Cancer Research, Victoria, Australia, and backcrossed to C57BL/6 in our facility. Myb+/- mice were provided by J. Frampton at Birmingham University Medical School. All mice were housed and bred under specific pathogen-free conditions in individually ventilated cages in a controlled day-night cycle at the ETH Phenomics Facility and were used for experiments on 6-12 weeks (adults) unless otherwise stated.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal experiments were performed according to Swiss animal protection law (TschG) and had been approved by the local animal ethics committee (cantonal veterinary office).
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.	Complied with the guidelines.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
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.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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.	NA
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.	NA

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	The mouse RNA-seq data first reported in this study are available at the Gene Expression Omnibus (GEO) repository under the accession number GSE140645 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE140645).
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).	NA
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).	NA
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.	NA

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.	No