EMBO reports

Chronic inflammation decreases HSC fitness by activating the druggable Jak/Stat3 signaling pathway

Srdjan Grusanovic, Petr Danek, Maria Kuzmina, Miroslava Adamcova, Monika Burocziova, Romana Mikyskova, Karolina Vanickova, Sladjana Kosanovic, Jana Pokorna, Milan Reinis, Tomas Brdicka, and Meritxell Alberich Jorda
DOI: 10.15252/embr.202254729

Corresponding author(s): Meritxell Alberich Jorda (alberich@img.cas.cz)

Review Timeline:

Submission Date:	25th Jan 22
Editorial Decision:	14th Feb 22
Revision Received:	13th Sep 22
Editorial Decision:	7th Oct 22
Revision Received:	13th Oct 22
Accepted:	24th Oct 22

Editor: Achim Breiling

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. 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 **14th Feb 2022**

Dear Dr. Alberich Jorda,

Thank you for the transfer of your research manuscript to EMBO reports. I have now received the reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, the referees think that these findings are of interest. However, all referees have several comments, concerns and suggestions, indicating that a major revision of the manuscript is necessary to allow publication of the study in EMBO reports. As the reports are below, and all their points need to be addressed, I will not detail them here.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript or in the detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision. Please contact me to discuss the revision should you need additional time.

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).

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and tables), but without the figures included. Please make sure that changes are highlighted to be clearly visible. Figure legends should be compiled at the end of the manuscript text.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures and EV figures. Please upload these as separate, individual files upon re-submission.

The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf file labeled Appendix. The Appendix should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

For more details, please refer to our guide to authors:
http://www.embopress.org/page/journal/14693178/authorguide#manuscriptpreparation

Please consult our guide for figure preparation:
http://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf

See also the guidelines for figure legend preparation:
https://www.embopress.org/page/journal/14693178/authorguide#figureformat

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.

Please also follow our guidelines for the use of living organisms, and the respective reporting guidelines:
http://www.embopress.org/page/journal/14693178/authorguide#livingorganisms

5) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq, structural and array data) are deposited in an appropriate public database. If no primary datasets have been deposited, please also state this in a dedicated section (e.g. 'No primary datasets have been generated and deposited'), see below.

See also: http://embor.embopress.org/authorguide#datadeposition

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Methods) that follows the model below. This is now mandatory (like the COI statement). 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])

*** Note - All links should resolve to a page where the data can be accessed. ***

Moreover, I have these editorial requests:

6) We strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

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

8) Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments were performed, if these were biological or technical replicates, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable, and also add a paragraph detailing this to the methods section. See: http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

9) Please also note our reference format:
http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

10) For microscopic images, please add scale bars of similar style and thickness to all the microscopic images, 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.

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 update your competing interests if necessary. Please name this section 'Disclosure and Competing Interests Statement'.

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

Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Yours sincerely,

Achim Breiling
Editor

EMBO Reports

Referee #1:

In this manuscript, Grusanovic and colleagues explore the effects of chronic sterile inflammation on hematopoietic stem cell (HSC) numbers and function and the molecular mechanisms underlying potential detrimental effects of inflammation on the hematopoietic system. To do so, they study a mouse model (CMO) of chronic recurrent multifocal osteomyelitis (CMO), an autoimmune condition known to induce long-term systemic inflammation. They report the following findings:
- Development of disease and chronic inflammation in CMO mice results in increased bone marrow (BM) cellularity, granulocyte content, as well as the relative frequencies and absolute numbers of HSPCs. Nonetheless, HSC function is significantly decreased as revealed by limiting dilution transplantation.
- Defective HSC function in CMO mice is partially induced by hematopoietic cells but also the stromal microenvironment in CMO mice. HSCs temporarily exposed to the BM of CMO mice exhibit impaired functionality in transplantation assays.
- MyD88 deficiency partially mitigates the effects of CMO on hematopoiesis, as it prevents increases in BM cellularity and granulopoiesis. However, elevated increases in HSC frequencies and numbers are partially preserved.
- RNAseq analysis reveals that HSCs exposed to the CMO BM microenvironment upon transplantation exhibit upregulated transcriptional programs related to inflammatory signatures, including the IL-6/Jak/Stat3 pathways.
- IL-6 is elevated in plasma of CMO mice. HSCs from CMO mice or transplanted into CMO BM have increased levels of pStat3. Pharmacological blockage of pStat3 normalizes HSC numbers in CMO mice.
- MLL-AF9 leukemic blast growth in vitro is enhanced in the presence of IL-6, while blockage of pStat3 decreases CFU of leukemic blasts. Transplantation of MLL-AF9 blasts into CMO mice results in accelerated development of AML

The study focuses on a relevant and interesting topic, namely the effects of chronic inflammation on HSCs. The methods are sound, and the manuscript is well written in a clear fashion. The data presented robustly demonstrate that in the chronic inflammatory setting of the BM of CMO mice, HSCs experience a gradual loss of function. These findings come to nicely add to previous evidence suggesting that long term exposure to inflammatory conditions impairs HSC fitness in non-sterile, infectious settings.
Nonetheless, there are some relevant problems with the manuscript as it stands.

• The fundamental and primary issue is that the authors fail to provide evidence for the principal premise of the paper as stated in the title, which is that the decreased HSC fitness in the CMO model is mediated via the IL-6/ Stat3 pathway. Indeed, evidence presented in this direction is only correlative but no direct demonstration that IL-6 nor pStat3 signaling are involved in the loss of HSC fitness is presented. The authors demonstrate that IL-6 and pStat3 are both increased in HSCs in the CMO BM and that in vivo blockage of pStat3 restores normal numbers of HSCs. However, whether IL-6 plays a direct and principal role in the observed effects remains unknown. Similarly if pStat3 signaling mediates the loss of HSC function is not addressed, and only effects of pStat3 blockage on HSC numbers are measured. To fully support their claims, the authors should, at a minimum demonstrate that in absence of IL-6 (IL6-/- mice, pharmacological blockage of IL-6 or through the use of HSCs defective in IL-6 signaling), HSC numbers and function are rescued in the CMO model. Given that Stat3 can be activated through numerous cytokine receptors, the authors should also demonstrate that increased Stat3 phosphorylation in HSCs of CMO mice is restored to normal levels in absence of IL-6 activation. Finally, the function of HSCs in mice developing CMO treated with Stattin should be tested in limiting dilution assays. Without these experiments the statement that HSC fitness is impaired through the IL-6/Jak/stat3 pathway is clearly not supported.

• Also related to the previous point, the authors should show in Figure 5 whether Stat3 inhibition rescues total BM cellularity and granulocyte counts in the BM of CMO mice. These effects seemed to be mechanistically decoupled from effects in HSCs as shown by experiments in Figure 2, so in principle Stattin treatment would potentially only target changes in HSCs. Since these experiments are done, it would be very informative to present the data.

• Given that the inflammatory signature is imprinted in HSCs upon temporal engraftment in CMO mice, the authors should determine whether IL-6 levels are increased in CMO BM upon transplantation.

• The authors claim that the effects on HSCs occur on an IL-b independent fashion based on the observations that in Myd88-/- mice, HSC numbers seem to be elevated. Yet, again, HSC function is not tested in these mice. Therefore, to claim that loss of HSC fitness in this model is independent of IL-1, would require testing the function of Myd88-deficient HSCs during CMO with limiting dilution transplantations. Also, do MyD88-/- mice have increased BM IL-1 levels during CMO?

• Related to this point, the data in Figure 3N seem to suggest that the increases of MPPs and HSCs elicited by CMO are mitigated in MyD88-deficient mice, even though the authors claim that no differences are observed between both cohorts. Statistical significance between CMO and CMOMyD88-/- groups, should be tested and shown.

• The authors use myeloablative conditioning for transplantation studies to address the specific role of the BM microenvironment. While control mice are always employed, it is unclear whether defective BM tissues in CMO mice or underlying inflammation may alter/delay the reconstitution of BM post transplantation and add confounding effects. While I understand this maybe difficult to

avoid and non-myeloablative transplantations can be technically challenging, it could be worth acknowledging this potential pitfall in the discussion.

• Finally, although the data presented in Figure 6 suggests that an accelerated development of leukemogenesis in CMO mice, it fails to support that this happens through IL-6 and/or Stat3 signaling. Which inflammatory mediators are responsible for this effect remains unknown and it is also unclear to what extent the effect depends on direct activation of leukemic cells or a decreased fitness of endogenous HSCs.

Other comments:

• In figure 2B instead of % of responders, the authors should consider showing chimerism levels for all individual recipient mice in both groups. How many mice were transplanted in these experiments?
• In Figure 5 A, only serum levels of IL-6 are shown, whereas levels of Il-6 in BM plasma would be more informative. A recent study shows that in fact during chronic LPS stimulation, CXCL12 abundant reticular cells of the BM are the main source of IL-6 acting on HSCs (Gerosa et al. Blood Advances 2021). Therefore, it would be important to confirm that IL-6 is also increased locally.
• The shifts in pStat3 signals in Figure 5C and 5E are extremely subtle, whereas the increased levels of pStat3 in progenitors is very clear as shown in the western blot in Figure S5B. Is the delta in the signal on LSK cells larger than for HSCs by flow cytometry?

Referee #2:

Inflammatory cytokines are well known to affect hematopoietic stem cells, with many studies showing an increase in HSC number, increase in cell division, and loss of self-renewal potential. In this paper Grusanovic et al evaluate the properties of HSCs in a mouse model of chronic inflammation, the CMO mouse. This is a particular model of inflammation that is reported to mimic human chronic multifocal osteomyelitis (CRMO) which is a poorly defined syndrome in humans without a clear molecular basis. IL1 and IL6 are persistently elevated in these mice. Consistent with prior studies, the authors find increased HSC numbers and reduced repopulation activity. While most of the paper confirms findings from prior studies, one contribution of the paper is showing that IL1 is not required to mediate effects on HSCs in this model; rather IL6-mediated Stat3 phosphorylation is critical to cause changes in HSC number. The authors go on to apply this finding to leukemia by studying MLL-AF9 driven leukemia in the CMO mouse. They show that the CMO background accelerates leukemia and shortens survival. This finding is rather preliminary and needs further studies to strengthen the connection with IL6. Overall, the paper mostly confirms the findings of prior reports but does separate out the effects of IL1 and IL6 in this very specific model of mouse inflammation.

Specific comments:

In figure 3M-N, the HSCs and MPPs remained expanded but the absolute number seems less. Is there a statistical difference between the CMO and CMO/MyD88?

In Figure 3, the increase in HSC number is somewhat intact even in the absence of MyD88, but is the functional defect still present? Transplants should be done, even if not limiting dilution.

Similar to the above comment, the Stattic treatment in Figure 5K reduces phenotypic HSC expansion, but does it also restore HSC self-renewal capacity? This is a more consequential outcome that the number of phenotypic HSCs. Transplants should be done to address this question.

Others have shown that IL6 can accelerate leukemia (see review https://www.frontiersin.org/articles/10.3389/fonc.2017.00265/full) The key point here is whether the MLL-AF9 driven leukemia is accelerated by IL6, or if this is an effect of some combination of cytokines elevated in the CMO mouse. Can STATIC rescue survival in the CMO mice with leukemia?

Referee #3:

Grusanovic et al. show that HSCs in the BM during chronic inflammation (CMO) have limited reconstitution potential and myeloid skewing. They propose that this occurs through IL6/STAT3 signaling axis. Indeed, by limiting STAT3 activation, they show that blood progenitors and marrow cellularity in CMO is mostly restored. The experiments are well-thought out, controlled, and statistically sound, however, the authors may have over interpreted several of the observations in the latter figures to fit a narrative. However, overall, this is sound body of work, but a few additional experiments and work would be encouraged to validate the significance and impact of these findings as well as strengthen the observations.

Comments to address:
Figure 1: What about MPP3 as these are myeloid biased as well as downstream GMPs?
Figure 3: Confirm that IL1b upregulated and that this signaling axis is found in HSPCs. MyD88 plays a role in several signaling axis and isn't unique to IL1b? In fact, substantial literature implicates the microbiota in inflammatory syndromes, perhaps this is the mechanism that's being blocked in MyD88 deficient cells?
Figure 3 is lacking controls for MyD88 deficient mice without CMO, that also have a distinct HSPC and myeloid phenotype.
Studying the marrow niche in irradiated mice is difficult, see Severe et al., Cell Stem Cell, 2019. Please explain how these data are interpreted in light of this destructive force to the niche.
Figure 5: C-F appear to be over interpreted based on the intracellular flow, could this data be supported with STAT3 target genes using qPCR for difference between WT and CMO exposure?
Figure 5 I: Stattic treatment of WT mice, does that also impact HSC numbers and pSTAT3 in HSPCs?
Figure 6: this seems to be an add on to the paper and distracts from the overall story. The complexity of MLL-AF9 HPCs, the microenvironment, and inflammatory cytokines confuse the goals of this manuscript. Might the authors consider removing this work for another submission?

Point-by-point response to reviewers' comments:

We appreciate the careful analysis of our manuscript by the reviewers and we want to thank them for the time they spent reviewing our manuscript. We believe that their constructive suggestions helped us to improve our manuscript. As requested by the reviewers, we have performed additional experiments and addressed their suggestions as described in detail below. With these revisions, we believe that our manuscript has been substantially strengthened.

Reviewer Comments:

Referee #1:

In this manuscript, Grusanovic and colleagues explore the effects of chronic sterile inflammation on hematopoietic stem cell (HSC) numbers and function and the molecular mechanisms underlying potential detrimental effects of inflammation on the hematopoietic system. To do so, they study a mouse model (CMO) of chronic recurrent multifocal osteomyelitis (CMO), an autoimmune condition known to induce long-term systemic inflammation. They report the following findings:

- Development of disease and chronic inflammation in CMO mice results in increased bone marrow (BM) cellularity, granulocyte content, as well as the relative frequencies and absolute numbers of HSPCs. Nonetheless, HSC function is significantly decreased as revealed by limiting dilution transplantation.

- Defective HSC function in CMO mice is partially induced by hematopoietic cells but also the stromal microenvironment in CMO mice. HSCs temporarily exposed to the BM of CMO mice exhibit impaired functionality in transplantation assays.

- MyD88 deficiency partially mitigates the effects of CMO on hematopoiesis, as it prevents increases in BM cellularity and granulopoiesis. However, elevated increases in HSC frequencies and numbers are partially preserved.

- RNAseq analysis reveals that HSCs exposed to the CMO BM microenvironment upon transplantation exhibit upregulated transcriptional programs related to inflammatory signatures, including the IL-6/Jak/Stat3 pathways.

- IL-6 is elevated in plasma of CMO mice. HSCs from CMO mice or transplanted into CMO BM have increased levels of pStat3. Pharmacological blockage of pStat3 normalizes HSC numbers in CMO mice.

- MLL-AF9 leukemic blast growth in vitro is enhanced in the presence of IL-6, while blockage of pStat3 decreases CFU of leukemic blasts. Transplantation of MLL-AF9 blasts into CMO mice results in accelerated development of AML

The study focuses on a relevant and interesting topic, namely the effects of chronic inflammation on HSCs. The methods are sound, and the manuscript is well written in a clear fashion. The data presented robustly demonstrate that in the chronic inflammatory setting of the BM of CMO mice, HSCs experience a gradual loss of function. These findings come to nicely add to previous evidence suggesting that long term exposure to inflammatory conditions impairs HSC fitness in non-sterile, infectious settings.

Nonetheless, there are some relevant problems with the manuscript as it stands.

• The fundamental and primary issue is that the authors fail to provide evidence for the principal premise of the paper as stated in the title, which is that the decreased HSC fitness in the CMO model is mediated via the IL-6/ Stat3 pathway. Indeed, evidence presented in this direction is only

correlative but no direct demonstration that IL-6 nor pStat3 signaling are involved in the loss of HSC fitness is presented. The authors demonstrate that IL-6 and pStat3 are both increased in HSCs in the CMO BM and that in vivo blockage of pStat3 restores normal numbers of HSCs. However, whether IL-6 plays a direct and principal role in the observed effects remains unknown. Similarly if pStat3 signaling mediates the loss of HSC function is not addressed, and only effects of pStat3 blockage on HSC numbers are measured. To fully support their claims, the authors should, at a minimum demonstrate that in absence of IL-6 (IL6-/- mice, pharmacological blockage of IL-6 or through the use of HSCs defective in IL-6 signaling), HSC numbers and function are rescued in the CMO model. Given that Stat3 can be activated through numerous cytokine receptors, the authors should also demonstrate that increased Stat3 phosphorylation in HSCs of CMO mice is restored to normal levels in absence of IL-6 activation. Finally, the function of HSCs in mice developing CMO treated with Stattin should be tested in limiting dilution assays. Without these experiments the statement that HSC fitness is impaired through the IL-6/Jak/stat3 pathway is clearly not supported.

We thank the reviewer for these comments and agree that these are key aspects in our manuscript. Thus, as suggested by the reviewer we addressed them experimentally.

On one hand, CMO mice were treated with Stattic (STAT3 inhibitor) and the HSC function was assessed in limiting dilution assays. We observed that CMO HSC function was restored when Stat3 activity was reduced. This result has been included in the revised Figure 7F-G and presented in the Results section (page 14).

On the other hand, CMO mice were treated with two distinct agents blocking IL-6 activity: (1) IL-6 blocking antibody and (2) IL-6 receptor blocking antibody. After treatment, animals were sacrificed, HSCs enumerated, transplanted in a limiting dilution manner into lethally irradiated WT recipient mice, and pStat3 levels were determined. We observed that both approaches were able to reduce the HSC numbers in CMO mice. However, blocking IL-6 activity was not sufficient to rescue the HSC fitness, as determined by limiting dilution transplantation assays. Accordingly, pStat3 levels remained elevated after treatment with IL-6 or IL-6 receptor blocking antibodies. These results have been included in Figure S7A-C and presented in the Results section (page 13).

Altogether, these new results support our statement that the decreased HSC fitness in the CMO model is mediated via the Jak/Stat3 signaling pathway, but did not support that IL-6 would be the unique cytokine activating Stat3 and mediating the reduced HSC fitness. Thus, we have incorporated this important conclusion in the Discussion section (page 17-18) and modified the title of the manuscript accordingly:

Old title: Chronic inflammation decreases HSC fitness via hyperactivation of the druggable IL-6/Jak/Stat3 signaling pathway

New title: Chronic inflammation decreases HSC fitness via hyperactivation of the druggable Jak/Stat3 signaling pathway

• Also related to the previous point, the authors should show in Figure 5 whether Stat3 inhibition rescues total BM cellularity and granulocyte counts in the BM of CMO mice. These effects seemed to be mechanistically decoupled from effects in HSCs as shown by experiments in Figure 2, so in principle

Stattin treatment would potentially only target changes in HSCs. Since these experiments are done, it would be very informative to present the data.

Indeed, as suggested by the reviewer, the BM cellularity and the number of granulocytes were not diminished upon Stattic treatment, supporting the idea that mechanistically these effects are mediated by a different pathway than the HSC expansion. These data have been included in the results section and revised Figure 7B-C and Figure S7D-E.

• Given that the inflammatory signature is imprinted in HSCs upon temporal engraftment in CMO mice, the authors should determine whether IL-6 levels are increased in CMO BM upon transplantation.

As suggested by the reviewer, we assessed IL-6 levels in CMO BM upon transplantation. We observed that, although statistically not significant, there was a tendency to preserve the increased IL-6 levels in CMO recipients. This observation also points out at the possibility that other cytokines are responsible for the activation of the Jak/Stat3 signaling pathway in HSCs under CMO conditions. These results have been included in Figure S6A, results, and discussion section (page 17-18).

• The authors claim that the effects on HSCs occur on an IL-b independent fashion based on the observations that in Myd88-/- mice, HSC numbers seem to be elevated. Yet, again, HSC function is not tested in these mice. Therefore, to claim that loss of HSC fitness in this model is independent of IL-1, would require testing the function of Myd88-deficient HSCs during CMO with limiting dilution transplantations. Also, do MyD88-/- mice have increased BM IL-1 levels during CMO?

We thank the reviewer for raising up this important point. As suggested, we performed limiting dilution transplantation assays using HSC isolated from CMO Myd88-deficient mice. In agreement with the elevated HSC numbers, we observed that the HSC functional defect is still present in CMO Myd88-deficient mice. These results support our statement that the effects on CMO HSCs occur in an IL1-b independent fashion, and have been included in Figure 4I-J, Figure S4C, and Results section page 10.

Increased IL-1β levels can be detected in paws, but not in BM, of CMO mice (see revised Figure S3A). Interestingly, CMO MyD88 deficient mice did not exhibited increased levels of IL-1β neither in BM or paws (see revised Figure S4A), and accordingly, all inflammatory signs were absent in CMO MyD88 KO mice. However, HSC numbers were not reduced in CMO MyD88 KO animals, supporting our claim that a different cytokine/s are mediating this expansion. These results have been included in the indicated figures and Results section page 9-10.

• Related to this point, the data in Figure 3N seem to suggest that the increases of MPPs and HSCs elicited by CMO are mitigated in MyD88-deficient mice, even though the authors claim that no differences are observed between both cohorts. Statistical significance between CMO and CMOMyD88-/- groups, should be tested and shown.

As suggested by the reviewer the statistical significance has been included in the new version of the figure. Please see revised Figure 4H and revised Figure S4B. Further, the text has been revised for accuracy (page 10).

• The authors use myeloablative conditioning for transplantation studies to address the specific role of the BM microenvironment. While control mice are always employed, it is unclear whether defective BM tissues in CMO mice or underlying inflammation may alter/delay the reconstitution of BM post transplantation and add confounding effects. While I understand this maybe difficult to avoid and non-myeloablative transplantations can be technically challenging, it could be worth acknowledging this potential pitfall in the discussion.

We agree with the reviewer and we are aware that the irradiation might introduce additional effects on our experimental setting. Therefore, as suggested by the reviewer this potential pitfall has been acknowledge in the Discussion (page 19).

• Finally, although the data presented in Figure 6 suggests that an accelerated development of leukemogenesis in CMO mice, it fails to support that this happens through IL-6 and/or Stat3 signaling. Which inflammatory mediators are responsible for this effect remains unknown and it is also unclear to what extent the effect depends on direct activation of leukemic cells or a decreased fitness of endogenous HSCs.

Based on this comment and the remarks from the other reviewers, we decided to exclude original Figure 6 from our revised manuscript. We agree with the reviewers that the leukemic part is not sufficiently developed and will be addressed in a future manuscript.

Other comments:

• In figure 2B instead of % of responders, the authors should consider showing chimerism levels for all individual recipient mice in both groups. How many mice were transplanted in these experiments?

As suggested by the reviewer Figure 2B has been updated to show engraftment of all individual transplanted mice (n=11 WT recipients and n=12 CMO recipients).

• In Figure 5 A, only serum levels of IL-6 are shown, whereas levels of Il-6 in BM plasma would be more informative. A recent study shows that in fact during chronic LPS stimulation, CXCL12 abundant reticular cells of the BM are the main source of IL-6 acting on HSCs (Gerosa et al. Blood Advances 2021). Therefore, it would be important to confirm that IL-6 is also increased locally.

We thank the reviewer for this comment, and as suggested we determined IL-6 levels in BM. In agreement with our IL-6 serum ELISA, we observed that the levels of IL-6 are locally increased in CMO BM. This result has been included in revised Figure 6A and Results section page 12.

• The shifts in pStat3 signals in Figure 5C and 5E are extremely subtle, whereas the increased levels of pStat3 in progenitors is very clear as shown in the western blot in Figure S5B. Is the delta in the signal on LSK cells larger than for HSCs by flow cytometry?

The delta signal was less pronounced in HSCs compared to cKit+ cells. Nevertheless, the poorly illustrative histograms in the original submission have been replaced by an appropriate representative plot. In the revised Figure 6C and revised Figure 6E we selected distinct samples to better illustrate the results. In addition, to support our observations, Stat3 target gene expression was determined by RT-PCR in WT LSKs exposed to WT and CMO recipients. In line with the increased pStat3 levels in cells exposed to CMO recipients, we observed increased expression of Stat3 target

genes (see revised Figure S6B).

Referee #2:

Inflammatory cytokines are well known to affect hematopoietic stem cells, with many studies showing an increase in HSC number, increase in cell division, and loss of self-renewal potential. In this paper Grusanovic et al evaluate the properties of HSCs in a mouse model of chronic inflammation, the CMO mouse. This is a particular model of inflammation that is reported to mimic human chronic multifocal osteomyelitis (CRMO) which is a poorly defined syndrome in humans without a clear molecular basis. IL1 and IL6 are persistently elevated in these mice. Consistent with prior studies, the authors find increased HSC numbers and reduced repopulation activity. While most of the paper confirms findings from prior studies, one contribution of the paper is showing that IL1 is not required to mediate effects on HSCs in this model; rather IL6-mediated Stat3 phosphorylation is critical to cause changes in HSC number. The authors go on to apply this finding to leukemia by studying MLL-AF9 driven leukemia in the CMO mouse. They show that the CMO background accelerates leukemia and shortens survival. This finding is rather preliminary and needs further studies to strengthen the connection with IL6. Overall, the paper mostly confirms the findings of prior reports but does separate out the effects of IL1 and IL6 in this very specific model of mouse inflammation.

Specific comments:

In figure 3M-N, the HSCs and MPPs remained expanded but the absolute number seems less. Is there a statistical difference between the CMO and CMO/MyD88?

We thank the reviewer for noticing the missing statistical analysis. As suggested, the analysis has been included in the new version of the figure. Please see revised Figure 4G and 4H and revised Figure S4B. Further, the text has been revised for accuracy (page 10).

In Figure 3, the increase in HSC number is somewhat intact even in the absence of MyD88, but is the functional defect still present? Transplants should be done, even if not limiting dilution.

We thank the reviewer for raising up this important point. As suggested, we performed limiting dilution transplantation assays using HSCs isolated from CMO Myd88-deficient mice. In agreement with the elevated HSC numbers, we observed that the HSC functional defect is still present in CMO Myd88-deficient mice. These results support our statement that the effects on CMO HSCs occur in an IL-1b independent fashion, and have been included in Figure 4I-J, Figure S4C, and Results section page 10.

Similar to the above comment, the Stattic treatment in Figure 5K reduces phenotypic HSC expansion, but does it also restore HSC self-renewal capacity? This is a more consequential outcome that the number of phenotypic HSCs. Transplants should be done to address this question.

We agree with the reviewer that this is an important point and we have performed the suggested experiment. CMO mice were treated with Stattic and the HSC function was assessed in limiting

dilution assays. We observed that CMO HSC function was restored when Stat3 activity was reduced. This result has been included in Figure 7F-G, presented in the Results section (page 14), and integrated in the Discussion (page 18).

Others have shown that IL6 can accelerate leukemia (see review https://www.frontiersin.org/articles/10.3389/fonc.2017.00265/full) The key point here is whether the MLL-AF9 driven leukemia is accelerated by IL6, or if this is an effect of some combination of cytokines elevated in the CMO mouse. Can STATIC rescue survival in the CMO mice with leukemia?

Based on this comment and the remarks from the other reviewers, we decided to exclude original Figure 6 from our revised manuscript. We agree with the reviewers that the leukemic part is not sufficiently developed and will be addressed in a future manuscript.

Referee #3:

Grusanovic et al. show that HSCs in the BM during chronic inflammation (CMO) have limited reconstitution potential and myeloid skewing. They propose that this occurs through IL6/STAT3 signaling axis. Indeed, by limiting STAT3 activation, they show that blood progenitors and marrow cellularity in CMO is mostly restored. The experiments are well-thought out, controlled, and statistically sound, however, the authors may have over interpreted several of the observations in the latter figures to fit a narrative. However, overall, this is sound body of work, but a few additional experiments and work would be encouraged to validate the significance and impact of these findings as well as strengthen the observations.

Comments to address:

Figure 1: What about MPP3 as these are myeloid biased as well as downstream GMPs?

We thank the reviewer for this comment and we performed flow cytometric analysis to address this point. In line with the increase of granulocytes shown in the CMO mice, we observed an expansion of the MPP3, the earliest myeloid precursor cells, and the downstream population GMP. These results have been included in revised Figure 1E-F, Figure S1A-B, and mentioned in the Results section (page 7).

Figure 3: Confirm that IL1b upregulated and that this signaling axis is found in HSPCs. MyD88 plays a role in several signaling axis and isn't unique to IL1b? In fact, substantial literature implicates the microbiota in inflammatory syndromes, perhaps this is the mechanism that's being blocked in MyD88 deficient cells?

Increased IL-1β levels can be detected in paws, but not in BM, of CMO mice (see revised Figure S3A), and accordingly WT and CMO HSCs exhibited similar levels of IkB-α, a negative modulator of the NF-KB signaling pathway (revised Figure S3B). Interestingly, paws from CMO MyD88 deficient mice do not exhibited increased levels of IL-1β (revised Figure S4A), and accordingly, all inflammatory signs are absent in CMO MyD88 KO mice. However, HSC numbers are not reduced in CMO MyD88 KO animals, supporting our claim that different cytokines are mediating this expansion. These results have been included in the revised Figure S3A-B, S4A, and the results section page 9-10.

Further, the reviewer brings up a very interesting point regarding the microbiota. A large body of evidence supports the role of microbiota in the pathogenesis of inflammatory disorders. For instance, IL-1 receptor antagonist knockout mice develop arthritis. Nevertheless, when mice were housed under germ free conditions the signs of arthritis were absent, but could be re-established by the reintroduction of specific microbes (Rogier et al, Microbiome, 2017). In the case of CMO mice, it was reported that they were protected from developing an inflammatory phenotype when fed with a diet high in fat and cholesterol, and that these effects were mediated by changes in the intestinal microbiota (Lukens et at, Nature, 2014). However, re-derivation of CMO mice under germ-free conditions is needed to provide conclusive proof that the microbiota is required to promote the inflammatory phenotype. In addition, whether the microbiota is involved in the HSC expansion and reduced function in the CMO mice, and whether the microbiota effects may occur in a MyD88 dependent manner, remains elusive. These aspects have been mentioned in the Discussion page 17-18.

Figure 3 is lacking controls for MyD88 deficient mice without CMO, that also have a distinct HSPC and myeloid phenotype.

We thank the reviewer for pointing out the absence of these controls, which have been included in the revised Figure 3 and revised Figure S3C. Remarkably, despite the fact that MyD88 deficiency in hematopoietic cells affects the myeloid compartment, CMO MyD88$^{flox/flox}$ VAVcre+ mice retained the expansion in the HSC compartment.

Studying the marrow niche in irradiated mice is difficult, see Severe et al., Cell Stem Cell, 2019. Please explain how these data are interpreted in light of this destructive force to the niche.

We agree with the reviewer that the irradiation introduces additional effects to our experimental setting. Therefore, as suggested this potential pitfall has been acknowledged in the Discussion (page 19).

Figure 5: C-F appear to be over interpreted based on the intracellular flow, could this data be supported with STAT3 target genes using qPCR for difference between WT and CMO exposure?

We thank the reviewer for this comment and as suggested we have added RT-PCR data on STAT3 target genes to make our observations stronger (see revised Figure S6B). In addition, we apologize for the poorly illustrative phospho-flow histograms in the original submission. In the revised Figure 6C and revised Figure 6E we selected distinct samples to better illustrate the results.

Figure 5 I: Stattic treatment of WT mice, does that also impact HSC numbers and pSTAT3 in HSPCs?

Indeed, this is an important control that was missing in our original submission and we thank the reviewer for pointing this out. We have now included treatment of WT mice with Stattic and analyzed HSC numbers and pSTAT3 activation. As shown in revised Figure S7H and S7J, the number of HSC was not significantly affected in WT mice treated with Stattic in comparison to WT mice treated with vehicle control. Accordingly, we observed that pSTAT3 levels were not affected in WT HSCs by the Stattic treatment (Figure S7K-L). The distinct effects of Stattic in WT and CMO mice can be explained by the different levels of Stat3 activation in WT and CMO HSCs, which would point at a therapeutic window. These results have been included in the indicated Figures, the results and discussion section (page 20).

Figure 6: this seems to be an add on to the paper and distracts from the overall story. The complexity of MLL-AF9 HPCs, the microenvironment, and inflammatory cytokines confuse the goals of this manuscript. Might the authors consider removing this work for another submission?

Based on this comment and the remarks from the other reviewers, we decided to exclude original Figure 6 from our revised manuscript. We agree with the reviewers that the leukemic part is not sufficiently developed and will be addressed in a future manuscript.

Dear Dr. Alberich Jorda

Thank you for the submission of your revised manuscript to our editorial offices. I have received the reports from the two referees that I asked to re-evaluate your study, you will find below. As you will see, both referees now support the publication of your study in EMBO reports. Referee #1 has some suggestions to improve the manuscript I ask you to address in a final revised version of the manuscript.

Moreover, I have these editorial requests:

- Please provide a more comprehensive title with not more than 100 characters (including spaces).

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

- Please add up to 5 key words to the title page, below the abstract.

- Please have your final manuscript carefully proofread by a native speaker (see also the comment by referee #1).

- 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 update your competing interests if necessary. Please name this section 'Disclosure and Competing Interests Statement' and put it after the Acknowledgements section.

- We now use CRediT to specify the contributions of each author in the journal submission system. CRediT replaces the author contribution section. Please use the free text box to provide more detailed descriptions. Thus, please remove the author contributions section from the manuscript text file. See also guide to authors:
https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines

- Please make sure your final manuscript file includes the "Data Availability section" (DAS - placed after Materials and Methods) that lists the deposited primary dataset, accession numbes and links to the database. Please remove any referee tokens and make sure the data is public latest upon publication of the manuscript.

- Please upload the supplemental figures (EV figures) as individual production quality figure files as .eps, .tif, .jpg (one file per figure) upon re-submission. Please name these Figure EVx and also use this as callout in the manuscript text file. Please combine the present 7 figures to have 5 final EV figures (like this, we do not need an Appendix file). Finally, please add the figure legends for these after the main figure legends (EV Figure legends).

- Please order the manuscript sections like this (using these names):
Title page - Abstract - Keywords - Introduction - Results - Discussion - Materials & Methods - DAS - Acknowledgements - Disclosure and Competing Interests Statement - References - Figure legends - Expanded View Figure legends.

- Table EV1 is a dataset. Please name this Dataset EV1 and upload the original excel file. Please put a title and a legend on the first TAB of the excel file. Finally, please change all callouts for this dataset to 'Dataset EV1' in the manuscript text file.

- Please make sure that the 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 figure legends (main, EV and Appendix figures), and that statistical testing has been done where applicable. Please avoid phrases like 'independent experiment', but clearly state if these were biological or technical replicates. Please add complete statistical testing to all diagrams (main and EV figures). Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant. In case n=2, please show the data as separate datapoints without error bars and statistics.

- Per journal policy, we do not allow 'data not shown', which is stated twice in the manuscript (pages 11 and 13). All data referred to in the paper should be displayed in the main or Expanded View figures, or an Appendix. Thus, please add these data (or change the text accordingly if these data are not central to the study). See:
http://embor.embopress.org/authorguide#unpublisheddata

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text. Please use the attached file as basis for further revisions and provide your final manuscript file with track changes, in order that we can see any modifications done.

In addition, I would need from you:
- a short, two-sentence summary of the manuscript (not more than 35 words).
- two to four short bullet points highlighting the key findings of your study (two lines each).

- a schematic summary figure (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.

Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Best,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

The authors have made a very good job in revising the manuscript, performing the requested experiments and reevaluating their conclusions in light of the new data generated. All my concerns have been adequately addressed.

I would still recommend:

1. A thorough revision to avoid grammatical mistakes (eg: "in line with these findings, CMO MyD88 KO mice did not **exhibited** increased levels of IL-1β"). Also, instead of "The CMO BM niche implants a myeloid..." I would use the term "imprints".

2. the authors could consider including updated citations to some relatively new studies on chronic inflammation and HSC fitness, in their initial paragraph of the discussion, along with other relevant manuscripts. For instance Bogeska et al. Cell Stem Cell 2022, or Isringhausen et al. JExpMed 2021

Referee #3:

This is an excellent example of a positive review process. The reviewers were able to make suggestions that significantly improved the manuscript and overall conclusions. The authors have addressed the concerns of this reviewer and many of the other comments as well. The manuscript should now be considered for publication in EMBO Reports.

The authors have addressed all minor editorial requests.

Dr. Meritxell Alberich Jorda
Institute of Molecular Genetics of the CAS
Hemato-oncology
Videnska 1083
Prague 4 142 20
Czech Republic

Dear Dr. Alberich Jorda,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case." Please note that the author checklist will still be published even if you opt out of the transparent process.

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Achim Breiling
Editor
EMBO Reports

****

THINGS TO DO NOW:

Please note that you will be contacted by Wiley Author Services to complete licensing and payment information. The required 'Page Charges Authorization Form' is available here: https://www.embopress.org/pb-assets/embo-site/er_apc.pdf - please download and complete the form and return to embopressproduction@wiley.com

EMBO Press participates in many Publish and Read agreements that allow authors to publish Open Access with reduced/no publication charges. Check your eligibility: https://authorservices.wiley.com/author-resources/Journal-Authors/open-access/affiliation-policies-payments/index.html

You will receive proofs by e-mail approximately 2-3 weeks after all relevant files have been sent to our Production Office; you should return your corrections within 2 days of receiving the proofs.

Please inform us if there is likely to be any difficulty in reaching you at the above address at that time. Failure to meet our deadlines may result in a delay of publication, or publication without your corrections.

All further communications concerning your paper should quote reference number EMBOR-2022-54729V3 and be addressed to emboreports@wiley.com.

Should you be planning a Press Release on your article, please get in contact with emboreports@wiley.com as early as possible, in order to coordinate publication and release dates.

EMBO Press Author Checklist

Corresponding Author Name: Meritxell Alberich-Jorda	**USEFUL LINKS FOR COMPLETING THIS FORM**
Journal Submitted to: EMBO Reports	The EMBO Journal - Author Guidelines
Manuscript Number: EMBOR-2022-54729	EMBO Reports - Author Guidelines
	Molecular Systems Biology - Author Guidelines
	EMBO Molecular Medicine - Author Guidelines

Reporting Checklist for Life Science Articles (updated January 2022)
This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.
Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for 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.
- 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.

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.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Material and Methods

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Not Applicable	

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Not Applicable	
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Yes	Material and Methods
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	Material and Methods

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Acknowledgements

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Figure legends and Material and Methods
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Yes	Material and Methods
Include a statement about **blinding** even if no blinding was done.	Yes	Material and Methods
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
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	Figure legends and Material and Methods

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figure legends
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figure legends

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
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	All experiments were approved by the ethical committee of the Institute
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	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
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.	Not Applicable	
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.	Not Applicable	

Data Availability

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)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Material and Methods
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	