EMBO reports

Type-2 innate signals are dispensable for skeletal muscle regeneration and pathology linked to DMD

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

Dear Kelly,

Thank you for the submission of your manuscript to EMBO reports. We have now received the full set of referee reports that is pasted below.

As you will see, all referees acknowledge that your findings are interesting and should be published. However, the referees also have several suggestions for how the study could be improved, and I would like to know whether you can address all of them? We can discuss the exact revision requirements further, also in a video chat, if you cannot or prefer not to address all comments. For example, I dont know how difficult it is to analyse the effect of eosinophil depletion or inhibiting STAT6 signaling in mouse models of muscle adipogenesis, as referee 2 suggests.

I am confident that we will be able to agree on a specific set of revisions, and I would thus like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of major revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (26th Sep 2024). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

You can either publish the study as a short report or as a full article. For short reports, the revised manuscript should not exceed 27,000 characters (including spaces but excluding materials & methods and references) and 5 main plus 5 expanded view figures. The results and discussion sections must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. For a normal article there are no length limitations, but it should have more than 5 main figures and the results and discussion sections must be separate. In both cases, the entire materials and methods must be included in the main manuscript file.

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When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure). See https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf for more info on how to prepare your figures.

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- the nature of the bars and error bars (s.d., s.e.m.),

- If the data are obtained from n<3, please use scatter blots showing all individual data points.

Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.

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12) All Materials and Methods need to be described in the main text using our 'Structured Methods' format, which is required for all research articles. According to this format, the Methods section includes a Reagents and Tools Table (listing key reagents, experimental models, software and relevant equipment and including their sources and relevant identifiers) followed by a Methods and Protocols section describing the methods using a step-by-step protocol format. The aim is to facilitate adoption of the methodologies across labs. More information on how to adhere to this format as well as a downloadable template (.docx) for the Reagents and Tools Table can be found in our author guidelines: https://www.embopress.org/page/journal/14693178/authorguide#structuredmethods.

An example of a Method paper with Structured Methods can be found here: https://www.embopress.org/doi/full/10.1038/s44320-024-00037-6#sec-4

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

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I look forward to seeing a revised form of your manuscript when it is ready.

Best regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

In the work by Messing et al, the authors re-investigate the role of eosinophils in skeletal muscle repair using two different murine model, one deficient in eosinophils (ΔdblGATA) and one deficient in IL-4/IL-13 signaling (Stat6-/-). The authors demonstrated with strong evidence that eosinophils and IL-4/IL-13 signaling are not necessary for muscle regenerative processes and are not involved in Duchenne muscular dystrophy pathophysiology, counteracting previous available data. The work is well structured and written.
However, I have some annotations:
in the line 48-50, the authors cited the type 1/2/3-immune responses. I suggest to better specifify the most important features of these 3 major types of innate and adaptive cell-mediated effector immunity, as highlighted for example in (10.1016/j.jaci.2014.11.001; J Allergy Clin Immunol. 2015 Mar;135(3):626-35. doi: 10.1016/j.jaci.2014.11.001.)
in the main figures 1 and 2, in the panel A and D the pictures related to T0 for both animal models is missing, as well as the experimental evidences concerning the body mass, TA mass/body mass, the cross sectional area (CSA), the number of SiglecF + cells per fibers. These data are fundamental to better evaluate the modifications of muscular phenotype/architecture in pathological mice after the muscular injury.
Similarly, in the last chapters of the results, the authors compare mdx:Stat6-/- mice to mdx:Stat6+/-. I suggest showing also the differences between mdx mice and mdx:Stat6-/- mice, in order to better understand the effects of the complete ablation of IL-4/IL-13 signaling in mice.

In the end, I have a curiosity. In a recently published paper from Jiang (Ital J Pediatr. 2023; 49: 83. 10.1186/s13052-023-01483-y), in a retrospective cohort study performed on 145 DMD patients between January 2012 and December 2020 the authors demonstrated that eosinophil count in peripheral blood was correlated with the severity of DMD. They suggest that these phenomena could indicate the therapeutic efficacy and prognosis of DMD patients to a certain extent, since eosinophils may be a potentially valuable biomarker or therapeutic target for DMD. Have you ever try to analyze the eosinophil count in peripheral blood of model mice and following the muscular injury?

Referee #2:

In their manuscript entitled, "Type-2 innate signaling is dispensable for skeletal muscle regeneration and pathology linked to Duchenne muscular dystrophy", Messing et al. set out to define the role of eosinophils and IL-4/IL-13 signaling in muscle regeneration and Duchenne muscular dystrophy (DMD). Using genetic approach to deplete eosinophils (dblGATA) or inhibit STAT6 signaling, they convincingly draw the conclusion that these two aspects of type 2 immunity do not influence muscle regeneration following acute injury nor the severity of muscle fibrosis in the mdx mouse model of DMD.

Overall, the experiments are well-designed to broadly assess the impact on muscle regeneration and fibrosis. Although the data are largely negative, it is still an important body of work to publish giving the complex role of type 2 innate immunity in tissue repair and fibrotic disease. The major concern with the manuscript is the over-generalized conclusion that type-2 innate immunity has no role when only two aspects (i.e. eosinophil and STAT6 signaling) of this complex immune response have been examined in this study. M2 macrophages, mast cells, ILC2s and Th2s are cells associated with type 2 immunity and have not

been addressed in this study. IL-4 can also activate PI3K and p38; these pathways were also not examined in the current study.

A further limitation of the study is an examination that was limited to regeneration and fibrosis. Other pathophysiological attributes of muscular dystrophy have not been assessed; membrane stability/repair, calcium homeostasis, and adipogenesis. The latter being particularly important given that prior studies have shown that eosinophils and type 2 cytokines inhibit adipogenic differentiation.

Before the manuscript can be considered further for publication, the authors need to revise the title of the manuscript to reflect what was studied (eosinophils and STAT6-signaling). Second, definitive statements of type 2 immunity and IL-4/IL-13 signaling throughout the manuscript need to be revised accordingly. Third, an examination of the effect of eosinophil depletion or inhibiting STAT6 signaling in mouse models of muscle adipogenesis (B6A/J mouse or glycerol-induced injury) is needed.

Further comments below:

1. The following statement does not take into consideration that other signal transduction pathways are activated downstream of the IL-4 and IL-13 receptor. In summary, we conclude that IL-4/IL-13 signaling is dispensable and does not play a significant role in muscle regeneration, inflammatory cell infiltration or muscle resident cell proliferation in response to injury. Limit the statement to STAT6 signaling.
2. Indicate in the figure legend the age of the mice when they underwent microdamage. How old were then when microdamage was initiated.
3. Some figure panels are not cited in the results (e.g. Figure 3E). Interestingly, a CSA analysis showed a small but significant reduction in the number of small fiber (CSA = 0-400 µm) in MD TAs of mdx:Stat6-/- compared to mdx:Stat6+/-.
4. A comparison of total STAT6 levels, phosphorylation levels and/or expression of STAT6 targets has not been done between STAT6 +/+ and STAT6+/- to rule out a gene dose effect (i.e. STAT6 are the same between STAT6 +/+ and STAT6+/- or is there an 'intermediate' loss of STAT6 that confounds the data).
5. 'Strings' appears to be a typo in line 283-284, linked to this hyper-responsive background may not be broadly extrapolatable to other mouse strings or human disease
6. This following is another example of overly interpreted statement, "type-2 innate signaling is likely not a relevant pathway for the development of targeted immunotherapeutics.
M2 macrophages and ILC2s are cellular components of the type 2 innate immunity, and they produce factors such as IGF1 and amphiregulin, which were not evaluated in this study. Thus, it is not appropriate to broadly conclude that type-2 innate signaling is likely not relevant in DMD. This can be easily addressed by limiting statements to what was specifically manipulated in this study (i.e. eosinophil and STAT6 signaling are likely not relevant).

Referee #3:

The manuscript titled Type-2 innate signaling is dispensable for skeletal muscle regeneration and
pathology linked to Duchenne muscular dystrophy described the absence of defect caused by the suppresion of eosinophil and IL4/IL13 signalling during muscle regeneration.

The authors show here, the dispensability of eosinophil/ IL4/IL13 signalling during regeneration in opposition with what Heredia et al. suggested in 2013. This previous work proposed that eosinophils plays a role in muscle repair by inhibiting FAPs differentiation throught IL4/IL13 signalling (Heredia et al., 2013). However this new work from Messing et al. brings opposite evidence showing that muscle repair in healthy and mdx context exerts no differences when eosinophils number is decrease or when IL4/IL13 signalling is downregulated.
For that purpose, the authors used different mice model, interestingly those models were also used in Heredia et al.,2013. The absence of eosinophils was visited with ΔdblGATA eosinophil-deficient mice as well as Stat6-/- mice were IL4/IL13 signalling was inhibited.
Surprisingly in both of those model WT or mdx background, the respective, deficiency in eosinophils and inhibition of Stat6 did not impact muscle regeneration. First, the authors presented regeneration characteristics of WT ΔdblGATA eosinophil-deficient mouse, where they validate a major decrease in eosinophil number without any significative impact on the regeneration. The same analysis structure has been reproduce using Stat6-/- mice where the same absence of effect has been shown. Messing et al., then proposed to investigated the importance of eosinophils or their signalling during pathological long-term inflammation/regeneration by crossing Stat6-/- mice with mdx background as well as using an α-IL5 neutralizing antibody to prevent differentiation and survival of eosinophils. Even if the mdx phenotype did not appeared very strong, the authors did not identify any major changes during muscle regeneration. The additonal injury by micro damaging the tissue as well as the aging process, are respectively in line with the previous observations and showed no differences with their respective controls. In this work Messing et al. brings strong evidences that eosinophils deficiency as well as the inhibition of their major downstream signalling does not affects muscle regeneration.

Overall, the work is well written and use a variety of differents technics that strengthens author's conclusion. I would also like to emphasize the structuration of the paper which helps with the understanding. The work provides good evidences and discussion in order to re-think the importance of eosinophil and their signalling during muscle regeneration. Hereafter are few comments that can clarify and maybe strenghens some of the conclusions.

Comments:

1. Overall the complete absence of effect on muscle regeneration is very surprising based on the impressive phenotype that Heredia et al. showed in their paper. One of the hypothesis proposed by the authors is the difference in term of mouse background between Balb/cJ and C57BL/6.
a. To what extent the authors believe that the mouse background has an importance on the phenotype differences? Do you have any supplementary data on Balb/c background which would support this hypothesis?

2. Based on the hypothesis that eosinophils might affect preferentially FAPs fate during muscle regeneration, are you able to quantify the fibrotic deposition in your different regenerative assay like the authors did in Figure 4G-H? Some of your images might show a slight increase which may be lowered because of C57BL/6 background.

3. According to literrature, eosinophils is one of the first population recruited on site (≃36h). It might be a good idea, to match your flow cytometry data, to add histological images at 3dpi or at an early time point to show a potential defect in early regeneration which might be compensate later at 7dpi by the global immune response.

4. In Figure 1F-G and 2F-G, this is surprinsing that only 50% of MP or FAPs are incorporating EdU especially at 3dpi. How can you explain the low amount of proliferating MP? Could that come from your previous gating VCAM/ITGa7 where VCAM signal seems dim? To strenghens the conclusion about MP and FAPs proliferation it might be interresting to show another way of quantification. Histological sections and a quantification of Pax7+ and PDGFRa+ cells numbers respectively can be implemented. EdU detection can also be done directly on the section after in vivo injection.

5. For both WT and mdx can the authors show the downregulation of Stat6 protein? Is Stat6-/- model a full body knock out? Can the defect in Stat6 expression affect MP and/or FAPs proliferation/fate independently of IL4/IL13 signalling? Does these populations have the same pattern of proliferation/differentiation in vitro than their respective control? Does this mice have a particular phenotype?

6. Globally, it would be nice if the author can present a WT control along with mdx mice to highlight the mdx phenotype. In this work, mdx background is used as chronic inflamatory model but no increase in eosinophils or macrophages number has been shown compare to the WT condition in Figure 2E and 3E.

7. According to the previous comment, the addition of an extra damage in mdx makes a lot of sense. Why the authors used a mild microdamage injury which might not trigger fully the inflammatory response and eosinophils activation (Desguerre et al., 2012)? Did the authors have data with stronger injury (cardiotoxin or BaCl2)? Do you think that the slight effect seen on fibers size might be more significant following that type of injury?

8. In Figure 3 and 4, in order to assess the regeneration process in MD mdx, α-IL5 treated mice and aging assay, it would be nice to show a quantification of MP and FAPs numbers, like the authors did in Figure 1 and 2.

Minor comments

1. The flow cytometry data presentation can be improve, please do not use density plot and add the used fluoropher next to the target.
2. In Figure 2d, the representative image of Stat6-/- at 28d staining might be improve in order to show the significant increase.

Reviewer comments and responses:

We thank the reviewers for their thoughtful review and comments on our manuscript. Below, we provide point-by-point responses to the reviews and have revised the manuscript accordingly. All major revisions in the manuscript are highlighted in red.

Referee #1:

In the work by Messing et al, the authors re-investigate the role of eosinophils in skeletal muscle repair using two different murine model, one deficient in eosinophils (ΔdblGATA) and one deficient in IL-4/IL-13 signaling (Stat6-/-). The authors demonstrated with strong evidence that eosinophils and IL-4/IL-13 signaling are not necessary for muscle regenerative processes and are not involved in Duchenne muscular dystrophy pathophysiology, counteracting previous available data. The work is well structured and written.

However, I have some annotations:

A- in the line 48-50, the authors cited the type 1/2/3-immune responses. I suggest to better specify the most important features of these 3 major types of innate and adaptive cell-mediated effector immunity, as highlighted for example in (10.1016/j.jaci.2014.11.001; J Allergy Clin Immunol. 2015 Mar;135(3):626-35. doi: 10.1016/j.jaci.2014.11.001.)

Thank you for this thoughtful suggestion. We have now adjusted the text, accordingly, starting on line 45.

B- in the main figures 1 and 2, in the panel A and D the pictures related to T0 for both animal models is missing, as well as the experimental evidences concerning the body mass, TA mass/body mass, the cross sectional area (CSA), the number of SiglecF+ cells per fibers. These data are fundamental to better evaluate the modifications of muscular phenotype/architecture in pathological mice after the muscular injury.

We have discussed this carefully among the investigators and in consultation with the EMBO Reports Handling Editor. Knowing that there was a ***lack of a clear phenotype post injury in our animal models***, we agreed collectively that it is highly unlikely that T0 will be different between animal models. Finally, because we are using a well-established injury model for which **all** timepoints have been published previously we noted that the T0 time point will likely not provide further information beyond what has been previously published (see Jung et. al. reference below). We sincerely hope the reviewer will agree that in the face of previous publications and the challenges linked with the timely evaluation of this control, our existing data is sufficient to support the conclusions of the manuscript.

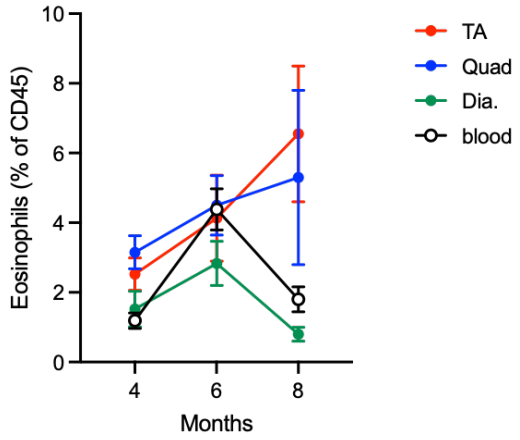
Jung HW, Choi JH, Jo T, Shin H, Suh JM. Systemic and Local Phenotypes of Barium Chloride Induced Skeletal Muscle Injury in Mice. Ann Geriatr Med Res. 2019 Jun;23(2):83-89. doi: 10.4235/agmr.19.0012. Epub 2019 Jun 30. PMID: 32743293; PMCID: PMC7387593.

C- Similarly, in the last chapters of the results, the authors compare mdx:Stat6-/- mice to mdx:Stat6+/-. I suggest showing also the differences between mdx mice and mdx:Stat6-/- mice, in order to better understand the effects of the complete ablation of IL-4/IL-13 signaling in mice.

We have now added Expanded View Figure 2 which shows a comparison of Wt vs Stat6+/- vs Stat6-/- for the acute model as well as mdx vs mdx:Stat6+/- vs mdx:Stat6-/- . We find no difference with regards to partial or complete ablation of STAT6 and muscle regeneration. We have added this additional information to the text (lines 172 and 233).

D- In the end, I have a curiosity. In a recently published paper from Jiang (Ital J Pediatr. 2023; 49: 83. 10.1186/s13052-023-01483-y), in a retrospective cohort study performed on 145 DMD patients between January 2012 and December 2020 the authors demonstrated that eosinophil count in peripheral blood was correlated with the severity of DMD. They suggest that these phenomena could indicate the therapeutic efficacy and prognosis of DMD patients to a certain extent, since eosinophils may be a potentially valuable biomarker or therapeutic target for DMD. Have you ever try to analyze the eosinophil count in peripheral blood of model mice and following the muscular injury?

Thank you for this intriguing question and the supporting reference (we actually have now added this to the manuscript as reference 41). We indeed examined this for mice at 4, 6 and 8 months of age (with a limited number of n/group). In our hands, blood eosinophils increase from age 4 months to age 6 months but then again drop at 8 months. Thus, in mice, this does not seem to not correlate well with disease progression. See the graph below:



Referee #2:
In their manuscript entitled, "Type-2 innate signaling is dispensable for skeletal muscle regeneration and pathology linked to Duchenne muscular dystrophy", Messing et al. set out to define the role of eosinophils and IL-4/IL-13 signaling in muscle regeneration and Duchenne muscular dystrophy (DMD). Using genetic approach to deplete eosinophils (dblGATA) or inhibit STAT6 signaling, they convincingly draw the conclusion that these two aspects of type 2 immunity do not influence muscle regeneration following acute injury nor the severity of muscle fibrosis in the mdx mouse model of DMD.

A-Overall, the experiments are well-designed to broadly assess the impact on muscle regeneration and fibrosis. Although the data are largely negative, it is still an important body of work to publish giving the complex role of type 2 innate immunity in tissue repair and fibrotic disease. The major concern with the manuscript is the over-generalized conclusion that type-2 innate immunity has no role when only two aspects (i.e. eosinophil and STAT6 signaling) of this complex immune response have been examined in this study. M2 macrophages, mast cells, ILC2s and Th2s are cells associated with type 2 immunity and have not been addressed in this study. IL-4 can also activate PI3K and p38; these pathways were also not examined in the current study.

Understood. We actually structured our manuscript in this way to match the previous Cell paper's phrasing and framing of their work. Indeed, we did not address many aspects of the type-2 innate immune cascade (nor did they). We have now adjusted the text in the manuscript to be more specific with respect to the pathways examined and our conclusions. In discussing this with the EMBO Handling Editor we agreed that, as a good compromise, it would be best to keep the title as is to match the Cell paper and provide a more granular explanation of the reviewers important point in the abstract and text of the manuscript. We have also now added a number of references (see below and references 16-19 in the manuscript) that show that the activation of Th2 immunity is dependent on STAT6. With respect to PI3K and p38 signalling we note that the published Cell manuscript also failed to see these alternative pathways activated by STAT6. Hence, for all the reasons above, we decided to keep our focus on STAT6-mediated TH2 immune response.

Kaplan MH, Schindler U, Smiley ST, Grusby MJ. Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. Immunity. 1996;4:313–319. doi: 10.1016/s1074-7613(00)80439-2

Akimoto T, Numata F, Tamura M, Takata Y, Higashida N, Takashi T, Takeda K, et al. Abrogation of bronchial eosinophilic inflammation and airway hyperreactivity in signal transducers and activators of transcription (STAT)6-deficient mice. J. Exp. Med. 1998;187:1537–1542. doi: 10.1084/jem.187.9.1537

Akimoto T, Numata F, Tamura M, Takata Y, Higashida N, Takashi T, Takeda K, et al. Abrogation of bronchial eosinophilic inflammation and airway hyperreactivity in signal transducers and activators of transcription (STAT)6-deficient mice. J. Exp. Med. 1998;187:1537–1542. doi: 10.1084/jem.187.9.1537

Takeda K, Tanaka T, Shi W, Matsumoto M, Minami M, Kashiwamura S, Nakanishi K, et al. Essential role of Stat6 in IL-4 signalling. Nature. 1996;380:627–630. doi: 10.1038/380627a0.

B- A further limitation of the study is an examination that was limited to regeneration and fibrosis. Other pathophysiological attributes of muscular dystrophy have not been assessed; membrane stability/repair, calcium homeostasis, and adipogenesis. The latter being particularly important given that prior studies have shown that eosinophils and type 2 cytokines inhibit adipogenic differentiation.

We were aware of the effects on adipogenic differentiation, but in all of our preliminary experiments we encountered only negative results investigating CSA, N/F, inflammation, and proliferation. We eventually ceased pursuing any of these other avenues and possibilities as we felt that those likely also will not show differences. We have now performed perilipin staining and additional collagen staining on a subset of our animals to probe whether we can see obvious differences in adipocytes and have added these data to a new Expanded View Figure 1. We found that, in line with all other assays performed, there is no striking difference in terms of adipocyte differentiation during muscle regeneration in our models.

C- Before the manuscript can be considered further for publication, the authors need to revise the title of the manuscript to reflect what was studied (eosinophils and STAT6-signaling). Second, definitive statements of type 2 immunity and IL-4/IL-13 signaling throughout the manuscript need to be revised accordingly. Third, an examination of the effect of eosinophil depletion or inhibiting STAT6 signaling in mouse models of muscle adipogenesis (B6A/J mouse or glycerol-induced injury) is needed.

As stated above, we agree with these points and, in consultation with the Editor, have revised the essential elements of text accordingly and have performed perilipin staining to assess adipogenesis. The addition of a new mouse model was deemed to be outside of the scope of this work and not warranted given that we do not see any differences in perilipin+ cell deposition and other aspects of muscle regeneration.

D- Further comments below:

1. The following statement does not take into consideration that other signal transduction pathways are activated downstream of the IL-4 and IL-13 receptor. In summary, we conclude that IL-4/IL-13 signaling is dispensable and does not play a significant role in muscle regeneration, inflammatory cell infiltration or muscle resident cell proliferation in response to injury. Limit the statement to STAT6 signaling.

We have now modified the text accordingly with regards to the significance of these other pathways. As stated above, we noted that the previous Cell manuscript also found no evidence of either PI3K or p38 pathway activation by IL4R/IL13R signalling.

2. Indicate in the figure legend the age of the mice when they underwent microdamage. How old were then when microdamage was initiated.

The mice were 12 weeks of age when the microdamage was started and 14 weeks of age when tissues were collected. We have now added this to the figure legend.

3. Some figure panels are not cited in the results (e.g. Figure 3E). Interestingly, a CSA analysis showed a small but significant reduction in the number of small fiber (CSA = 0-400 μm) in MD TAs of mdx:Stat6-/- compared to mdx:Stat6+/-.

Thank you for pointing this out. We also added this to the text (line 170).

4. A comparison of total STAT6 levels, phosphorylation levels and/or expression of STAT6 targets has not been done between STAT6 +/+ and STAT6+/- to rule out a gene dose effect (i.e. STAT6 are the same between STAT6 +/+ and STAT6+/- or is there an 'intermediate' loss of STAT6 that confounds the data).

We have added Expanded View Figure 2 which shows a comparison of Wt vs Stat6+/- vs Stat6-/- for the acute model as well as mdx vs mdx:Stat6+/- vs mdx:Stat6-/-. We show that there is no difference with regards to partial or complete ablation of STAT6 and muscle regeneration. We have now added this in the text accordingly (lines 172 and 233). Due to an absence of and “intermediate” loss effect of STAT6 deletion, levels of phosphorylation have not been investigated.

5. 'Strings' appears to be a typo in line 283-284, linked to this hyper-responsive background may not be broadly extrapolatable to other mouse strings or human disease.

Apologies. This has been corrected.

6. This following is another example of overly interpreted statement, "type-2 innate signaling is likely not a relevant pathway for the development of targeted immunotherapeutics. M2 macrophages and ILC2s are cellular components of the type 2 innate immunity, and they produce factors such as IGF1 and amphiregulin, which were not evaluated in this study. Thus, it is not appropriate to broadly conclude that type-2 innate signaling is likely not relevant in DMD. This can be easily addressed by limiting statements to what was specifically manipulated in this study (i.e. eosinophil and STAT6 signaling are likely not relevant).

We have adjusted the text accordingly (line 348) to be more moderate and narrower with regards to the conclusions on this point.

Referee #3:

The manuscript titled Type-2 innate signaling is dispensable for skeletal muscle regeneration and pathology linked to Duchenne muscular dystrophy described the absence of defect caused by the suppression of eosinophil and IL4/IL13 signaling during muscle regeneration.

The authors show here, the dispensability of eosinophil/ IL4/IL13 signaling during regeneration in opposition with what Heredia et al. suggested in 2013. This previous work proposed that eosinophils plays a role in muscle repair by inhibiting FAPs differentiation through IL4/IL13 signaling (Heredia et al., 2013). However this new work from Messing et al. brings opposite evidence showing that muscle repair in healthy and mdx context exerts no differences when eosinophils number is decreased or when IL4/IL13 signaling is downregulated.
For that purpose, the authors used different mice models, interestingly those models were also used in Heredia et al.,2013. The absence of eosinophils was visited with ΔdblGATA eosinophil-deficient mice as well as Stat6-/- mice were IL4/IL13 signaling was inhibited. Surprisingly in both of those model WT or mdx background, the respective deficiency in eosinophils and inhibition of Stat6 did not impact muscle regeneration. First, the authors presented regeneration characteristics of WT ΔdblGATA eosinophil-deficient mouse, where they validate a major decrease in eosinophil number without any significative impact on the regeneration. The same analysis structure has been reproduced using Stat6-/- mice where the same absence of effect has been shown. Messing et al., then proposed to investigated the importance of eosinophils or their signaling during pathological long-term inflammation/regeneration by crossing Stat6-/- mice with mdx background as well as using an α-IL5 neutralizing antibody to prevent differentiation and survival of eosinophils. Even if the mdx phenotype did not appear very strong, the authors did not identify any major changes during muscle regeneration. The additional injury by micro damaging the tissue as well as the aging process, are respectively in line with the previous observations and showed no differences with their respective controls. In this work Messing et al. brings strong evidence that eosinophils deficiency as well as the inhibition of their major downstream signaling does not affect muscle regeneration.

Overall, the work is well written and use a variety of different techniques that strengthens author's conclusion. I would also like to emphasize the structuration of the paper which helps with the understanding. The work provides good evidences and discussion in order to re-think the importance of eosinophil and their signalling during muscle regeneration. Hereafter are few comments that can clarify and maybe strenghens some of the conclusions.

Comments:

1. Overall the complete absence of effect on muscle regeneration is very surprising based on the impressive phenotype that Heredia et al. showed in their paper. One of the hypothesis proposed by the authors is the difference in term of mouse background between Balb/cJ and C57BL/6.
a. To what extent the authors believe that the mouse background has an importance on the phenotype differences? Do you have any supplementary data on Balb/c background which would support this hypothesis?

We have now performed acute muscle injury in BALB/c mice compared to C57BL/6 mice and have now added this data as an additional main figure, Figure 5. We have evaluated body mass, TA mass (normalized to BM), CSA, N/F, adipocyte accumulation, collagen deposition and number of SiglecF+ cells. We have also added these results to the text starting in line 238. The results indicate that there is indeed a difference in the ability of these two strains to regenerate muscle after injury. The CSA analysis indicates a delay in regeneration in the BALB/c mice and we have also observed a trend toward more adipocyte accumulation (not significant) and significantly more collagen deposition at day 28 after injury. Further, we observed significantly more SiglecF+ cells (eosinophils) at 7 days post injury. We think this demonstrate that even without any other genetic manipulation, the two strains already show differences which may be exacerbated by the absence of eosinophils or IL-4 signaling, perhaps to the point of an inability of BALB/c

muscle to regenerate entirely (as suggested by Heredia et al.). We do not have the required mouse strains to explore this further (i.e., all the mutant strains on a BALB/c background), but we feel this additional data strengthens our hypothesis. This underscores that utilizing various mouse models with different genetic backgrounds, as done by Heredia et al., likely complicates the interpretation of results and introduces confusion regarding the roles of immune cells and pathways studied.

2. Based on the hypothesis that eosinophils might affect preferentially FAPs fate during muscle regeneration, are you able to quantify the fibrotic deposition in your different regenerative assay like the authors did in Figure 4G-H? Some of your images might show a slight increase which may be lowered because of C57BL/6 background.

We have now added collagen quantifications of a subset of our animals to Expanded View Figure 1. We observe no significant difference in collagen deposition across all models tested (beyond the differences we see on the BALB/c background mentioned above).

3. According to literature, eosinophils is one of the first population recruited on site (≃36h). It might be a good idea, to match your flow cytometry data, to add histological images at 3dpi or at an early time point to show a potential defect in early regeneration which might be compensated later at 7dpi by the global immune response.

We were unable to generate mice to perform histology at 3dpi in a timely manner. However, we argue that even if there is a defect in early regeneration, it is not significant enough to cause long term effects (as suggested by Heredia et. al, who argue that eosinophils/IL-4 signaling is essential for muscle regeneration). The lack of differences between both number and proliferation of MPs and FAPs at 3dpi supports the conclusion that that there are no impactful differences in regeneration even at an earlier time point.

4. In Figure 1F-G and 2F-G, this is surprising that only 50% of MP or FAPs are incorporating EdU especially at 3dpi. How can you explain the low amount of proliferating MP? Could that come from your previous gating VCAM/ITGa7 where VCAM signal seems dim? To strengthen the conclusion about MP and FAPs proliferation it might be interesting to show another way of quantification. Histological sections and a quantification of Pax7+ and PDGFRa+ cells numbers respectively can be implemented. EdU detection can also be done directly on the section after in vivo injection.

As mentioned above, we were not able to generate additional mice to perform further measurements (such as Pax7+ or PDGFRa+ staining) at 3dpi. However, when comparing our results to previously published work (Hardy et al., 2016), we note that the % of proliferating MPs seems to be typical for the injury model that we used (BaCl2). In the reference below (Hardy et al, 2016), about 50% of MPs are Ki67+ at 4dpi (as shown in Figure 1). Since, beyond a lack of differences in proliferation, we also do not observe a difference in regeneration and the % proliferation which matches the previous work, we are confident in these results and hope that the reviewer will agree.

Hardy D, Besnard A, Latil M, Jouvion G, Briand D, Thépenier C, Pascal Q, Guguin A, Gayraud-Morel B, Cavaillon JM, Tajbakhsh S, Rocheteau P, Chrétien F. Comparative Study of Injury Models for Studying Muscle Regeneration in Mice. PLoS One. 2016 Jan 25;11(1):e0147198. doi: 10.1371/journal.pone.0147198. PMID: 26807982; PMCID: PMC4726569.

5. For both WT and mdx can the authors show the downregulation of Stat6 protein? Is Stat6-/- model a full body knock out? Can the defect in Stat6 expression affect MP and/or FAPs proliferation/fate independently of IL4/IL13 signalling? Does these populations have the same pattern of proliferation/differentiation in vitro than their respective control? Does this mice have a particular phenotype?

The model we are using is a full body KO and these mice have no phenotype at steady state. When challenged with some form of type-2 immune inducing agent (various allergens for example), these mice do show defects in the ability to mount an appropriate type-2 immune response (see references below and reference 16-19 in the manuscript). Since we do not observe any obvious phenotype in the absence of STAT6 in our mice, we do not believe that STAT6 expression affects MPs or FAPs. If there is an effect, it is likely too subtle to detect. We have also added an additional Expanded View Figure 2 that does explore Wt, vs HEMI vs full KO of STAT6 and show that a partial loss of STAT6 also does not affect muscle regeneration nor confound our findings.

Kaplan MH, Schindler U, Smiley ST, Grusby MJ. Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. Immunity. 1996;4:313–319. doi: 10.1016/s1074-7613(00)80439-2

Akimoto T, Numata F, Tamura M, Takata Y, Higashida N, Takashi T, Takeda K, et al. Abrogation of bronchial eosinophilic inflammation and airway hyperreactivity in signal transducers and activators of transcription (STAT)6-deficient mice. J. Exp. Med. 1998;187:1537–1542. doi: 10.1084/jem.187.9.1537

Akimoto T, Numata F, Tamura M, Takata Y, Higashida N, Takashi T, Takeda K, et al. Abrogation of bronchial eosinophilic inflammation and airway hyperreactivity in signal transducers and activators of transcription (STAT)6-deficient mice. J. Exp. Med. 1998;187:1537–1542. doi: 10.1084/jem.187.9.1537

Takeda K, Tanaka T, Shi W, Matsumoto M, Minami M, Kashiwamura S, Nakanishi K, et al. Essential role of Stat6 in IL-4 signalling. Nature. 1996;380:627–630. doi: 10.1038/380627a0.

6. Globally, it would be nice if the author can present a WT control along with mdx mice to highlight the mdx phenotype. In this work, mdx background is used as chronic inflamatory model but no increase in eosinophils or macrophages number has been shown compare to the WT condition in Figure 2E and 3E.

This comparison has been recently published by the laboratory of Dr. Armando Villalta (see reference below Kastenschmidt et al). In this work, the authors compared eosinophil counts in Wt compared to mdx mice and show that there is a consistent elevation of eosinophils at 4, 12 and 52 weeks of age. We have added a sentence our manuscript pointing this out and also added the very relevant reference (line 284, reference 38).

Kastenschmidt JM, Coulis G, Farahat PK, Pham P, Rios R, Cristal TT, Mannaa AH, Ayer RE, Yahia R, Deshpande AA, Hughes BS. A stromal progenitor and ILC2 niche promotes muscle eosinophilia and fibrosis-associated gene expression. Cell reports. 2021 Apr 13;35(2).

7. According to the previous comment, the addition of an extra damage in mdx makes a lot of sense. Why the authors used a mild microdamage injury which might not trigger fully the inflammatory response and eosinophils activation (Desguerre et al., 2012)? Did the authors have data with stronger injury (cardiotoxin or BaCl2)? Do you think that the slight effect seen on fibers size might be more significant following that type of injury?

Our goal was to induce persistent chronic damage and fibrosis deposition (rather than a one-time acute injury reminiscent of the injection of BaCl2). While we do not have the data requested, we show that with advanced disease (aged mdx mice), even though there is significantly more inflammation and fibrosis, there continues to be no phenotypical changes in STAT6+/- vs STAT6-/- mdx mice.

8. In Figure 3 and 4, in order to assess the regeneration process in MD mdx, α-IL5 treated mice and aging assay, it would be nice to show a quantification of MP and FAPs numbers, like the authors did in Figure 1 and 2.

Unfortunately, at the time of the experiment for the a-IL5 treated mice, we did not record the number of FAPs and MPs. With regard to the aged mdx mice experiment, since there are no changes in collagen deposition, or myofiber CSA, we hypothesised that there would not be any changes in MP and FAP number. In addition, even if there were changes, we have shown that it has no effect on muscle histopathology.

Minor comments

1. The flow cytometry data presentation can be improve, please do not use density plot and add the used fluoropher next to the target.

We have added the fluorophore to the images. We, respectfully, would like to keep the density plot style as it as it better represents cell population separation.

2. In Figure 2d, the representative image of Stat6-/- at 28d staining might be improve in order to show the significant increase.

We have adjusted the image accordingly.

Dear Kelly, and happy new year !

Thank you for the submission of your revised manuscript. We have now received the enclosed reports from the referees and I am happy to say that all support its publication now. Only a few editorial requests will need to be addressed before we can proceed with the official acceptance of your manuscript:

- Your ms has 5 main figures but is laid out as a full article with separate results and discussion sections. Please either add one more main figure or combine the results and discussion sections to publish your paper as a short report. The character count will need to be reduced a little, which usually occurs naturally when results and discussion are combined.

- Please add up to 5 keywords to your ms file.

- Please add the direct URL for the deposited dataset to the DAS.

- Please rename the conflict of interest subheading to "Disclosure Statement and Competing Interests"

- Please remove the author credits from the ms file. All credits are entered during online ms submission.

- Please correct the REFERENCE FORMAT to the EMBO reports format: It needs to be alphabetical, not numerical; et al needs to be used after 10 author names; DOIs should only be used for preprints and datasets that have not been published yet

- The author checklist Data Availability section has not been completed. Please send us a new completed checklist.

- The FUNDING INFO needs to be part of the Acknowledgments.

- Please add a callout for Fig. 5E.

- Materials and Methods should be "Methods".

- Our systematic figure check of accepted ms detected a possible partial image reuse within Fig 1A (2 images on the left) and a potential full image reuse between Fig 1D (WT) and Fig 2D (STAT6-/-). Can you please explain what happened?

Figure Legends - Comments

- Please note that the legend for figure EV 1d is missing in the manuscript. This needs to be rectified.
- Please note that the legend for figures 1f-g; 2f-g are mislabeled as 1d-e; 2f-g in the manuscript. This needs to be rectified.
- Please note that the exact p values are not provided in the legends of figures 1d-e; 2d; 3b, d-h; 4a-c, e-f, h-i; 5c-e.
- Please note that in figures 1d-e; 2d; 4a-c, e-f, h-i; 4a-c, e-f, h-i; 5c-e; there is a mismatch between the annotated p values in the figure legend and the annotated p values in the figure file that should be corrected.
- Please note that the error bars are not defined in the legends of figures 1b-g, 2b-g; 3a-b, d-h; 4a-c, e-f, h-i; 5b-e; EV 1a-d; EV 2a-b.

I would like to suggest some changes to the abstract that needs to be written in present tense:

Immune responses play an integral role in skeletal muscle regeneration. In the genetically inherited muscle disease Duchenne muscular dystrophy (DMD), muscle regeneration is disrupted, leading to chronic inflammation, fibrosis, and early mortality. Previously, it has been suggested that type-2 innate immune cells, particularly eosinophils and their production of IL-4, play an essential role in effective muscle regeneration after acute injury. We here re-investigate the role of eosinophils in skeletal muscle repair using mice deficient in eosinophils (ΔdblGATA), or deficient in IL-4R/IL-13R signaling through STAT6 (Stat6-/-). We show that neither deficiency has an impact on skeletal muscle regeneration in response to acute injury as quantified by fiber size, immune cell infiltration, or muscle-resident stem cell proliferation. We also investigate the role of STAT6 signaling in mdx:Stat6-/- mice, a model of DMD and, again, find that ablation of STAT6 signaling has no effect on the rate or severity of fibrotic scar formation or disease progression. In contrast to previous models, our data suggest a negligible role for eosinophils and STAT6 signaling in skeletal muscle regeneration after acute or chronic injury.

I think the title is fine, given that "Type-2 innate signaling" as meant here is specified in the abstract.

EMBO press papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is exactly 550 pixels wide and 200-600 pixels high (the

height is variable). The synopsis image should provide a sketch of the major findings, like a graphical abstract. Please note that text needs to be readable at the final size. Please send us this information along with the final manuscript.

I look forward to seeing a final version of your manuscript as soon as possible.

Best,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

According to the concerns raised in the last revision, the authors improved significantly the manuscript.

Referee #2:

While the authors' response adequately addresses most of my concerns, I still do not agree with the title in the current form. We run the risk that readers will make hasty conclusions by reading the title of the manuscript, without a thorough evaluation of the great work presented in this manuscript or abstract. Given that this manuscript has not evaluated all elements of type 2 innate immunity, we run a dangerous course in which the field may abandon future studies aimed at exploring facets of type 2 immunity that have been explored in the current manuscript. I strongly recommend a revision of the title, whose final acceptance I defer to the discretion of the editors.

Referee #3:

The authors have satisfactorily addressed the issues raised by the reviewers.

1. Your ms has 5 main figures but is laid out as a full article with separate results and discussion sections. Please either add one more main figure or combine the results and discussion sections to publish your paper as a short report. The character count will need to be reduced a little, which usually occurs naturally when results and discussion are combined. We decided to add one additional figure (Figure 3 was split into Figures 3 and 4).

2. Change title re latest Editor Email. Completed

3. Please add up to 5 keywords to your ms file. Completed

4. Please add the direct URL for the deposited dataset to the DAS. Completed

5. Please rename the conflict of interest subheading to "Disclosure Statement and Competing Interests" Completed

6. Please remove the author credits from the ms file. All credits are entered during online ms submission. Completed

7. Please correct the REFERENCE FORMAT to the EMBO reports format: It needs to be alphabetical, not numerical; et al needs to be used after 10 author names; DOIs should only be used for preprints and datasets that have not been published yet Completed

8. The author checklist Data Availability section has not been completed. Please send us a new completed checklist. Completed

9. The FUNDING INFO needs to be part of the Acknowledgments. Completed

10. Please add a callout for Fig. 5E. Completed

11. Materials and Methods should be "Methods". Completed

12. Our systematic figure check of accepted ms detected a possible partial image reuse within Fig 1A (2 images on the left) and a potential full image reuse between Fig 1D (WT) and Fig 2D (STAT6-/-). Can you please explain what happened? We carefully reviewed the figures but do not notice an accidental duplication between the indicated groups/images. Is it possible that this can happen when the phenotypes are very similar?

    Figure Legends - Comments

13. Please note that the legend for figure EV 1d is missing in the manuscript. This needs to be rectified. Completed

14. Please note that the legend for figures 1f-g; 2f-g are mislabeled as 1d-e; 2f-g in the manuscript. This needs to be rectified. Completed

15. Please note that the exact p values are not provided in the legends of figures 1d-e; 2d; 3b, d-h; 4a-c, e-f, h-i; 5c-e. Completed

16. Please note that in figures 1d-e; 2d; 4a-c, e-f, h-i; 4a-c, e-f, h-i; 5c-e; there is a mismatch between the annotated p values in the figure legend and the annotated p values in the figure file that should be corrected. Completed

17. Please note that the error bars are not defined in the legends of figures 1b-g, 2b-g; 3a-b, d-h; 4a-c, e-f, h-i; 5b-e; EV 1a-d; EV 2a-b. Completed

18. I would like to suggest some changes to the abstract that needs to be written in present tense:

    Immune responses play an integral role in skeletal muscle regeneration. In the genetically inherited muscle disease Duchenne muscular dystrophy (DMD), muscle regeneration is disrupted, leading to chronic inflammation, fibrosis, and early mortality. Previously, it has been suggested that type-2 innate immune cells, particularly eosinophils and their production of IL-4, play an essential role in effective muscle regeneration after acute injury. We here re-investigate the role of eosinophils in skeletal muscle repair using mice deficient in eosinophils (ΔdblGATA), or deficient in IL-4R/IL-13R signaling through STAT6 (Stat6-/-). We show that neither deficiency has an impact on skeletal muscle regeneration in response to acute injury as quantified by fiber size, immune cell infiltration, or muscle-resident stem cell proliferation. We also investigate the role of STAT6 signaling in mdx:Stat6-/- mice, a model of DMD and, again, find that ablation of STAT6 signaling has no effect on the rate or severity of fibrotic scar formation or disease progression. In contrast to previous models, our data suggest a negligible role for eosinophils and STAT6 signaling in skeletal muscle regeneration after acute or chronic injury. Completed

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Canada

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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
- 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

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.

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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	

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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	Reagents and Tools Table (EV Table 1)

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Short novel DNA or RNA including primers, probes: provide the sequences.	Not Applicable	

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Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Not Applicable	
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Animal observed in or captured from the field: Provide species, sex, and age where possible**.**	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	Methods

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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	
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If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Acknowledgement section

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)
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Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Methods and Figure Legends
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?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Yes	Methods and Figure Legends
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	Methods and Figure Legends

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	
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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	Data Availability Section
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	