

Retinoic acid-induced type 1 regulatory T cells suppress autoimmunity

Mathilde Raverdeau, Maria Christofi, Anna Malara, Mieszko M. Wilk, Alicja Misiak, Lucia Kuffova, Tian Yu, Aoife M. McGinley, Shauna Quinn, Chandirasegaran Massilamany, Jay Reddy, John V. Forrester and Kingston H.G. Mills

Review timeline:

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

1st Editorial Decision

8 November 2018

Thank you for the submission of your research manuscript to EMBO reports. We have now received reports from two referees that were asked to evaluate your study, which can be found at the end of this email. I apologize for the delay in getting back to you, but we originally had a third referee, that promised twice to return his/her report soon, but in the end declared not to be able to assess the study due to time constraints.

As you will see, both referees think that the manuscript requires a major revision to allow publication in EMBO reports. All three referees have a number of concerns and/or suggestions to improve the manuscript, which we ask you to address in a revised manuscript. As the reports are below, I will not detail them here.

Given the constructive referee comments, I would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript and/or in a 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 or rejection 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; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

Supplementary/additional data: 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 labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature.

For more details please refer to our guide to authors:

<http://embor.embopress.org/authorguide#manuscriptpreparation>

Important: All materials and methods should be included in the main manuscript file.

See also our guide for figure preparation:

http://www.embopress.org/sites/default/files/EMBOPress_Figure_Guidelines_061115.pdf

Regarding data quantification and statistics, please specify, where applicable, the number "n" for how many independent experiments (biological replicates) were performed, 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 add a paragraph to the methods section detailing the statistical testing used throughout the manuscript. See:

<http://embor.embopress.org/authorguide#statisticalanalysis>

We now 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. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

Finally, please also follow our guidelines for the use of living organisms, and the respective reporting guidelines: <http://embor.embopress.org/authorguide#livingorganisms>

When submitting your revised manuscript, we will require:

- a complete author checklist, which you can download from our author guidelines (<http://embor.embopress.org/authorguide#revision>). Please insert page numbers in the checklist to indicate where the requested information can be found.
- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted single figure files in high resolution (for main figures and EV figures)

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.

REFeree REPORTS

Referee #1:

In this manuscript the authors have used a combination of antigen, retinoic acid (RA) and IL-2 to increase the production of cells able to prevent onset of EAE and also in a different antigenic system prevent EAU. The authors show that such immunisation and cells generated by such immunisation inhibits the production of pro-inflammatory cytokines IFN γ and IL-17. In the EAE experiments they show reduced numbers of cells expressing proinflammatory cytokines in the spinal cord following their immunisation protocol with antigen, RA and IL-2. They also show increased production of IL-10 in vitro in the EAE experiments.

While in the EAU experiments using a double transgenic mouse system they show that IL-10 is being made CD4+T cells critically there is no evidence that IL-10 is being made by the CD4+T cells induced by MOG, RA and IL-2. The cultures showing IL-10 production are of spleen cells from immunised mice (Fig 1). In Fig 4 evidence is presented showing that co-culture of "iTregs" (in fact LN cells from immunised mice) with cells from EAE primed mice manifest reduced production of pro-inflammatory cytokines.

There is no hard evidence that this is due to IL-10 and indeed it could be contact mediated. Likewise the inhibition of induced EAE by expanded "iTregs" has not been formally attributed to IL-10. Therefore, while this is a very interesting set of experiments, it does not clarify the mechanisms of inhibition of autoimmune pathology by the cells induced by RA and IL-2. Is IL-10 involved?

For this an anti-IL-10R blockade should be done in vitro and in vivo. Is it cell contact mediated? The in vitro experiments could be carried out using a membrane to separate populations. Is IL-10 made by the cells induced by antigen, RA and IL-2? The EAU experiments suggest yes, but there is no evidence from the EAE experiments.

Referee #3:

The authors show that immunization with peptide/protein along with RA and IL-2 induces the development of antigen specific IL-10 secreting T-cells expressing markers associated to TR1 cells. These TR1-like cells block autoimmunity onset in an induced model of EAE, uveitis and in a TCR transgenic spontaneous model of uveitis.

Adoptive transfer of T-cells from tolerized mice prevents or delay disease onset in the host.
 This is a short paper suggesting a potential new immunotherapy based on the expansion of antigen specific TR1 cells.

Major points:

The title should reflect the TR1 (or IL10 secreting) nature of the Tregs described in this study, as it is now shown it seems the authors refer to Foxp3+ cells

Other more advanced immunotherapeutic approaches involving TR1 (or IL-10 secreting) cells have been described, these approaches should have been cited, and the present study should be put in the context of these alternative therapeutic approaches (in the discussion).

The potential mechanisms responsible for the therapeutic effect of RA (TR1 induction) must be included in the discussion.

Fig1A flow cytometry staining of Foxp3+ cells is not clear, it seems the authors should reduce the number of events displayed to clearly show a Foxp3+ T-cell population

Fig 1C. The gating strategy (the flow plots) should be shown, as these are relevant to define TR1 cells.

Fig1D the cells are from spleen while in the main text are lymphnode derived.

Fig 2. From the dextramer+ cells, the authors should show the TR1 associated markers (PD-1, CD49b, LAG3 etc) and compare with dextramer negative population, after treatment but before induction of disease and after induction of disease. Also a negative control of non-treated non-immunized (or even better, treated with another peptide) stained with dextramer-MOG must be included.

Also in Fig 2, IL-10 secretion must be shown (including the post treatment -before induction- of the dextramer + subset (vs. negative).

Fig EVE3, The authors should also compare CD44+ from peptide or antigen treated mice without TR1 inducers (What does FMO mean?)

Fig5E IL-10 Elisa should be shown, from total CD4+ TCR transgenic cells

The authors should show that TR1 induced protection is antigen specific or not (bystander suppression), they may use different peptides to induce disease in both autoimmune models.

The authors should adoptive transfer CD4+ T cells (or even better dextramer+ cells) from treated mice, and determine their potential to block disease progression, without having to expand/induce them in vitro. In the present experimental setting, It is not clear which cells the authors inject as a control, are they non-antigen specific treated with RA+IL-2? Or antigen specific not treated with RA+IL-2, it is not clear if the induction of TR1 (with potential to block disease progression) is really induced in vitro.

The autoimmune models proposed are contrived in the sense that the autoimmune response is of

monoclonal nature. To address this issue It would be very, interesting to show the potential of this therapy in a non TCR-transgenic not induced spontaneous an polyclonal model of autoimmunity like the NOD mouse (or other suitable models), using already describe insulin, 2.5mi epitope, IGRP... epitopes.

1st Revision - authors' response

29 January 2019

Replies to Referees comments

Referee #1:

In this manuscript the authors have used a combination of antigen, retinoic acid (RA) and IL-2 to increase the production of cells able to prevent onset of EAE and also in a different antigenic system prevent EAU.

The authors show that such immunisation and cells generated by such immunisation inhibits the production of pro-inflammatory cytokines IFN γ and IL-17. In the EAE experiments they show reduced numbers of cells expressing proinflammatory cytokines in the spinal cord following their immunisation protocol with antigen, RA and IL-2. They also show increased production of IL-10 in vitro in the EAE experiments.

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Author response: We have now shown that IL-10 production by CD4 T cells from mice immunized with MOG, RA and IL-2. This data is presented in Fig 2A&B of the revised manuscript.

There is no hard evidence that this is due to IL-10 and indeed it could be contact mediated. Likewise the inhibition of induced EAE by expanded "iTregs" has not been formally attributed to IL-10. Therefore, while this is a very interesting set of experiments, it does not clarify the mechanisms of inhibition of autoimmune pathology by the cells induced by RA and IL-2. Is IL-10 involved? For this an anti-IL-10R blockade should be done in vitro and in vivo. Is it cell contact mediated? The in vitro experiments could be carried out using a membrane to separate populations. Is IL-10 made by the cells induced by antigen, RA and IL-2? The EAU experiments suggest yes, but there is no evidence from the EAE experiments.

Author response: We have now demonstrated IL-10 production by CD4 T cells from mice immunized with MOG, RA and IL-2. This data is shown in Fig 2A&B of the revised manuscript. We also have examined the role of IL-10 in attenuation of EAE induced by immunization with MOG + RA + IL-2. The data shown in Fig 4G of the revised manuscript demonstrate that protection against EAE induced by this immunization protocol is maintained in IL-10-defective mice, suggesting that the induced Treg cells do not suppress EAE via IL-10 production. We have now discussed alternative IL-10-independent mechanisms of suppression by Tr1 cells.

Referee #3:

The authors show that immunization with peptide/protein along with RA and IL-2 induces the development of antigen specific IL-10 secreting T-cells expressing markers associated to TR1 cells. These TR1-like cells block autoimmunity onset in an induced model of EAE, uveitis and in a TCR transgenic spontaneous model of uveitis.

Adoptive transfer of T-cells from tolerized mice prevents or delay disease onset in the host.

This is a short paper suggesting a potential new immunotherapy based on the expansion of antigen specific TR1 cells.

Major points:

The title should reflect the TR1 (or IL10 secreting) nature of the Tregs described in this study, as it is now shown it seems the authors refer to Foxp3+ cells.

Author response: We have modified the title to: 'Type 1 regulatory T cells induced with autoantigen, retinoic acid and IL-2 suppress the development of autoimmunity'. We have also clarified in the abstract and text of the that the Treg cells are Tr1 cells.

Other more advanced immunotherapeutic approaches involving TR1 (or IL-10 secreting) cells have been described, these approaches should have been cited, and the present study should be put in the context of these alternative therapeutic approaches (in the discussion).

Author response: We have now cited and discussed other more advanced therapeutic approaches involving Tr1 and IL-10-secreting Treg cells. Since this is a brief report restricted by EMBO reports to 25,000 characters we could not include extensive discussion of the literature.

Fig1A flow cytometry staining of Foxp3+ cells is not clear, it seems the authors should reduce the number of events displayed to clearly show a Foxp3+ T-cell population.

Author response: We reduced the number of events displayed in the plot to make the Foxp+ T cell population clearer.

Fig 1C. The gating strategy (the flow plots) should be shown, as these are relevant to define TR1 cells.

Author response: We have now included the gating strategy in Figure EV1.

Fig1D the cells are from spleen while in the main text are lymph node derived.

Author response: This error has been corrected.

Fig 2. From the dextramer+ cells, the authors should show the TR1 associated markers (PD-1, CD49b, LAG3 etc) and compare with dextramer negative population, after treatment but before induction of disease and after induction of disease. Also a negative control of non-treated non-immunized (or even better, treated with another peptide) stained with dextramer-MOG must be included. Also in Fig 2, IL-10 secretion must be shown (including the post treatment -before induction- of the dextramer + subset (vs. negative)).

Author response: We have now included data for dextramer+ cells in naïve mice (Fig 2D), as well as PD-1, ICOS and CD49d expression on dextramer+ cells from mice immunized on day 3 of EAE (Fig 2E). The MHC class II dextramers are notoriously difficult to work with. Multicolour flow cytometry for intracellular cytokine or even surface markers along with dextramer staining is very challenging. The fixation and permeabilization in the staining procedures can make the dextramers detach from the cell surface. Therefore, IL-10 secretion on dextramer+ cells is technically not possible. However, we have now demonstrated IL-10 production by CD4 T cells from mice immunized with MOG + RA + IL-2; shown in Fig 2A&B of the revised manuscript.

Fig EVE3, The authors should also compare CD44+ from peptide or antigen treated mice without TR1 inducers (What does FMO mean?)

Author response: We have now examined CD62L, CD49b, ICOS and PD-1 on CD44⁺ CD4 T cells from mice immunized with MOG without the Tr1-inducers RA and IL-2 (Fig EV4C in revised manuscript). FMO is Fluorescence Minus One, a control used to identify and gate cells in the context of data spread due to the multiple fluorochromes in a given panel. We have spelled out FMO in the legend to Figure 2 of the revised manuscript.

Fig 5E IL-10 Elisa should be shown, from total CD4+ TCR transgenic cells

Author response: The generation of this data from the EAU model would take several months, because of the elaborate and very strict animal license rules in the UK. The group in Aberdeen who did the EAU models are in the process of applying for a new animal license. This has to be completed submitted and approved before any new work can be performed in this model and this will take many months. We have now provided convincing evidence of IL-10 production by CD4 T cells from mice immunized with MOG, RA and IL-2 in the EAE model based on intracellular cytokine staining and flow cytometry (Fig 2 A,B).

The authors should show that TR1 induced protection is antigen specific or not (bystander suppression), they may use different peptides to induce disease in both autoimmune models.

Author response: We have now included data from the EAE model showing that protection is antigen-specific; attenuation of EAE was induced by immunization with MOG and the Tr1-inducers but not with OVA and Tr1-inducers. This data is shown in Fig EV5 of the revised manuscript.

The authors should adoptive transfer CD4+ T cells (or even better dextramer+ cells) from treated mice, and determine their potential to block disease progression, without having to expand/induce them in vitro. In the present experimental setting, It is not clear which cells the authors inject as a control, are they non-antigen specific treated with RA+IL-2? Or antigen specific not treated with RA+IL-2, it is not clear if the induction of TR1 (with potential to block disease progression) is really induced in vitro.

Author response: The suggestion of transferring dextramer+ cells is a good one, but is logistically not possible, because of technical challenges or working with dextramers (we have consulted with our collaborator and co-author Prof Jay Reddy who is a leading expert in the area) and the very low numbers of antigen-specific cells in immunized mice. However, we did carry out transfer experiments with T cells enriched for antigen-specific CD4 T cells by in vitro stimulation with specific antigen presented by MHC class II. The control cells that we injected in the transfer experiment were lymphocytes from naïve mice. We have clarified this in the revised manuscript. Amplifying the cells in vitro with antigen not only gives us more cells to work with but also ensure that the majority of these cells are antigen-specific Treg cells. Culture of T cells from naïve unimmunized mice with RA and IL-2 does not expand antigen-specific T cells (Fig 2B). Furthermore, cells from mice immunized with MOG alone and cultured *in vitro* with MOG or MOG, RA and IL-2 did not exhibit the same level of expression of CD49b, ICOS and PD-1 (New data presented in Fig EV4C). This demonstrates that immunization with MOG, RA and IL-2 is required to generate Tr1 cells, which can be expanded in vitro by re-stimulation with MOG in the presence of RA and IL-2.

The autoimmune models proposed are contrived in the sense that the autoimmune response is of monoclonal nature. To address this issue It would be very, interesting to show the potential of this therapy in a non TCR-transgenic not induced spontaneous an polyclonal model of autoimmunity like the NOD mouse (or other suitable models), using already describe insulin, 2.5mi epitope, IGRP... epitopes.

Author response: We have already shown that our novel immunization protocol that induces antigen-specific Treg cells protects in the MOG-induced EAE model, in an antigen-induced EAU model and in a spontaneous EAU model. This work has taken more than 2 years to complete. We do not have the NOD mice or diabetes model running in our animal facility and respectfully suggests that this request is beyond the scope of the current brief report.

2nd Editorial Decision

12 February 2019

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the two referees that were asked to re-evaluate your study, you will find below. As you will see, the referees now support the publication of your manuscript in EMBO reports.

Before we can proceed with formal acceptance, I have these few editorial requests, which we ask you to address in a final revised version of the manuscript:

- The title is presently too long. Could you please provide a more concise title (without punctuation marks) with not more than 100 characters (including spaces)
- Please provide the abstract written in present tense.
- Please call out the different panels of Fig. EV3 in the manuscript text.
- 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, and some queries, we ask you to address. Please provide your final manuscript file with track changes, in order that we can see the modifications done.

Further, I would need from you:

- a short, two-sentence summary of the manuscript
- two to three bullet points highlighting the key findings of your study
- 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.

REFEREE REPORTS

Referee #1:

The authors have amended the manuscript in the light of this reviewer's comments. They have clearly now shown that IL-10 does NOT play a role in the suppression of autoimmunity by antigen in the presence of RA and IL-2. They have furthermore amended the discussion to somewhat accommodate this new finding.

Referee #3:

Most of the question have been answered. That's fine with me for publication

2nd Revision - authors' response

15 February 2019

The authors performed all minor editorial changes.

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Kingston H.G. Mills

Journal Submitted to: EMBO reports

Manuscript Number: EMBOR-2018-47121V1

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if $n < 5$, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
 - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
 - are tests one-sided or two-sided?
 - are there adjustments for multiple comparisons?
 - exact statistical test results, e.g., P values = x but not P values < x;
 - definition of 'center values' as median or average;
 - definition of error bars as s.d. or s.e.m.

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

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself.

Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

USEFUL LINKS FOR COMPLETING THIS FORM

<http://www.antibodypedia.com>

<http://1degreebio.org>

<http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo>

<http://grants.nih.gov/grants/olaw/olaw.htm>

<http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm>

<http://ClinicalTrials.gov>

<http://www.consort-statement.org>

<http://www.consort-statement.org/checklists/view/32-consort/66-title>

<http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tur>

<http://datadryad.org>

<http://figshare.com>

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

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

<http://biomodels.net/>

<http://biomodels.net/miriam/>

<http://jij.biochem.sun.ac.za>

http://oba.od.nih.gov/biosecurity/biosecurity_documents.html

<http://www.selectagents.gov/>

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	All data was generated from animal experiments; see reply to 1b below.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Samples sizes were based on power calculations carried out using PASS13 software and were based on a two sample t-test as this is the usual method in the literature in this research area for comparison of treatment effects. Relevant sample mean and SD for the power calculations were derived from the literature and validated by a statistician. Overall treatment effects were examined by ANOVA.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples were excluded from analysis, except in one experiment where there were technical problems with the i.v. injection of cells, which can be a very difficult technique in black mice.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Animals were randomly assigned to each treatment group.
For animal studies, include a statement about randomization even if no randomization was used.	We have included a statement about randomization in the Material and Methods section of the text.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Animals were randomly assigned to each treatment group.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Other than randomly assigning animals to each treatment group no blinding was done.
5. For every figure, are statistical tests justified as appropriate?	Appropriate statistical tests have been used and detailed in each figure legend.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Prism software and the R software were used for all statistical analysis and the choice of test was based on the distribution of the data and whether comparing two or more treatment groups.
Is there an estimate of variation within each group of data?	Yes
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

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

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

D- Animal Models

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

E- Human Subjects

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

F- Data Accessibility

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

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No.
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