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HIV-1 infection of CD4 T cells impairs antigen-specific B cell function

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

1st Editorial Decision 16th Jul 2020

Dear Olivier,

Thank you for submitting your manuscript to The EMBO Journal. I am terribly sorry for the delay in getting back to you with a decision. This delay occurred due to an unresponsive referee and the need to involve a another referee late in the game.

I have now received the input from two good experts in the field and their comments are provided below. As you can see both referees find the analysis interesting and support publication here. They raise a number of relative minor concerns that should be straight forward enough to address. Let me know if we need to discuss any of them further. Happy to do so!

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

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

Please check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
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IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
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Referee #2:

The work of Kaw et.al. is focused on the mechanisms of impaired antibody immune response in HIV-1 infected patients. While such an impairment is a part of general immune deficiency, HIV does not infect B cells and thus such a mechanism should include T-B cell interactions. The authors performed experiments to dissect these interactions in the context of HIV infection. They used various experimental mouse models as well as human lymphoid tissues. The authors tested their hypothesis that NEF is a key viral protein that impairs T helper function necessary for B cells to respond to an antigen by production of specific antibodies.

In my opinion the authors proved this point and, in part, deciphered molecular mechanism of the effect of NEF that involves actin dynamics dysregulation. All these NEF effects on T-B cell interactions resulted in decrease in formation of germinal centers. Importantly that either delta-nef HIV or HIV with specific deletions in nef gene do not impair these processes.

In general, this is a convincing study and my critical remarks are few and are of editorial nature:

1, The authors should comment on equally efficient replication of WT and delta NEF HIV-1 isolates in tissues, as it is generally considered that NEF impairs HIV replication. Did they apply similar inoculums?
2. The authors should comment in their discussion the exclusion of tissues from 3 donors that did not respond to WT HIV infection by the suppression of antibody production.

3. The impairment of B cells' ability to respond to tetanus toxoid in the context of HIV infected human lymphoid tissue was reported in Glushakova et.al., Nature Med 1988. However, not all WT HIV isolates impaired B cell function.

Referee #3:

Kaw and colleagues investigate the impact of the HIV-1 Nef protein on B cell function. The authors

used two models, a mouse system of HEL immunization and human tonsil explants, with a combination of methods, including live imaging, transcriptomic analysis and functional assays to demonstrate that Nef induces B cell dysfunction. The set of data is impressive, well presented and the claims are well supported by experimental evidence. The major strength of the paper, the in use of an in vivo model, also represents its limitation, as most of the findings are obtained in a non-infectious heterologous model (effect of a human retroviral protein in mice). The use of tonsil samples elegantly completes the manuscript. Some additional data would improve the ms.

Minor comments:

1. A control experiment without immunizations may be added in the presentation of the B-HEL model.

2. Does Nef alter the viability and the activation status of CD4 T cell adoptively transferred before and after immunization?

3. The experiments with (SIR)-actin could be performed in T cells to determine whether F-actin polymerization is also decreased in T cells.

4. Quantification of cytokine production is usually made by ELISA rather than FACS. Did the authors quantify IL-4 in the supernatants?

5. Does exogenous IL-4 rescue B cell proliferation in the presence of Nef?

6. The fig 4 could be split to create a novel figure 5 dedicated to tonsil results and include additional data in the explant, such as B cell proliferation, IL4 quantification/or microscopy. Additional validations with infectious HIV-1 and human cells would improve the ms.

7. Most of live imaging experiments are performed at day 7. At this time point, most of the Ag provided by the immunization should to be cleared. Thus, contacts are likely to be increased due to the overall increased activation of T cells rather than to a direct interaction between processed Ag on MHC-II B cells and TCR on T cells as suggested in the ms. Could the authors discuss this point?

Referee #2:

The work of Kaw et.al. is focused on the mechanisms of impaired antibody immune response in HIV-1 infected patients. While such an impairment is a part of general immune deficiency, HIV does not infect B cells and thus such a mechanism should include T-B cell interactions. The authors performed experiments to dissect these interactions in the context of HIV infection. They used various experimental mouse models as well as human lymphoid tissues. The authors tested their hypothesis that NEF is a key viral protein that impairs T helper function necessary for B cells to respond to an antigen by production of specific antibodies.

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In general, this is a convincing study and my critical remarks are few and are of editorial nature:

Reply: Thank you for this positive assessment of the overall quality and relevance of our study.

1. The authors should comment on equally efficient replication of WT and delta NEF HIV-1 isolates in tissues, as it is generally considered that NEF impairs HIV replication. Did they apply similar inoculums?
Reply: This is correct: as specified in the materials and methods section, infections in tonsil tissue were performed using equivalent amounts of infectious virus (as titered on Tzm-bl reporter cells). When using equivalent amounts of virus particles instead, Nef exerts a positive effect on virion infectivity that is particularly apparent in the first round of infection and results in different replication kinetics (Nef+ viruses grow better than Nef- viruses) Glushakova et al., 1999 JVI; Glushakova et al., 2001 JVI, Homann et al., 2009 Retrovirology). This normalization to similar amounts of infectious and not physical particles was hence important for our experiment as we wanted to compare the impact of Nef on T cell help in infections with comparable amounts of infected cells and not study effects resulting from an increased number of infected cells in WT over ΔNef infections. This is now specifically explained in the revised text (lines 357-364).
2. The authors should comment in their discussion the exclusion of tissues from 3 donors that did not respond to WT HIV infection by the suppression of antibody production.
Reply: Donor-to-donor variability in the responsiveness of tonsil tissue to interference of antibody production is now discussed in the revised manuscript (lines 406-410).
3. The impairment of B cells' ability to respond to tetanus toxoid in the context of HIV infected human lymphoid tissue was reported in Glushakova et.al., Nature Med 1988. However, not all WT HIV isolates impaired B cell function.
Reply: The description of HIV-mediated interference with antibody production by the Margolis lab in tonsil tissue is now emphasized in the revised text (lines 354-355).

Referee #3:

Kaw and colleagues investigate the impact of the HIV-1 Nef protein on B cell function. The authors used two models, a mouse system of HEL immunization and human tonsil explants, with a

combination of methods, including live imaging, transcriptomic analysis and functional assays to demonstrate that Nef induces B cell dysfunction. The set of data is impressive, well presented and the claims are well supported by experimental evidence. The major strength of the paper, the in use of an in vivo model, also represents its limitation, as most of the findings are obtained in a non-infectious heterologous model (effect of a human retroviral protein in mice). The use of tonsil samples elegantly completes the manuscript. Some additional data would improve the ms.

Reply: We are glad to hear that the reviewer appreciates our experimental strategy and considers that the data presented support our main conclusions.

Minor comments:

1. A control experiment without immunizations may be added in the presentation of the B-HEL model.

Reply: We always included a "no-T cell control" to our immunization experiments to define the unspecific background by B_{HEL} cells (shown e.g. in Fig1B). We would argue that this is the more relevant control than "no immunization" for Ab production as it displays the amount of background anti-HEL antibody production triggered by immunization in the absence of specific T cell help but only by HEL specific B cells. A "No-immunization" control may be more relevant for the cell contact analyses in vivo, since the ability to undergo transient cell-cell interactions does not per se depend on the presence of specific antigen. We therefore included new intravital imaging data to compare the effect of Nef on T-B cell interactions in vivo and included animals immunized with adjuvants only as control. The results are shown as revised Figs. 5 and S3.

2. Does Nef alter the viability and the activation status of CD4 T cell adoptively transferred before and after immunization?

Reply: Figs. 4F-G and EV4 D-H already contains data showing that Nef-expressing and control cells expand equally well following adoptive transfer, indicating that their viability is not compromised. We now added new and consistent data on additional activation markers as revised Fig. EV4J-O. Since the question of effects of Nef on the activation status of CD4 T cells may be most relevant at the time of adoptive transfer, we also added new data as Fig. EV2D and E demonstrating that Nef did not affect the basal activation state of transferred CD4 T cells.

3. The experiments with (SIR)-actin could be performed in T cells to determine whether F-actin polymerization is also decreased in T cells.

Reply: Our previous text was apparently not clear enough in this regard: We and others have documented comprehensively in the past that Nef impairs actin polymerization on the T cell side of the IS and such data are shown in Fig. 2B and D. The SIR-actin experiment was only required to illustrate specifically actin dynamics in the B cells, which was not analyzed in previous studies since they did not use specific antigen and hence, actin polymerization at the B cell side of the IS was not triggered. We modified the text for clarification (lines 162-173).

4. Quantification of cytokine production is usually made by ELISA rather than FACS. Did the authors quantify IL-4 in the supernatants?

Reply: As requested, we quantified the effect of Nef on bulk cytokine production in the supernatant of our cultures. As shown in revised Figs. S2 G-J, expression of Nef did not have statistically significant effects on the amounts of IL-4 but also not on other cytokines in the cell culture supernatant. It remains unclear if the effects of Nef are highly localized and only affect the polarized secretion to B cells at the IS or whether the frequency of contacts between Nef expressing T cells with B cells in the experiments is too low to appreciate alteration in bulk cytokine production of these cultures. This is now discussed in the revised text (lines 268-271).

5. Does exogenous IL-4 rescue B cell proliferation in the presence of Nef?

Reply: We did not observe this in a single preliminary experiment but based on the lack of effect of Nef on bulk IL-4 levels, such a rescue might also not be expected. It is also difficult to know which concentration would need to be applied and whether systemic addition of IL-4 is qualitatively equivalent to polarized secretion at the IS. In addition, we noted in some experiments that Nef can have an effect on IS recruitment of important receptors such as CD4 and CD28. This effect is subject to experimental variation, which is why we do not present it in the manuscript, but it illustrates that the effects of Nef at the IS leading to the impairment of B cell activation are likely more complex than just IL-4 secretion. This is now discussed in the revised manuscript (lines 447-454).

6. The fig 4 could be split to create a novel figure 5 dedicated to tonsil results and include additional data in the explant, such as B cell proliferation, IL4 quantification/or microscopy. Additional validations with infectious HIV-1 and human cells would improve the ms.

Reply: As suggested, we split the figure into three new figures: revised Fig. 4 presents the lymph node and germinal center analysis, revised Fig. 5 a much extended in vivo imaging analysis of T cell-B cell interactions (see point 7) and Fig.6 the HIV infection data in human tonsil tissue. We fully agree that assessing if Nef impairs e.g. polarized IL-4 secretion at the IS in infected primary human cells would add value to the study. Because of the need for antigen specificity, this could currently only be done in the tonsil explant system and we have tried extensively to do this. The difficulty was that the number of productively infected cells per time point in these cultures is low (2-3%) and the number of Ag-specific cells even lower (below 1%). Although we developed 2PM microscopy on tonsil blocks in the lab to detect fluorescent reporters in infected cells, it remained extremely challenging to find individual ISs between HIV infected and TT-specific B cells. The specificity of the few individual conjugates observed was then impossible to judge and a solid quantification not feasible. These technical limitations thus far prevented us from analyzing the effect of Nef on polarized IL-4 secretion in the context of HIV-infected human cells. This limitation is now specifically stated and discussed in the revised manuscript (lines 454-458).

7. Most of live imaging experiments are performed at day 7. At this time point, most of the Ag provided by the immunizaion should to be cleared. Thus, contacts are likely to be increased due to the overall increased activation of T cells rather than to a direct interaction between processed Ag on MHC-II B cells and TCR on T cells as suggested in the ms. Could the authors discuss this point?

Reply: We believe that this is to some extend a misunderstanding arising from the lack of clarity of our text. As is also established in the literature, most of the Ag has certainly been cleared by day 7 but T-B interactions remain Ag-specific. To illustrate this point, we added new 2PM analyses including quantifications of the 2PM analysis at day 7 (revised Fig. 5 D) and included a control in which immunization with CFA only reveals the lack of prolonged cell-cell interaction in the absence of specific antigen (Fig S3). Moreover, we carried out analogous analyses of cell-cell interactions early post adoptive transfer, which also revealed a potent effect of Nef on T cell – B cell interactions. These new data are presented as revised figure 5A,B.

1st Revision - Editorial Decision 15th Sep 2020

Dear Olivier,

Thank you for submitting your revised manuscript to The EMBO Journal. I have now had a chance to take a look at it and I appreciate the introduced changes. I am therefore very pleased to let you know that we will accept the manuscript for publication here. Before sending you the formal acceptance letter there are just a few things to sort out in final revision:

- You have at the moment 6 keywords, but can only have 5

- Please relabel Declaration of Interests as Conflict of Interest

- The legend for the table should be added as a separate tab to the table and remove them from the appendix. The tables should be re-labelled as Dataset EV1 and 2. Make sure to correct callout in MS text as well.

- The movie legend should be zipped with the movie file and removed from the Appendix

- The supplementary information file should be re-labeled as appendix and the figures labeled as appendix figure S1 etc. Please also correct callouts in MS text

- For citations please cut author list after 10 authors followed by et al.

- We include a synopsis of the paper (see http://emboj.embopress.org/). Please provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper.

- We also need a summary figure for the synopsis. The size should be 550 wide by [200-400] high (pixels). You can also use something from the figures if that is easier.

- We encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. It would be great if you could provide me with a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figure? The PDF files should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation could be useful but is not essential. The PDF files will be published online with the article as supplementary "Source Data" files.

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Congratulations on a nice study

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

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- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
- Expanded View files (replacing Supplementary Information)
Please see out instructions to authors
https://www.embopress.org/page/journal/14602075/authorguide#expandedview

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The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 14th Dec 2020.

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

2nd Revision - Editorial Decision 1st Oct 2020

Dear Oliver,

Thanks for submitting your revised manuscript to The EMBO Journal. I have now had a chance to take a look at everything and all looks good.

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

Congratulation on a nice study!

Best Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2020-105594

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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 χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	All experiments were repeated to ensure reproducibility. The exact repeat times of the experiments are provided in the figure legends. For imaging experiments, the number cells were selected (see Materials and methods) based on experience from previous work that ensured generation of satistical relevant data.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For each immunization experiment, 2-3 mice per group (except negative control) were used to make it feasible for processing. Data obtained from each mouse is represented as filled circles along the average in all in vivo experiments.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Those experiments were excluded from the study where positive or negative controls didn't work, e.g in ex vivo B cell proliferation assays and HIV infection of tonsillar histocultures (see Materials and methods).
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.	All the images were acquired randomly within a sample group. For live imaging of immune synapse, two investigators did the quantification where one investigator was not aware of the sample ID.
For animal studies, include a statement about randomization even if no randomization was used.	All animal groups were treated in the same manner to avoid subjective bias. Selection of the animals were solely based on genotype and sex.
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.	Blinding was not done.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Blinding was not done.
5. For every figure, are statistical tests justified as appropriate?	Yes. Statistical tests used are mentioned in figure legends and in the material and method section.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	All the statistical analysis was conducted using Prism version 5.0 (GraphPad). In general, Mann-Whitney test or unpaired t-test was used. For multiple condition comparisos, one-way ANOVA and Dunn's multiple comparison test was used. Details of the specific test used for each experiment are described in the figure legends.
Is there an estimate of variation within each group of data?	Yes and is indicated in the figure legends.
Is the variance similar between the groups that are being statistically compared?	Yes, tested within Graphpad Prism.

C- Reagents

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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).	*Purified anti-mouse CD16/32, Clone 93, BioLegend, catalog number 101302, https://www.biolegend.com/de-de/products/purified-anti-mouse-cd16-32-antibody-190 *Biotin anti-mouse IgD, Clone 11-26c.2a, BioLegend, catalog number 405733, https://www.biolegend.com/de-de/products/biotin-anti-mouse-igd-9573 *Alexa Fluor® 488 anti-mouse GL7, Clone GL7, catalog number 144612, https://www.biolegend.com/de-de/products/alexa-fluor-488-anti-mouse-human-gl7-antigen-t-and-b-cell-activation-marker-antibody-9579 *Alexa Fluor® 647 anti-mouse CD21/CD35, Clone 7E9, catalog number 123424, https://www.biolegend.com/de-de/products/alexa-fluor-647-anti-mouse-cd21-cd35-cr2-cr1-antibody-13441 *PE anti-mouse CD45.2, Clone 104, catalog number 109808, https://www.biolegend.com/de-de/products/pe-anti-mouse-cd45-2-antibody-7 *Brilliant Violet 650™ anti-mouse B220, Clone RA3-6B2, catalog number 103241, https://www.biolegend.com/de-de/products/brilliant-violet-650-anti-mouse-human-cd45r-b220-antibody-7844 *PE/Cyanine7 anti-mouse PD-L1, Clone 10F.9G2, catalog number 124314, https://www.biolegend.com/de-de/products/pe-cyanine7-anti-mouse-cd274-b7-h1--pd-l1-antibody-6721 *Biotin anti-Mouse CD95, Clone Jo2, catalog number 554256, https://www.bdbiosciences.com/eu/applications/research/t-cell-immunology/regulatory-t-cells/surface-markers/mouse/biotin-hamster-anti-mouse-cd95-jo2/p/554256 *BV711 anti-Mouse CD3e, Clone 500A2, catalog number 740665,
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7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HEK-293T and Jurkat cell lines are commercially available and were routinely tested for mycoplasma contamination.

* 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.	Mouse strains and their source are reported 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.	All experiments with mice were carried out in accordance with the standards approved by central animal facility of the University of Heidelberg (G252/14, G300/19, T45/14, T30/17 and T39/19).
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 complied with all the guidelines.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	All procedures using human sample were in accordance with standards approved by the Heidelberg University Hospital ethics committee (ethics vote S-123/2014). Tonsils were surgically removed during standard tonsillectomy of anonymous donors with informed consent.
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.	See above.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	This section has been included in the manuscript now.
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).	Microarry data has been deposited in the NCBI GEO repository (GSE156100) https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE156100
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

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