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Multiplexed CRISPR/CAS9-Mediated Engineering of Pre-clinical Mouse Models Bearing Native Human B Cell Receptors

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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 22nd Jul 2020

Dear Facundo,

Thanks for submitting your manuscript to The EMBO Journal. Your study has now been seen by two referees who also had access to the referee comments from a previous journal as well as your response.

As you can see from the comments below, the referees feel a bit mixed if the added value of targeting simultaneous IgH and IgL loci using CRISPR/Cas9 methodology is enough of a technological advance in order to consider publication here. While Referee #2 is supportive of the work and find it an important step forward, referee #1 is a bit more hesitant if we gain enough new insight.

I have discussed the referee comments further with my colleagues and while we do see the point raised by referee #1 we also find the described approach useful and of value to the field. I would therefore like to invite you to submit a revised version - only text changes and clarifications are needed.

Regarding the previous referee comments - do you have data on hand to address the comment raised by referee #1 to including SHM analyses for the lowest precursor frequency... (2nd to last point). If you have this data then it would be good to include it.

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:
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That should be all. Let me know if we need to discuss anything further.

best Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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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)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
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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 20th Oct 2020.

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Referee #1:

The manuscript by Bautista and colleagues describes a methodology to generate mice with knock-in V-region sequences in both the heavy and light chain loci. The technology uses CRISPR/Cas9 and leads to efficient modification of the endogenous IgH and IgL loci. Such B cells respond well to antigen, undergo CSR and somatically mutate their V regions. The paper is clear, well written, and the results are convincing.

My only major concern is the novelty and the main conceptual advance being reported here? Knocking in sequences with CRISPR/Cas9 is routinely used by many, including commercial sources, and was previously reported by the authors to knockin V-regions into the IgH locus. What is being reported here is that they can accomplish this at both the IgH and IgL loci simultaneously. In addition, it has already been reported by many groups that mice with pre-rearranged V-regions that have been knocked into the IgH locus undergo CSR and SHM.

While these mice will represent a good tool to perhaps develop more potent bNAbs, or study the biological response to HIV in mice, the technological advance, at least to me, is not very striking.

Referee #2:

The study of Wang et is technically very sound, theoretically and practically interesting and will be

of significant interest to those studying B cell biology, particularly against complex immunogens that are ultimately designed for human use. I note also that the authors have made many revisions in light of 3 thorough reviews. While I am reluctant to add to their existing efforts, there are three things I would suggest they address for clarity.

1. Lines 359-360. This was quite confusing as the wording here suggests the transferred frequency was 1 in 10^4 CD45.2 cells, with the frequency of eOD-GT8 binding cells within this group being variably 14-63% depending on the genotype of the relevant B cells (line 317-318). Later, however, this experimental procedure is written in such a way that it infers the frequency of eOD-specific B cells is 1 in 10^4 etc. This should be clarified to be either strictly referring to CD45.2 cells or actually calculate the frequencies of the antigen specific B cells for the different naïve populations that were transferred. These values could influence the kinetics of the resultant responses, whereas now it could appear to the casual reader that the same number of precursors were used for each construct.
2. Line 393, (Figure S7A is missing a closing parenthesis.
3. Line 432 "silent to silent-mutations". One of those should be "non-silent".
4. "accruement" is a most unusual word. Would "accrual" have wider recognition?
5. Line 452 missing a comma after (Jardine et al., 2016b)
6. Line 410-411, it is potentially misleading to say that IgG1 cells appeared in the GC at d36, when no assessment was done between days 15 and 36 and only day 36 data are shown. Please be more precise in referring to what was done - IgG1 cells were only assessed on day 36? IgG1 cells were absent on day 15? It is a small but important point.
7. How many samples were analyzed for the data in S2 and S3? Statistics would be best, but in their absence the number of samples of which this is representative should be given.

Point by point response to the Reviewers

Reviewer#1(R1):

The manuscript by Batista and colleagues describes a methodology to generate mice with knock-in V-region sequences in both the heavy and light chain loci. The technology uses CRISPR/Cas9 and leads to efficient modification of the endogenous IgH and IgL loci. Such B cells respond well to antigen, undergo CSR and somatically mutate their V regions. The paper is clear, well written, and the results are convincing.

My only major concern is the novelty and the main conceptual advance being reported here? Knocking in sequences with CRISPR/Cas9 is routinely used by many, including commercial sources, and was previously reported by the authors to knockin V-regions into the IgH locus. What is being reported here is that they can accomplish this at both the IgH and IgL loci simultaneously. In addition, it has already been reported by many groups that mice with pre-rearranged V-regions that have been knocked into the IgH locus undergo CSR and SHM.

While these mice will represent a good tool to perhaps develop more potent bNAbs, or study the biological response to HIV in mice, the technological advance, at least to me, is not very striking.

Response: We thank R1 for his/her positive comments on our manuscript highlighting that "***The paper is clear, well written, and the results are convincing***". R1 expressed some concerns regarding the novelty to which we respectfully disagree for the following reasons: As we have mentioned in our Discussion section, line 501-505, to the best of our knowledge, ours is the first *in vivo* report of multiple HDR-mediated targeted insertions – this is unprecedented

1. As far as B cell repertoire knock-in (KI) mice are concerned, although CRISPR/Cas9 has been used to generate mice bearing pre-rearranged V-regions into the Ig H locus, ours is the first report of CRISPR/Cas9-mediated KI of LC immunoglobulin variable regions into the κ locus of fertilized zygotes. Previously, this has proved to be very difficult.
2. Our CRISPR/Cas9-mediated LC KI models are novel because in the past insertions at the κ locus have either relied on ES cells (Pelanda et al., Immunity, 1997) or non-physiological approaches such as the targeting of bicistronic Ig H and L chains to the Ig H locus (Jacobsen et al., Journal of Experimental Medicine, 2018).
3. In regard to the relevance of the VRC01-class KI mice to the HIV field, it is known that for this class of bnAbs it is of critical importance to knock in both HC and LC sequences at their native loci to study response to germline targeting immunogens. This is because while some HIV-1 bnAbs only require HCs to neutralize the virus, VRC01 bnAbs are dependent on both HC and LCs. For example, in the VRC01 subclass bnAbs, a 5-aa LC is indispensable for bnAb function. Thus it cannot be more strongly emphasized that mouse models with concomitant HC and LC VRC01-subclass KI sequence expression at their native loci are necessary for the studies of CSR, SHM and antibody evolution in response to immunization.
4. Finally, the mouse models reported here are based on the KI of bona fide native human BCR sequences, which are important in order to assess how these B cells will respond to germline-targeting immunogens. In contrast, in the past testing germline-targeting immunogens has often relied on KI mouse models expressing inferred germline (iGL) reverted precursors of known bnAbs.

Taken together, we believe that the scientific contributions of this work are significant not only in terms of multiplexed in vivo HDR KI mouse generation, but also in providing valuable preclinical BCR KI models for immunogen evaluation.

Reviewer#2 (R2):

The study of Wang et is technically very sound, theoretically and practically interesting and will be of significant interest to those studying B cell biology, particularly against complex immunogens that are ultimately designed for human use. I note also that the authors have made many revisions in light of 3 thorough reviews. While I am reluctant to add to their existing efforts, there are three things I would suggest they address for clarity.

Response: We thank R2 for his/her positive comments pointing out that our manuscript is "*is technically very sound, theoretically and practically interesting and will be of significant interest to those studying B cell biology*". We also thank R2 for her/his constructive suggestions to improve clarity and readability of our manuscript which we have fully address it below:

Major comments:

R2 states: *Lines 359-360. This was quite confusing as the wording here suggests the transferred frequency was 1 in 10^4 CD45.2 cells, with the frequency of eOD-GT8 binding cells within this group being variably 14-63% depending on the genotype of the relevant B cells (line 317-318). Later, however, this experimental procedure is written in such a way that it infers the frequency of eOD-specific B cells is 1 in 10^4 etc. This should be clarified to be either strictly referring to CD45.2 cells or actually calculate the frequencies of the antigen specific B cells for the different naïve populations that were transferred. These values could influence the kinetics of the resultant responses, whereas now it could appear to the casual reader that the same number of precursors were used for each construct.*

Response: We apologize for the confusion regarding the cell frequency used and thank R2 for pointing this out. In our manuscript, all the transferred frequencies were calculated as described in experimental procedure as (Number of CD45.2 binders)/(Number of B cells). We have now corrected it in our revised manuscript. Please see line 362.

R2 states: *Line 393, (Figure S7A is missing a closing parenthesis.*

Response: We have added the missing parenthesis in line 396.

R2 states: *Line 432 "silent to silent-mutations". One of those should be "non-silent".*

Response: We have corrected it in our revised manuscript. Please see line 439.

R2 states: *"accruement" is a most unusual word. Would "accrual" have wider recognition?*

Response: As suggested, we have replaced accruement with accrual, line 448.

R2 states: *Line 452 missing a comma after (Jardine et al., 2016b)*

Response: We have added the missing comma, line 461.

R2 states: *Line 410-411, it is potentially misleading to say that IgG1 cells appeared in the GC at d36, when no assessment was done between days 15 and 36 and only day 36 data are shown. Please be more precise in referring to what was done - IgG1 cells were only assessed on day 36? IgG1 cells were absent on day 15? It is a small but important point.*

Response: We thank R2 for pointing this out. We assessed IgG1 cells only on day 36 and not on day 15. We have corrected this information to draw a precise conclusion in this revised version. Please see line 414-416.

R2 states: *How many samples were analyzed for the data in S2 and S3? Statistics would be best, but in their absence the number of samples of which this is representative should be given.*

Response: A representative F1 breeder from each line was tested for T and B lymphocyte development and the positive progenies from the breeder were screened for persistent breeding. We have added this information in figure legends of S2 and S3 in Appendix.

1st Revision - Editorial Decision 23rd Oct 2020

Dear Facundo,

Thanks for submitting your revised manuscript.

I have now had a chance to take a careful look at the revised version and I appreciate the introduced changes and the inclusion of the SHM analyses for the lowest precursor frequency (Appendix Figure S10). I am therefore very pleased to accept the manuscript for publication here.

Congratulations on a very valuable resource!

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Corresponding Author Name: Facundo D. Batista
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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 iustified
- ➔ Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

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

- ➔ a specification of the experimental system investigated (eg cell line, species name).
- ➔ the assay(s) and method(s) used to carry out the reported observations and measurements
- ➔ an explicit mention of the biological and chemical entity(ies) that are being measured.
- ➔ an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- ➔ the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- ➔ a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- ➔ a statement of how many times the experiment shown was independently replicated in the laboratory.
- ➔ definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

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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?	Sample sizes were chosen on the basis of published work in which similar phenotypical characterization and similar defects were reported. For the immunocharacterization experiments, we used at least 3 mice to detect an effect size of 5% with power of 95% in specific cells population. Statistical analyses were performed using Prism (GraphPad Software)
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Sample sizes were chosen on the basis of published work in which similar phenotypical characterization and similar defects were reported.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No animals were excluded from analysis. Criteria for analyses were pre-established for all types of experiments.
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.	Mice were randomly chosen to perform assays.
For animal studies, include a statement about randomization even if no randomization was used.	Mice were randomly chosen to perform assays.
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' of investigators to sample identity was not done in this study.
4.b. For animal studies, include a statement about blinding even if no blinding was done	'Blinding' of investigators to sample identity was not done in this study.
5. For every figure, are statistical tests justified as appropriate?	Statistical parameters including the exact value of n with the description of what n represents, the mean, the SEM and the p value are reported in the Figures and the Figure Legends. Statistical analyses were performed using Prism (GraphPad Software) and compared with Student's t-test, p values < 0.05 were considered significant.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Normal distribution of samples was assumed on the basis of published studies with analysis similar to ours.
Is there an estimate of variation within each group of data?	All data are provided with standard error of the mean, as specified in the respective figure legend section in the text.
Is the variance similar between the groups that are being statistically compared?	N/A

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).	Reported in Matherials 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.	Reported in Materials and Methods section . Mice used in this work were housed at the the Ragon Institute's animal facility.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Reported in Material and Methods section . All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Harvard University and the Massachusetts General Hospital and conducted in accordance with the regulations of the American Association for the Accreditation of Laboratory Animal Care (AAALAC).
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.	Our work in in compliance with 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	Our study does not include datasets.
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).	N/A
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.	N/A