EMBO
Molecular Medicine

Efficacy of simultaneous VEGF-A/ANG-2 neutralization in suppressing spontaneous choroidal neovascularization

Richard H Foxton, Sabine Uhles, Sabine Grüner, Franco Revelant, and Christoph Ullmer

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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 4 January 2019

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now received the enclosed report from a referee who refereed the initial Regula et al paper and was aware of the situation. As you will see, this reviewer is globally supportive and I am pleased to inform you that we will be able to accept your manuscript pending the following final amendments:

- Please address referee 1 comments, reword the introduction as suggested, and perform the recommended analysis to improve the study.

Please submit your revised manuscript as soon as possible.

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System for Author):

For my further criticism on the JR5558 model see comments below.

Referee #1 (Remarks for Author):

This paper partially recapitulates previously published (flawed) data. Here, this dataset seems to be solid and enough experiments have been carried out for a proper statistical analysis. The proper single controls for the dual-specific VEGF-A/ANG-2 molecule were also provided. In summary, the paper shows that inhibiting VEGF-A/ANG-2 with a single dual molecule prevents neovasularization and microglia reactivity in JR5558 mice.

A major point of criticism is that JR5558 mice used in the experiments were described here exclusively as spontaneous CNV model and that only one reference related to a single lab (bias!) is cited. However, this model was previously described by two groups. The first report (Nagai, cited here) indicated that the ectopic vascular vessels were choroidal in origin and infiltrated the RPE and intraretinal space. The second group (Hasegawa et al., PLOS One 20014) reported that the model captures early stages of retinal angiomatous proliferation (RAP), with intraretinal vessels diving into the subretinal space but not breaching the RPE as required for a CNV.

I think that the authors should correct this bias here and expand the introduction by including the RAP paper. Consequently, the title term "posterior segment vascularization" could be used instead of "CNV".

The manuscript could be further improved if the available Iba1 staining would not only be used to count the absolute number of cells but also to perform a ramification analysis, e.g. by using the grid-cross method or other tools to describe the morphological phenotype of amoeboid versus ramified cells.

1st Revision - authors' response 18 January 2019

A major point of criticism is that JR5558 mice used in the experiments were described here exclusively as spontaneous CNV model and that only one reference related to a single lab (bias!) is cited. However, this model was previously described by two groups. The first report (Nagai, cited here) indicated that the ectopic vascular vessels were choroidal in origin and infiltrated the RPE and intraretinal space. The second group (Hasegawa et al., PLOS One 2014) reported that the model captures early stages of retinal angiomatous proliferation (RAP), with intraretinal vessels diving into the subretinal space but not breaching the RPE as required for a CNV.

I think that the authors should correct this bias here and expand the introduction by including the RAP paper. Consequently, the title term "posterior segment vascularization" could be used instead of "CNV".

We thank the reviewer for this comment and agree that they bring up a valid point. We are aware of the difference of opinion of the vessel origin. Since the intention of this correspondence was the exact replication of the Regula et al. 2016 data, the JR5558 mice used here were originated from the same colony as used in the studies by Regula et al., 2016 and Nagai et al., 2014, which were described as having CNV. For further clarity, we have mentioned the origin.
Page 3, line 30: JR5558 mice were supplied from a colony that was also used by Regula et al.
In addition, we have mentioned the Hasegawa paper in the introduction and that the phenotypes are described differently.

The following change has been made, in order to correct an error in the previous manuscript. Page 3, line 9 and 11: change of ‘retinal leakage’ to ‘neovascular leakage’.

The manuscript could be further improved if the available Iba1 staining would not only be used to count the absolute number of cells but also to perform a ramification analysis, e.g. by using the grid-cross method or other tools to describe the morphological phenotype of amoeboid versus ramified cells.

We performed confocal 20x image acquisitions with maximum projection of 5-7 image frames in z-direction (spanning ~7-10 µm) of the available RPE/choroidal Iba1 staining and conducted a semi-automated microglia morphology analysis applying an in-house generated image analysis software program. We concluded that not every Iba1 positive cell could be morphologically characterized. Due to an increased Iba1 positive cell density at or close to the lesions of untreated and IgG controls with numerous clumping cells, it was impossible to identify single cells to assess a ramified/amoeboid shape. Only manually selected single Iba1 positive cells peripheral to the lesions could be analysed which does not comply with an unbiased analysis. Therefore, we expected no clear conclusion from such an analysis since only selected Iba1 positive cells would be analysed per lesion and the number of Iba1 positive cells varied among the different treatment groups, being lowest in the VEGF-A/ANG-2 group.

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Christoph Ullmer
Journal Submitted to: EMBO Molecular Medicine
Manuscript Number: EMM-2018-10204

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 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.

USEFUL LINKS FOR COMPLETING THIS FORM

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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)

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	The sample size was chosen based on previous experiments and experience with this mouse strain and treatment protocol. A statement regarding this is included in the materials and methods.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	See above answer to 1.a.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	The JR5558 mouse strain develops lesions spontaneously, and with variable numbers and patterns. Some mice were excluded based on numbers of lesions, that is less than 10 in both eye, which was pre-established before the treatments were given. For analysis, some eyes were excluded according to Chauvenet's criteria (+/- 1.75 SD), which again was established prior to analysis.
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 assigned to treatment groups so that each group received mice with statistically identical numbers of lesions prior to treatment start (see figure 1B). Once treatment groups were assigned, another operator not involved in randomisation assigned the groups, which were kept masked from other users involved in analysis, until all statistical analysis was complete.
For animal studies, include a statement about randomization even if no randomization was used.	Mice were assigned to treatment groups according to lesion numbers, so that there were statistically equal numbers of lesions per mouse in each group (figure 1B). This was done prior to group allocation, which was then carried out by another investigator.
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.	Yes, in both cases investigators were blinded. Group allocation was done by investigators who were blinded to randomisation of mice. Investigators assessing results and involved in quantifcation were blind to treatment groups until all analysis (including statistics) were complete.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Investigators involved in randomisation, quantification and analysis of results were all blinded to treatment groups until analysis was complete.
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. D'Agostino & Pearson normality test was used and found to be normally distributed. Only exception is Iba1 staining where the sample size used was prohibitive for conclusive testing.
Is there an estimate of variation within each group of data?	This has not been included.
Is the variance similar between the groups that are being statistically compared?	Yes, less than 3-fold difference between treatment groups. Lower variance can be seen with anti-VEGF, anti-ANG2 and combination treatment, since these reduce the lesion numbers and size, which also reduced variability in some cases. Source data are reported in the manuscript to compare variance.

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).	Antibodies used for animal treatments were generated in house and are identical to those used in the study by Regula et al EMM (2016). Furthermore, the anti-VEGF-A antibody is published in Liang WC, Wu X, Peale FV, Lee CV, Meng YG, Gutierrez J, Fu L, Malik AK, Gerber HP, Ferrara N et al (2006) Cross-species vascular endothelial growth factor (VEGF)-blocking antibodies completely inhibit the growth of human tumor xenografts and measure the contribution of stromal VEGF. J Biol Chem 281: 951–961, and https://www.ncbi.nlm.nih.gov/pubmed/23405099.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and 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.	These are described in the paper.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	These are described in the paper.
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 comply with these guidelines

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
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.	NA
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	NA
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).	We have included datasets as supplementary documents
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