The retinal pigment epithelium as a gateway for monocyte trafficking into the eye

Inbal Benhar, Kitty Reemst, Vyacheslav Kalchenko and Michal Schwartz

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Review timeline:

Submission date: 01 November 2015
Editorial Decision: 21 December 2015
Resubmission: 25 February 2016
Accepted: 21 March 2016

Editor: Karin Dumstrei

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 21 December 2015

Thank you for submitting your manuscript to The EMBO Journal. I am very sorry about the delay in getting back to you with a decision, but I have now received the two reports on the manuscript.

As you can see below, the reports are a bit mixed. While referee #1 is clearly supportive, s/he also provides a super brief report with relative little substance which is not very helpful. Referee #2 offers very constrictive comments. While the referee finds the findings interesting, s/he also brings up concerns that preclude publication here at this stage. I have a looked carefully at the comments and I agree with them, we would need such level of insight for consideration here. Since it is unclear if you can address them in full by inclusion of additional experiments and the timeframe needed for such experiments, I am afraid that I can't commit to consider a revised version. I therefore see no other choice, but to reject the manuscript at this stage.

I am very sorry for the lengthy review process.

REFEEEREE REPORTS

Referee #1:

The purpose of the submitted manuscript was to demonstrate that like the choroid plexus epithelium in other regions of the central nervous system, the retinal pigment epithelium (RPE) acts as the primary entry point for infiltrating monocytes during retinal insult. This is a novel area of research with respect to the eye, and the authors nicely demonstrate VCAM-1-dependent infiltration of monocytes following optic nerve crush and the requirement of monocytes for abrogating pro-
inflammatory signaling initiated by retinal ganglion cell axon injury.

Specific criticisms are enumerated below:
1. Introduction and discussion sections need to be more concise. The discussion in particular seems to repeat much of the information presented elsewhere in the manuscript.

Referee #2:

Benhar et al examine the role of RPE on the polarization of myeloid cell (monocyte/macrophage) infiltrating the eye following a remote injury (optic nerve crush, ONC) or glutamate elicited excitotoxicity in the adult mouse retina. It is proposed that blood-derived monocytes trafficking to the retina/eye following injury are in close contact with RPE cells that express the integrin ligand VCAM. Evidence is provided that blocking of VCAM following ONC skews retina infiltrating monocytes toward a pro-inflammatory phenotype. While some interesting findings are presented in the current study, e.g. the imaging of myeloid cells into the retina/eye, there are a number of concerns that preclude publication in the current format.

Major points:
Figure 3 -Cell type distinction is not clear (infiltrating macrophages or residential microglia). Residential microglia (Ly6C low) could be proliferating in response to injury. I would like to see the time course experiment repeated with the Cx3CR1-GFP BM chimeras.

Figure 3 -Not convinced by the data and with conclusion about VCAMs controlling activation phenotype. Uneven cell composition between the isotype and anti-VCAM treated eyes could explain differences in cytokine profiles. For example, a decrease in macrophage infiltration could result in the majority of immune cells being pro-inflammatory microglia. They would need to specifically sort CD11b, Cx3CR1 cells from the eyes treated with and without anti-VCAM and then run real-time PRC for accurate comparison. Or culture BM macrophages in vitro and treat with VCAM ligand and measure cytokine profiles.

Figure 4 - Again the time of retina dissection/staining should be at the point where immune cell accumulation is the highest to see if high infiltration corresponds with BRB breakdown. At 7 days the cell number is the highest (400) whereas after one day there are just 100 CD11b cells in the retina.

Figure 4C - Many GFP-positive cells are associated with the optic nerve. This could be a potential route of entry. This is important because the route of immune cell trafficking is the main objective of this study. Have the authors considered to section the eye with the nerve attached and then monitor the number of Cx3CR1-GFP immune cells following ONC. Include these data with figure 3 to show the time course of GFP cells in the nerve, RPE, and retina overtime. This could provide a direct connection between the remote ONC injury and immune trafficking.

Figure 4D and 4E - quantification for IL10, TGFb, IGF1 and BDNF+ cells are all missing.

Figure 5 - Are the RPE cells actually the ones expressing these immune molecules? Confirm with in situ hybridization.

Minor points:
Figure 3B - The scale on the y-axis for the retina is misleading.

Figure 3D - Retinas were collected after only 2 days (legend). Time course experiments should be aligned for proper comparison. Also, according to figure 3A, the number of CD11b cells in the retina at 3 days is much lower than the numbers shown at 2 days under similar conditions in figure 3D.
Point-by-point response:

Referee #1:

The purpose of the submitted manuscript was to demonstrate that like the choroid plexus epithelium in other regions of the central nervous system, the retinal pigment epithelium (RPE) acts as the primary entry point for infiltrating monocytes during retinal insult. This is a novel area of research with respect to the eye, and the authors nicely demonstrate VCAM-1-dependent infiltration of monocytes following optic nerve crush and the requirement of monocytes for abrogating pro-inflammatory signaling initiated by retinal ganglion cell axon injury.

Specific criticisms are enumerated below:
1. Introduction and discussion sections need to be more concise. The discussion in particular seems to repeat much of the information presented elsewhere in the manuscript.

We thank the reviewer for his/her comments and for acknowledging the novelty of our present work. In the revised manuscript we have edited the text to make it more concise and clear.

Referee #2:

Benhar et al examine the role of RPE on the polarization of myeloid cell (monocyte/macrophage) infiltrating the eye following a remote injury (optic nerve crush, ONC) or glutamate elicited excitotoxicity in the adult mouse retina. It is proposed that blood-derived monocytes trafficking to the retina/eye following injury are in close contact with RPE cells that express the integrin ligand VCAM. Evidence is provided that blocking of VCAM following ONC skews retina infiltrating monocytes toward a pro-inflammatory phenotype. While some interesting findings are presented in the current study, e.g. the imaging of myeloid cells into the retina/eye, there are a number of concerns that preclude publication in the current format.

Major points:
Figure 3 - Cell type distinction is not clear (infiltrating macrophages or residential microglia). Residential microglia (Ly6C low) could be proliferating in response to injury. I would like to see the time course experiment repeated with the Cx3CR1-GFP BM chimeras.

We agree that the Ly6C-low cells detected in the retina might include proliferating microglia. Perhaps we did not make it sufficiently clear. Importantly, in Fig. 1, we made the distinction between resident microglia and infiltrating macrophages based on CD45 expression levels, and verified the infiltration of monocytes to the retina using Cx3CR1-GFP BM chimeras (Fig. 1E, originally 1B). In Fig. 5 (originally Fig. 4), we additionally showed in actin-GFP chimeras that peripheral cell infiltration is restricted to the injured eye, emphasizing the presence of infiltrating peripheral myeloid cells beyond the proliferation of resident microglia. Nevertheless, in the revised manuscript, we have rephrased the relevant parts in the text and discussed the possibility that the late increase in retinal of Ly6C-low myeloid cells may also include resident microglia.

Figure 3- Not convinced by the data and with conclusion about VCAMs controlling activation phenotype. Uneven cell composition between the isotype and anti-VCAM treated eyes could explain differences in cytokine profiles. For example, a decrease in macrophage infiltration could result in the majority of immune cells being pro-inflammatory microglia. They would need to specifically sort CD11b, Cx3CR1 cells from the eyes treated with and without anti-VCAM and then run real-time PRC for accurate comparison. Or culture BM macrophages in vitro and treat with VCAM ligand and measure cytokine profiles.

We regret if our conclusions with regard to the VCAM-1 results were not clearly stated; we did not intend to claim that VCAM-1 skews the macrophage phenotype, but rather, that it is involved in restoration of local immune homeostasis via facilitating the infiltration of monocyte-derived
macrophages to the retina. We agree that the pro-inflammatory cytokine profile could very well result from the activity of pro-inflammatory microglia, as we know from our previous work (Shechter et al., PloS Med 2009; London et al., J Exp Med 2011), in which we showed that when monocyte-derived macrophage infiltration is inhibited, the microglial response is exacerbated, and the local milieu remains pro-inflammatory. We have presented this data in the revised manuscript in a clearer way (pages 16-17).

Figure 4 - Again the time of retina dissection/staining should be at the point where immune cell accumulation is the highest to see if high infiltration corresponds with BRB breakdown. At 7 days the cell number is the highest (400) whereas after one day there are just 100 CD11b cells in the retina.

As requested, in the revised manuscript we have provided staining for the BRB from day 7 after the injury (Fig. EV1), which shows that there are no signs of iBRB disruption at this time point as well. Notably, however, we initially chose to assess BRB breakdown in sections of the eye from d1 after the injury based on our previous experience in the counterpart barriers of the brain and the spinal cord (Shechter et al., Plos Med 2009; Shechter et al., Immunity 2013; Kunis et al., Brain 2013; Raposo et al., J Neurosci 2014). Activation of the choroid plexus epithelium, similar to what we have found in the current study with regard to the RPE, was found to occur as early as 8 hours after spinal cord injury, whereas the peak accumulation of anti-inflammatory monocyte-derived macrophages within the neuronal tissue is seen only several days later. This is related to the fact that the cells enter through a remote gateway and then traffic within the CNS to the site of neuronal damage. Thus, the number of cells at later time points reflects accumulation rather than entry. The blood-brain barrier undergoes rapid sealing in the CNS, and no immunological leakage (e.g., in the form of perivascular cuffs) is seen unless inflammation is not resolved (Shechter and Schwartz, Trends Mol Med 2012).

Figure 4C - Many GFP-positive cells are associated with the optic nerve. This could be a potential route of entry. This is important because the route of immune cell trafficking is the main objective of this study. Have the authors considered to section the eye with the nerve attached and then monitor the number of Cx3CR1-GFP immune cells following ONC. Include these data with figure 3 to show the time course of GFP cells in the nerve, RPE, and retina overtime. This could provide a direct connection between the remote ONC injury and immune trafficking.

This is an important comment. Since the optic nerve is the site of injury, we expected to see monocytes recruited to it as well. As requested, we have now added data to Fig. 1 (Now Fig. 1A-C), showing the recruitment of monocytes (and T cells) to injured optic nerves. Notably, as stated in the text, we do not negate the possibility that infiltration into the retina could occur via this route as well (page 16). Nevertheless, the close association of peripheral monocytes with the RPE, as shown in our results (Fig. 5), including in areas remote from the optic nerve head, as well as the activation of this tissue for leukocyte trafficking (Fig. 2), strengthens the present contention that the RPE is involved in monocyte trafficking into the retina in the models we studied.

Figure 4D and 4E - quantification for IL10, TGFb, IGF1 and BDNF+ cells are all missing.

Staining for the above-mentioned markers was performed to study the ability of i.v.t.-injected monocytes to differentiate into macrophages that secrete these factors, which are related to neuroprotection and inflammatory resolution. Quantifications were added to the text in the revised manuscript (pages 13-14).

Figure 5 - Are the RPE cells actually the ones expressing these immune molecules? Confirm with in situ hybridization.

We accept the referee's comment, asking to confirm expression of immune trafficking molecules by the RPE. In the revised manuscript, we have included immunofluorescence staining for ICAM-1 and VCAM-1 after both optic nerve and glutamate injuries, to show their expression at the protein level by cells of the RPE (Now Fig. 2B and 6E).

Minor points:
**Figure 3B - The scale on the y-axis for the retina is misleading.**

Since different y-axes were used for analyzing the RPE and the retina, we have restructured the figure to avoid any misleading interpretation.

**Figure 3D - Retinas were collected after only 2 days (legend). Time course experiments should be aligned for proper comparison.**

We realized that the way the original figure was constructed may be have been confusing. In the revised manuscript, we have separated the data presented in the original figure into two figures, now Fig. 3 and Fig. 4, as they describe two distinct experiments with different purposes.

**Also, according to figure 3A, the number of CD11b cells in the retina at 3 days is much lower than the numbers shown at 2 days under similar conditions in figure 3D.**

There is some variance between experiments, possibly due to alterations in tissue processing, reagent quality, FACS voltages, etc. Notably, more cells were also recorded in the noninjured control in Fig. 3D (now 4A) as compared to 3A, while the ratio between injured and non-injured remained similar, pointing to the fact that any technical variance probably affected the experiment as a whole. Overall, the dynamics and pattern of infiltration after injury repeated across several experiments, at different time points.

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21 March 2016

Thanks for submitting your revised manuscript to The EMBO Journal. Your study has now been re-reviewed by referee #2. As you can see below, this referee appreciates the introduced changes and supports publication here. I am therefore very pleased to accept the manuscript for publication here.

**REFEREE REPORT**

Referee #2:

The authors have addressed the comments raised by this reviewer. One minor comment: in Figure 6E, the scale bar is missing.
2014.

Please follow the journal’s authorship guidelines in preparing your manuscript. The guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to complete each section in the methods section for statistics, reagents, animal models and human subjects.

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 only for independent experiments and sample sizes where the application of statistical tests is warranted. Error bars should not be shown for technical replicates.
- when n is small (n < 5), the individual data points from each experiment should be plotted alongside an error bar.

Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author guidelines on Data Presentation (see link list at top right).

2. Captions
Each figure caption should contain the following information, for each panel where they are relevant:
- a specification of the experimental system investigated (e.g. 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 absent/variable/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 p-value; 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 value = x but P value < q;
- * definition of 'center values', such 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.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to provide a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B. Statistics and general methods

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<td>1. Are the sample sizes chosen to ensure adequate power to detect a pre-specified effect size?</td>
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<td>2. Are animal studies, include a statement about sample size estimate even if no statistical methods were used.</td>
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<td>6. For animal studies, include a statement about blinding only if no blinding was done</td>
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C. Reagents

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D. Animal Models

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<td>2. For experiments involving in vivo experiments, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.</td>
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<td>3. Are the animal models consistent with the ARRIVE guidelines (please cite the ARRIVE guidelines) to ensure that other relevant aspects of animal studies are adequately reported. See authors guidelines, under ‘Reporting Guidelines’.</td>
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E. Human Subjects

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F- Data Accessibility

16. Provide accession codes for deposited data. See author guidelines, under ‘Data Deposition’ (see link list at top right).
   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

17. 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.

18. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Examples:
   a. Primary Data
      Reference Data:
      Huang L, Brown AE, Le M (2012). Crystal structure of the TRBD domain of TERT and the CRAT of TR. Protein Data Bank 4O26

19. 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, standardised format (SBML, CellML) should be used instead of scripts (e.g., MATLAB).

G- Dual use research of concern

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.

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23. 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 (AMRIS/DIC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.