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Mapping the origin and fate of myeloid cells in compartments of the eye by single-cell profiling

Peter Wieghofer, Nora Hagemeyer, Roman Sankowski, Anja Schlecht, Ori Staszewski, Lukas Amann, Markus Gruber, Jana Koch, Annika Hausmann, Peipei Zhang, Stefaniya Boneva, Takahiro Masuda, Ingo Hilgendorf, Tobias Goldmann, Chotima Böttcher, Josef Priller, Fabio Rossi, Clemens Lange, and Marco Prinz

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1st Editorial Decision 8th May 2020

Dear Marco,

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see the referees appreciate the reported findings and are overall supportive of the study. The referees raise really good points and addressing them that would strengthen the analysis. Some of them include to better compare this dataset with previously published ones and to understand if retinal microglia are different from brain microglia.

I think it would be helpful to discuss the raised points further and we can do so via email or skype.

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 discussing the revisions further

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

Please also 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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- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
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- 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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Referee #1:

In this manuscript, Wieghofer et al investigate the myeloid cell compartment in the eye by single-cell RNAseq, providing unique information about novel cell populations and their changes during development and in response to injury. The advent of scRNAseq has led to a reevaluation of cell-diversity in multiple diseases targeting the CNS. Indeed, the authors have made unique contributes to the analysis of myeloid cells in the CNS in the context of MS. Thus, these studies are timely and of broad interest to the field. These studies are properly controlled and described. I only have minor comments to improve an already exceptional work.

1. Fig 1d could be indicated if in the legend, the authors would indicate which clusters belong to the microglia, macrophages and monos/granulos/lymphos group.

2. Since the authors recently provided a comprehensive characterization of myeloid cells in the CNS by scRNA-seq in high impact papers in Science and Nature, it would be extremely useful if the authors could compare the myeloid cell population identified in the CNS and the eye to determine what populations are shared and show similar responses to insult and which populations show a more tissue-specific response.

Referee #2:

Wieghofer et al aimed to delineate the origin, function and fate of the myeloid cells of the of the eye during health and disease. They combined single cell RNA-seq analysis, fate mapping and

parabiosis to analyze the distributions of myeloid cells from the retina, the ciliary body and the cornea of adult mice. They found that retinal microglia and ciliary body macrophages are long lived cells that have negligible input from blood monocytes, while cornea macrophages are initially composed of yolk-sac derived cells but are gradually replaced by blood derived cells. Additionally, they used a neovascularization model to study the role of retinal myeloid populations in age-related macular degeneration, and observed infiltrating immune populations and phenotypical changes in the retinal microglia population.

Main conclusions
The authors use multiple state of the art techniques and elegant genetic lineage tracing strategies to address the myeloid composition of eye in health and disease, the manuscript is therefore interesting for macrophage and eye diseases researchers. However, several aspects need to be addressed before this manuscript could become eligible for publication in EMBO.

The authors have not compared any of these results to human data. Last year, 2 manuscripts were published that performed single cell RNA-seq of the human retina.

https://www.nature.com/articles/s41467-019-12780-8
https://www.embopress.org/doi/10.15252/embj.2018100811

The authors should compare their populations to these human counterparts. Are the physiological mouse populations preserved in human tissue?

What about the disease associated populations? In Menon et al., 2019, it was shown that retinal microglia exhibit enrichment for age related macular degeneration associated genetic variants. Is this also thing true for retinal mouse microglia, and does this differ between physiological microglia, and microglia from the neovascularization model?

Another major point is the novelty and significance of the results is not really clear from the current manuscript. Key questions that deserve more attention are:

Are retinal microglia different from brain microglia?

Are they in any way involved in retinal specific functions? The same thing for ciliary body and cornea macrophages?

The proteomic markers they use to validate their results are the same markers as that have been used for a long time in microglia, macrophage field, such as P2ry12, Tmem19, and CD74/HLA genes. These are very general markers of microglia and or macrophages. They should consider adding other proteomics markers for retinal specific microglia and or eye specific macrophage markers.

Also, here it would be appreciated if they tried to validate some of these markers in human tissues.

The number of cells sequenced, and more importantly retained after filtering, is not given. Based on Fig 1F, I assume {plus minus}400 cells in normal retina, and approximately 500 cells in the CNV experiments? Some of the clusters are very small, in wild type retina, 400 cells in 16 clusters, that is an average of 25 cells/cluster if evenly distributed. How reliable are these clusters. The data seem overclustered. In Fig 1b, 3 microglia subclusters are identified of 30-40 cells each? What are these subsets, what discriminates them, any cluster-enriched genes?

Are the 3 microglia clusters presented in Fig 1d also present in Fig 5? Why were the microglia from these 2 sets of experiments not co-clustered/analyzed together? How do subpopulations described in Fig1 behave/change in experiments described in Fig 5?

The Cx3CR1-CreER mouse has reported leakiness issues: tam-independent excision. That is an issue for lineage tracing experiments.
See:
Louise Chappell-Maor, Masha Kolesnikov, Jonathan Grozovski, Jung-Seok Kim, Anat Shemer, Zhana Haimon, Sigalit Boura-Halfon, Takahiro Masuda, Marco Prinz, Steffen Jung (2020). Comparative analysis of CreER transgenic mice for the study of brain macrophages: A case study. Eur J Immunol, 50(3), 353-362. doi:10.1002/eji.201948342.

Fonseca, M. I., Chu, S. H., Hernandez, M. X., Fang, M. J., Modarresi, L., Selvan, P., . . . Tenner, A. J. (2017). Cell-specific deletion of C1qa identifies microglia as the dominant source of C1q in mouse brain. J Neuroinflammation, 14(1), 48. doi:10.1186/s12974-017-0814-9

Only in fig 2, -TAM controls are included. A lot of reporter lines are used in this study, and turn-over conclusions are drawn based on these models.

The level of chimerism of the parabiosis animals is not shown, in blood for instance. 11,3 % van choroid MF is derived from parabiosis animal. Fig 2b/c depicts that in the cornea the % original (YFP+) cells drops from 74 to 16 %. Of the 60% replaced cells, roughly a quarter is symbiont-derived.
Are these numbers expected, high, please elaborate.

Minor issues
• The authors state: 'we identified 17 distinct clusters using the t-stochastic neighbor embedding algorithm'. tSNE algorithm is a data dimensionality-reduction method (used for visualization), not a clustering algorithm.

• no significance testing plotted in 2C, 3C, 3E, 4D, 4E, please add.

• Fig 4f is cited in text prior to 4c-4d-4e

• In legend fig s3, gene expression differences between cell types are referred to as up- or down-regulated. Strictly speaking that is not correct, they are more/less abundantly expressed in respective cell types.

Referee #3:

This study presented a comprehensive characterisation of myeloid cells in the cornea, ciliary body and retina using single cell profiling, detailed analysis on the origin of these myeloid cells, and characterisation of the pathological changes in rMG in an AMD neovascularization model. The strength of the study is the inclusion of multiple mouse models for lineage tracing and neovascularization model, and multiple assays used to confirm the findings in this study. This is a high quality, carefully designed study, the dataset presented would be of high value to the eye field. My main issue is the single cell dataset profiled a relatively small number of cells compared to the popular 10X method. i.e. previous study has profiled ~10k retinal microglia using 10x (PMID:

30850344). Thus, I would like to get some reassurance that the findings are consistent between biological samples. i.e. are the clusters well represented across different animals? Also, the identification of disease-associated rMG in the AMD model is novel and very interesting, I would like to see a bit more analysis on them. The following comments aim to improve on the readability of the manuscript:

Major comments:

1. Previous studies showed that some retinal microglia subpopulations showed variable expression of some genes, such as IL34, CD11c, CD11b, and TLR4. Do expression of these genes correlate to some of the microglia clusters identified in the single cell profiling?
2. Some clusters expressed both microglia and photoreceptor genes are explained as a result of phagocytosis of photoreceptor debris (figure 1: cluster 13, 14, 15; figure 5, cluster 9 and 14). Is it possible these are doublets of photoreceptors & microglia? In particular, the dissected samples contained some photoreceptors (supplementary figure 1, 5). Also, GO analysis did not highlight enrichment of phagocytosis genes in cluster 14?
3. Can the authors clarify which rMG clusters are associated with disease progression? Are there marker genes that can distinguish individual disease-associated rMG clusters?
4. Page 13: 'Both P2ry12 and Tmem119 expression decreased with disease progression and could only be detected in the clusters 3, 4 and 5, but was lost in cluster 6.' This highlight differences in the disease-associated rMG clusters in CNV progression, are there other signaling pathways that are altered in these disease-associated rMG clusters ? i.e. GO analysis
5. In the psuedo time trajectory analysis, the CNVD7 condition is split into two branches: one close to the control which is indicative of loss of reactivity, but there is also another branch on its own. Is this branch consist of particular rMG clusters? What is the author's interpretation on the cellular state of these rMG in CNVD7?

Minor comment:

6. Can the authors add the total cell number profiled after QC using single cell RNAseq for different libraries in the text? i.e. Figure 5f showed >500 cells total, but Figure 5g showed ~400 cells?
7. Page 8: 'In conclusion, all investigated eye macrophage populations (rMG, cbMΦ and cMΦ) are, at least primarily, of prenatal origin....'; only ~20-30% of microglia are from prenatal origin in corena and ciliary epithelium, perhaps partially of prenatal origin is a better description?
8. Figure 2B: the label 'E9-P0' is confusing as the timepoint is specifically P0
9. I think the abbreviation YS was used once only and seems unnecessary
10. In the results section, the authors referred to cluster numbers very regularly and I found it very difficult to follow, as I have to regularly cross-check the identity of the clusters. Adding labels for the cell type identity to the clusters in the text and figures would help improve clarity e.g. Figure 1F, 1G, 5F, 5H.
11. Figure 4 d + e: the left panel bar charts and the right panel graph are displaying the same data, I think one of them would be sufficient.

Referee #1:

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We would like to thank for this very positive and encouraging statement.

1. Fig 1d could be indicated if in the legend, the authors would indicate which clusters belong to the microglia, macrophages and monos/granulos/lymphos group.

We appreciate this suggestion and implemented the cluster identities in the respective figure legend which makes it easier for the reader to follow the content of Figure 1.

2. Since the authors recently provided a comprehensive characterization of myeloid cells in the CNS by scRNA-seq in high impact papers in Science and Nature, it would be extremely useful if the authors could compare the myeloid cell population identified in the CNS and the eye to determine what populations are shared and show similar responses to insult and which populations show a more tissue-specific response.

We thank the reviewer for this suggestion and performed the respective analysis that can be now seen in a ***novel Supplemental Figure 3****. The analysis allows the conclusion that retinal and brain microglia share very similar expression signatures. Several clusters are composed of cells from all compartments while three clusters appear to be significantly enriched with cells from the ciliary body by hypergeometric testing. Differentially regulated genes can be found in the corresponding heatmap and the figure includes t-SNE plots reflecting certain myeloid cell expression signatures that were used before in Jordao et al. 2019, e.g."microglia" or "boarder-associated macrophages".*

Referee #2:

Wieghofer et al aimed to delineate the origin, function and fate of the myeloid cells of the of the eye during health and disease. They combined single cell RNA-seq analysis, fate mapping and parabiosis to analyze the distributions of myeloid cells from the retina, the ciliary body and the cornea of adult mice. They found that retinal microglia and ciliary body macrophages are long lived cells that have negligible input from blood monocytes, while cornea macrophages are initially composed of yolk-sac derived cells but are gradually replaced by blood derived cells. Additionally, they used a neovascularization model to study the

role of retinal myeloid populations in age-related macular degeneration, and observed infiltrating immune populations and phenotypical changes in the retinal microglia population.

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The authors have not compared any of these results to human data. Last year, 2 manuscripts were published that performed single cell RNA-seq of the human retina.

https://www.nature.com/articles/s41467-019-12780-8
https://www.embopress.org/doi/10.15252/embj.2018100811

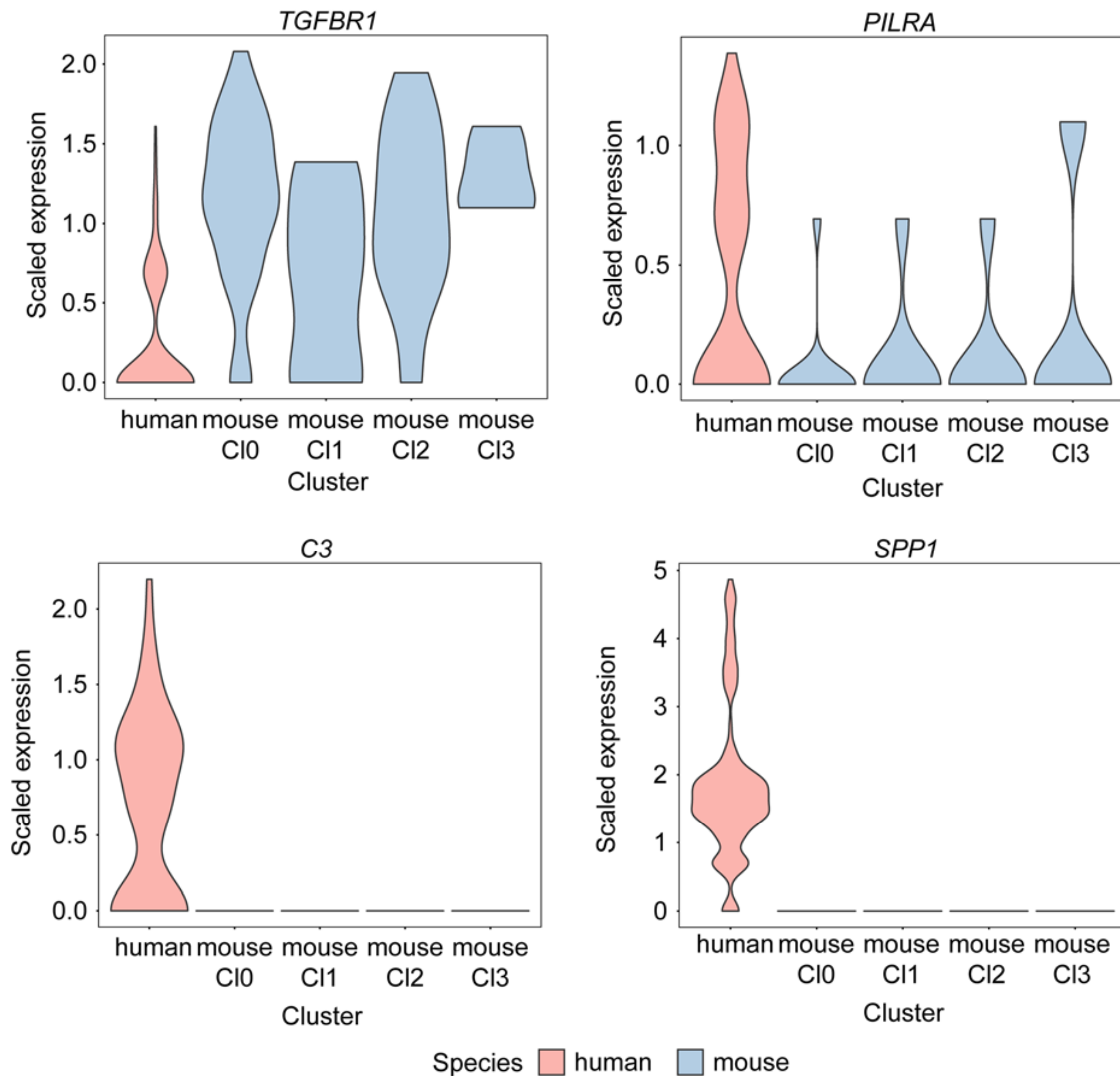
The authors should compare their populations to these human counterparts. Are the physiological mouse populations preserved in human tissue?

The reviewer is right about the intention to ultimately compare findings made in mice to the human situation. We therefore attempted a comparison of the availabe human data to our data sets. However, both studies have a focus on neuronal and glial cells beside the vasculature (wet AMD) resulting in relatively low numbers of microglia.

*Regarding the Lukowski paper, three HLA-related genes are shown as representative microglia genes. In line with this, we added a **novel Figure 7** including HLA-DRA which we investigated in more detail by bulk RNA-Seq and immunohistochemistry in human samples and compared it to the rodent situation.*

The study by Menon et al. 2019 focused on neuronal and glial cells. However, some genes that are associated with AMD as genetic variants promoting disease initiation/progression were found to be expressed by native microglia in the human situation.

We therefore analyzed the human retinal microglial cells (only one cluster) from the Menon manuscript with the murine microglia to compare the expression of the genes mentioned in the manuscript, namely C3, TGFBR1 and PILRA. The data are presented below as Violin Plot showing differential expression of these genes between the murine and human cluster. In addition, we show SPP1 which can be regarded as an activation marker. As we have shown before, the SPP1 expression significantly increases in (brain) microglia with age in the human situation under steady state conditions (see Sankowski et al. 2019). We therefore believe that human microglia are per se in a more advanced activated state than freshly isolated murine microglia making a direct comparison not useful.



From our point of view, the CNV model is suitable to investigate certain hallmarks of AMD in mice, like neovascularization and the composition of myeloid cells. However, the mode of development of the CNV between mice and humans follows substantially different kinetics. Genetic risk variants found by association studies, like in the Menon manuscript, are likely modulators of disease initiation and progression over decades ultimately leading to an emergence of CNV membranes in humans but are not necessarily regulated in the same manner in murine CNV-associated myeloid subsets caused by an acute laser burn over the course of one week. With respect to the aforementioned reasons and in combination with relatively low cell numbers in the human retinal microglia data set we would like to present this data set only in the rebuttal letter and do not implement it in the manuscript.

Another major point is the novelty and significance of the results is not really clear from the current manuscript. Key questions that deserve more attention are:

Are retinal microglia different from brain microglia?

*We apologize that we didn't put enough emphasis on this very important question in the submitted manuscript version. Some typical microglia signature genes were already shown but only by bulk RNA-Seq in the supplementals. In the revised version, we used our available scRNA-Seq data from the eye and extended the analysis with data from the brain (Jordao et al. 2019) which resulted in a **novel Supplemental Figure 3** addressing this question in more detail.*

Are they in any way involved in retinal specific functions? The same thing for ciliary body and cornea macrophages?

We now introduced compartment-specific macrophage functions in more detail in the introduction (page 4).

The proteomic markers they use to validate their results are the same markers as that have been used for a long time in microglia, macrophage field, such as P2ry12, Tmem19, and CD74/HLA genes. These are very general markers of microglia and or macrophages. They should consider adding other proteomics markers for retinal specific microglia and or eye specific macrophage markers.

*We thank the reviewer for this comment. P2ry12, Tmem119, Cd74 and H2-Aa (MHCII) were among the most regulated genes in all clusters together (Fig. 1g) and showed a high expression pattern on both mRNA and protein level. In the frame of our revision, we attempted an extension of our validation for proteomic markers. In the revised **Suppl. Fig. 4**, we now included a CD206 immunolabelling in Cx3cr1-GFP mice besides the analysis of Iba1+ cells in Itgax-GFP (CD11c) mice and newly generated Hexb-tdTomato mice.*

Also, here it would be appreciated if they tried to validate some of these markers in human tissues.

*We addressed this question and analyzed the presence of MHCII in mice and the respective orthologous gene HLA-DR in the human situation in the CNV model, both on mRNA and protein level in parallel (please see **novel Figure 7**).*

The number of cells sequenced, and more importantly retained after filtering, is not given. Based on Fig 1F, I assume {plus minus}400 cells in normal retina, and approximately 500 cells in the CNV experiments? Some of the clusters are very small, in wild type retina, 400 cells in 16 clusters, that is an average of 25 cells/cluster if evenly distributed. How reliable are these clusters? The data seem overclustered. In Fig 1b, 3 microglia subclusters are identified of 30-40 cells each? What are these subsets, what discriminates them, any cluster-enriched genes?

The data were analyzed using the RaceID package as we have done seccesfully in our previous studies before (Jordao et al. Science, 2019, Masuda et al. Nature 2019, Sankwoski et al. Nat Neurosci 2019). The RaceID package was originally desinged to identify rare cell subsets [10.1038/nature14966] and we are therefore convinced that the number of isolated cells is sufficient for the method used.

Are the 3 microglia clusters presented in Fig 1d also present in Fig 5? Why were the microglia from these 2 sets of experiments not co-clustered/analyzed together? How do subpopulations described in Fig1 behave/change in experiments described in Fig 5?

We thank the reviewer for this comment. We now improved parts the main text, the figure legend and methods section to make it more transparent to the readership.

The homeostatic rMG (clusters 13, 14, 15) in Figure 1 represent the same data set like the non-lasered controls in the CNV experiments. However, there can´t be an exact match between the clusters in Figure 1 and 5 for some reasons. First, the native non-lasered control microglia were analyzed together with the CNV d3 and d7 cells which leads to a novel clustering. Second, both CNV d3 and d7 contain a certain amount of unaffected microglia likely derived from the areas around the lesions. This leads to the clusters 4 and 5 significantly enriched for the control condition but still containing cells from the diseased conditions. Consequently, the control microglia clusters 4 and 5 are not directly comparable to the homeostatic clusters from Figure 1.

The Cx3CR1-CreER mouse has reported leakiness issues: tam-independent excision. That is an issue for lineage tracing experiments.

See:
Louise Chappell-Maor, Masha Kolesnikov, Jonathan Grozovski, Jung-Seok Kim, Anat Shemer, Zhana Haimon, Sigalit Boura-Halfon, Takahiro Masuda, Marco Prinz, Steffen Jung (2020). Comparative analysis of CreER transgenic mice for the study of brain macrophages: A case study. Eur J Immunol, 50(3), 353-362. doi:10.1002/eji.201948342.

Fonseca, M. I., Chu, S. H., Hernandez, M. X., Fang, M. J., Modarresi, L., Selvan, P., . . . Tenner, A. J. (2017). Cell-specific deletion of C1qa identifies microglia as the dominant source of C1q in mouse brain. J Neuroinflammation, 14(1), 48. doi:10.1186/s12974-017-0814-9

Only in fig 2, -TAM controls are included. A lot of reporter lines are used in this study, and turn-over conclusions are drawn based on these models.

The reviewer is right. We are fully aware about the drawbacks of this line and we were co-auhtors on some of these publications.

We now included new data on the TAM-independent recombination of retinal microglia in ***Figure 4g****. Here, we found around 50 % Tomato-expressing positive rMG by flow cytometry but more importantly, no recombination in peripheral blood monocytes. We hereby conclude that there are spontaneous recombination events in retinal microglia but these remain limited to that cell population and do not affect the prerequisition of our experimental setup: a high recombination in rMG versus virtually no in monocytes after TAM treatment.*

Regarding the turnover experiments shown in ***Figure 3*** *performed with the Cx3cr1CreER:Rosa26-fl-stop-fl-YFP reporter mice, we now did FACS experiments with untreated mice. We found 9.2 % spontaneous YFP labelling in rMG (n=4), 0.22 %*

in cornea macrophages (n=4), 0 % in monocytes (n=4) in the absence of TAM. Conclusively, the TAM-independent recombination in YFP reporter mice is negligible and far below the lowest recombination rate that we measured in the TAM treated mice in the turnover experiments.

The level of chimerism of the parabiosis animals is not shown, in blood for instance.

*We added the chimerism as percentage of Ly6ChiGFP+ monocytes in the wildtype parabiotic partner in the figure legend (**Figure 3e**).*

11,3 % van choroid MF is derived from parabiosis animal. Fig 2b/c depicts that in the cornea the % original (YFP+) cells drops from 74 to 16 %. Of the 60 % replaced cells, roughly a quarter is symbiont-derived. Are these numbers expected, high, please elaborate.

We used two different models for the measurement of myeloid cell turnover: The first one (Cx3cr1CreER:Rosa26-YFP) labels the tissue resident macrophages to investigate the replenishment by non-labeled peripheral blood-derived cells. The second model of parabiosis allows to label peripheral blood cells exclusively to prove the contribution of these cells to the resident cornea macrophage pool which increaes over time. In this model, it can be assumed that only around half of the cells are detected depending on the blood chimerism of GFP+ cells in the wildtype partner, thereby underestimating the true turnover. Both models are complementary to each other but the percentages of cell laballing can´t be compared directly.

Minor issues

- **The authors state: 'we identified 17 distinct clusters using the t-stochastic neighbor embedding algorithm'. tSNE algorithm is a data dimensionality-reduction method (used for visualization), not a clustering algorithm.**

This is true and we changed the wording accordingly.

- **no significance testing plotted in 2C, 3C, 3E, 4D, 4E, please add.**

We applied statistics to the data sets in the respective figures, figure legends and the statistics statement in the methods.

- **Fig 4f is cited in text prior to 4c-4d-4e**

We changed the order in the text accordingly.

- **In legend fig s3, gene expression differences between cell types are referred to**

as up- or down-regulated. Strictly speaking that is not correct, they are more/less abundantly expressed in respective cell types.

The wording is indeed misleading and we improved the figure legend according to the reviewers suggestion.

Referee #3:

This study presented a comprehensive characterisation of myeloid cells in the cornea, ciliary body and retina using single cell profiling, detailed analysis on the origin of these myeloid cells, and characterisation of the pathological changes in rMG in an AMD neovascularization model. The strength of the study is the inclusion of multiple mouse models for lineage tracing and neovascularization model, and multiple assays used to confirm the findings in this study. This is a high quality, carefully designed study, the dataset presented would be of high value to the eye field.

We are grateful for this enthusiastic statement.

My main issue is the single cell dataset profiled a relatively small number of cells compared to the popular 10X method. i.e. previous study has profiled ~10k retinal microglia using 10x (PMID: 30850344). Thus, I would like to get some reassurance that the findings are consistent between biological samples. i.e. are the clusters well represented across different animals?

*As several animals were pooled prior to sorting thereby averaged per cluster, the presence of each cluster in each animal is challenging. However, as retinal microglia integrate well with brain microglia from our previous publications (Masuda et al. Nature 2019), it can be expected that each animal shows similar transcriptional states of microglia. The same holds true with overlapping expression signatures between eye and brain macrophages (please see **novel Figure S3**). Furthermore, our single cell data were analyzed using the RaceID package as we have done seccesfully in our previous studies before (Jordao et al. Science, 2019, Masuda et al. Nature 2019, Sankwoski et al. Nat Neurosci 2019). The RaceID package was originally desinged to identify rare cell subsets [10.1038/nature14966] and we are therefore convinced that the number of isolated cells is sufficient for the method used.*

Also, the identification of disease-associated rMG in the AMD model is novel and very interesting, I would like to see a bit more analysis on them. The following comments aim to improve on the readability of the manuscript:

Major comments:

1. Previous studies showed that some retinal microglia subpopulations showed variable expression of some genes, such as IL34, CD11c, CD11b, and TLR4. Do

expression of these genes correlate to some of the microglia clusters identified in the single cell profiling?

We would like to thank the reviewer for these suggestions. In 2019, O'Koren et al. found a reduction of microglia numbers specifically in the inner plexiform layer but not in the outer plexiform layer under IL-34 KO conditions. The source of IL-34 was idenitified as retinal ganglion cells leading to a specific IL-34-dependent microglial subpopulation. However, we addressed this question but could not detect an expression of Il34 on RNA level in retinal microglia.

*Regarding the integrin expression, we found no obvious differences in the expression of Itgam (CD11b) in the distinct microglia clusters. However, we included CD11c-GFP mice in our extended protein validation analysis (**Suppl. Figure S4**). Here, we found a low fraction of GFP-positive cells which is in line with our bulk RNA-Seq results of a moderate Itgax gene expression, encoding CD11c.*

The Tlr4-/- mouse was reported to show a microglial-related phenotype (PMID: 30873007). We therefore checked the expression of Tlr4 but this gene could not be found among the uniquely expressed genes of the homeostatic or CNV-related clusters.

2. Some clusters expressed both microglia and photoreceptor genes are explained as a result of phagocytosis of photoreceptor debris (figure 1: cluster 13, 14, 15; figure 5, cluster 9 and 14). Is it possible these are doublets of photoreceptors & microglia? In particular, the dissected samples contained some photoreceptors (supplementary figure 1, 5). Also, GO analysis did not highlight enrichment of phagocytosis genes in cluster 14?

We would like to thank the reviewer for this comment. In our setup, we enriched the leukocyte fraction by CD45+ and, at the same time, performed a depletion of neutrophils, B and T cells from the same fraction (CD3- CD19- Ly6G-). However, the discrimination of CD45 wasn't absolute and a certain amount of non-leukocyte cells were sorted as well. We therefore focused on the myeloid cells in our analysis but provided the original data as a supplementary data set.

*The applied doublet-exclusion (Fsc-W and Fsc-A vs. Fsc-H) of viable cells during flow cytometry-based purification reduces the inclusion of doublets to a minimum. However, we can not completely rule out that cell debris of undergoing neuronal cells eventually attached to the microglial cells or underwent phagocytosis. It is true that only cluster 13 and 15 are positive for the GO term "phagocytosis" in **Suppl. Figure 2** but at the same time have a higher log2fold change of genes expressed in photoreceptors, like Rho and Gnat1 in the heatmap in Fig. 1g, in comparison to cluster 14 assigned as non-phagocytotic.*

*In the CNV data set where a strong laser burn is applied, we are convinced that myeloid cells take up cell debris from dying photoreceptor cells close to the lesion formation, represented by cluster 9 and 14. For example, cluster 9 has a high proportion of cells from CNV d3 that we regard as the peak of inflammation in this model, corroborated by the composition of cells (**Figure 5g**) and the minimal spanning tree (**Figure 6a**). However, only cluster 14 is assigned as phagocytic in the novel supplemental figure 7 but on the other hand cluster 9 expresses genes typically present in APCs, like Cd74 and several H2 genes, and be therefore regarded as phagocytes.*

3. Can the authors clarify which rMG clusters are associated with disease progression? Are there marker genes that can distinguish individual disease-associated rMG clusters?

We thank the reviewer for this interesting question. The hypergeometric testing that we applied to the CNV data set revealed cluster 4 and 5 to be significantly associated with the control condition while cluster 6 is exclusively associated with CNV d7 and the clusters 11, 12 and 14 are related to CNV d7.

*However, there is not one marker gene that can clearly distinguish one from the other cluster/population. All CNV-associated rMG clusters express more or the less the same genes but to a different extent. For example, P2ry12 still appears in all CNV-associated gene expression profiles but is relatively lower to the high expressing population(s) or even not detectable anymore on protein level (**Figure 6b**). In contrast, Sparc or Hexb are more stably expressed across the rMG clusters (**Figure 5i**). These findings were the reason why we applied the pseudotime trajectory analysis to identify transcriptionally similar gene modules in rMG showing a resolution of the highly activated state at CNV d3 to CNV d7 in the minimum spanning tree (**Figure 6a**).*

4. Page 13: 'Both P2ry12 and Tmem119 expression decreased with disease progression and could only be detected in the clusters 3, 4 and 5, but was lost in cluster 6.' This highlights differences in the disease-associated rMG clusters in CNV progression, are there other signaling pathways that are altered in these disease-associated rMG clusters ? i.e. GO analysis

*To address specific functional states of the particular microglial clusters and other myeloid cells we provide a **novel Suppl. Figure 7** containing a GO term analysis.*

5. In the psuedo time trajectory analysis, the CNVD7 condition is split into two branches: one close to the control which is indicative of loss of reactivity, but there is also another branch on its own. Is this branch consisting of particular rMG clusters? What is the author's interpretation on the cellular state of these rMG in CNVD7?

The minimal spanning tree in combination with the heatmap above shows a closer relationship of CNV d7 to the control than CNV d3 to the control. Our interpretation is a peak of the inflammatory state at CNV d3 that turns into an ongoing resolution until CNV d7, thereby reflected by one CNV d7 population close to the CNV d3 representing the lower branch and a second population similar to the control condition.

Minor comments:

6. Can the authors add the total cell number profiled after QC using single cell RNAseq for different libraries in the text? i.e. Figure 5f showed >500 cells total, but Figure 5g showed ~400 cells?

*We now included the total cell number of 511 cells in the legend of **Figure 5f** and the numbers of cells per condition (control, CNV d3, CNV d7). The discrepancy between*

Figures 5f *and* ***5g*** *can be explained by a reduced number of cells due to the automated cell type assignment thereby exluding unassigned cells (grey cells in* ***Figure 5d****).*

7. Page 8: 'In conclusion, all investigated eye macrophage populations (rMG, cbMΦ and cMΦ) are, at least primarily, of prenatal origin....'; only ~20-30% of microglia are from prenatal origin in corena and ciliary epithelium, perhaps partially of prenatal origin is a better description?

We changed the sentence accordingly to: 'In conclusion, all investigated compartments of the murine eye contained macrophage populations (rMG, cbMΦ and cMΦ) of prenatal origin...".

8. Figure 2B: the label 'E9-P0' is confusing as the timepoint is specifically P0

The used label is indeed misleading and was changed as suggested.

9. I think the abbreviation YS was used once only and seems unnecessary

We removed the abbreviation "YS" for yolk sac from the manuscript.

10. In the results section, the authors referred to cluster numbers very regularly and I found it very difficult to follow, as I have to regularly cross-check the identity of the clusters. Adding labels for the cell type identity to the clusters in the text and figures would help improve clarity e.g. Figure 1F, 1G, 5F, 5H.

We would like to thank the reviewer for this comment and considered to change the nomenclature in the suggested way. However, only microglial clusters are exclusively derived from one compartment, the retina, while mostly all other clusters are either enriched of ciliary body- or cornea-derived cells. Among these clusters a few are not significantly enriched with cells from one or the other compartment. Consequently, a common scheme, can't be applied for all clusters and would be misleading.

11. Figure 4 d + e: the left panel bar charts and the right panel graph are displaying the same data, I think one of them would be sufficient.

Here, we intended to show the ratio of microglia and peripheral blood monocytes in the left graph (percentages). In the right graph, we show the absolute numbers of cells (mean values) present inside the lesions which is not included in the left graphs but gives an idea about the kinetics of cell types. To make it more clear to the reader, we changed the labels of the axes accordingly.

1st Revision - Editorial Decision 23rd Oct 2020

Dear Marco,

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been seen by referee #3 and the comments are provided below.

As you can see the referee appreciates the introduced changes and has one remaining minor comment to be addressed. Given this input, I am very pleased to let you know that we will accept the manuscript for publication here. Before sending you the formal accept letter there are just a few remaining editorial points to resolve in a final revisions.

- Some of the figures has many panels like for example figure 4 and I almost wonder if some of them should be split into two, but I will leave that up to you. OK to have 9 main figures. You can only have 6 EV figures though and the rest would have to be added into an appendix - see author guidelines.

- you can only have 5 keywords (you have at the moment 8)

- the contributions of Lukas Amann is missing in the author contribution section

- please enter funding info into the online system as well

- reference format should alphabetically with author list cut after 10

- please add the method references to the main reference list

- the accession numbers should be added into a data availability section

- can you please confirm that there is an image in the Fig 1H => P2RY12 bottom row panel (red channel)

- I have asked our publisher to do their pre-publication checks on the paper. They will send me the file within the next few days. Please wait to upload the revised version until you have received their comments.

- 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.

That should be all - let me know if you have any further questions.

Congratulations on a nice study!

With best wishes

Karin

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:
https://bit.ly/EMBOPressFigurePreparationGuideline

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 21st Jan 2021.

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

Referee #3:

The authors have addressed all my questions in this revision, except for one point which was not reflected in the manuscript; I am not sure the author's response to my comment 3 was incorporated into the revised manuscript and it would be informative to include the following in a brief format: 'However, there is not one marker gene that can clearly distinguish one from the other cluster/population. All CNV-associated rMG clusters express more or the less the same genes but to a different extent. For example, P2ry12 still appears in all CNVassociated gene expression profiles but is relatively lower to the high expressing population(s) or even not detectable anymore on protein level (Figure 6b). In contrast,Sparc or Hexb are more stably expressed across the rMG clusters (Figure 5i). These findings were the reason why we applied the pseudotime trajectory analysis to identify transcriptionally similar gene modules in rMG showing a resolution of the highly

activated state at CNV d3 to CNV d7 in the minimum spanning tree (Figure 6a).'

Congratulations on an interesting study!

point-by-point reply:

- Some of the figures has many panels like for example figure 4 and I almost wonder if some of them should be split into two, but I will leave that up to you. OK to have 9 main figures. You can only have 6 EV figures though and the rest would have to be added into an appendix - see author guidelines.

We changed the figures accordingly and created 9 main and 6 extended view figures. We used the graphical abstract a s atemplate for the summary and removed it from the former supplemental figures. In this scenario we avoid a one-figure-appendix and a redundant graphical abstract.

- you can only have 5 keywords (you have at the moment 8)

We reduced the number of keywords.

- the contributions of Lukas Amann is missing in the author contribution section

We added Lukas Amann to the author contributions

- please enter funding info into the online system as well

We thank for making us aware of this and entered the funding in the system

- reference format should alphabetically with author list cut after 10
- please add the method references to the main reference list

We implemented the reference style from EMBO Journal and fused the methods references with the ones from the main body of the manuscript in an alphabetical order

- the accession numbers should be added into a data availability section

The accession can be found in a new "data availability" section

- can you please confirm that there is an image in the Fig 1H => P2RY12 bottom row panel (red channel)

We hereby confirm that there is an image in fig. 1h showing cornea macrophages completely devoid of P2RY12 expression in direct comparison microglia

- I have asked our publisher to do their pre-publication checks on the paper. They will send me the file within the next few days. Please wait to upload the revised version until you have received their comments.

We included all changes in the version that we received from the publisher

- 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 provide a general summary and 4 bullet points in a comparable way to recent publications in EMBO Journal.

- 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 created summary figure with elements from the former graphical abstract.

Referee #3:

The authors have addressed all my questions in this revision, except for one point which was not reflected in the manuscript; I am not sure the author's response to my comment 3 was incorporated into the revised manuscript and it would be informative to include the following in a brief format:
'However, there is not one marker gene that can clearly distinguish one from the other cluster/population. All CNV-associated rMG clusters express more or the less the same genes but to a different extent. For example, P2ry12 still appears in all CNVassociated gene expression profiles but is relatively lower to the high expressing population(s) or even not detectable anymore on protein level (Figure 6b). In contrast,Sparc or Hexb are more stably expressed across the rMG clusters (Figure 5i). These findings were the reason why we applied the pseudotime trajectory analysis to identify transcriptionally similar gene modules in rMG showing a resolution of the highly activated state at CNV d3 to CNV d7 in the minimum spanning tree (Figure 6a).'

We thank the reviewer for his comment to not only reply to his question but to implement it in the main text. We apologize that we missed this and included a sentence explaining the reasons behind the pseudotemporal ordering based on only relative expression changes of single genes across clusters.

2nd Revision - Editorial Decision 18th Dec 2020

Dear Marco,

Thank you for submitting your revised manuscript to The EMBO Journal. I have now had the chance to take a look at the revised version and I appreciate the introduced changes.

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

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. If you do NOT want this, you will need to inform the Editorial Office via email immediately. More information is available here: https://emboj.embopress.org/about#Transparent_Process

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Prof. Dr. Marco Prinz
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2020-105123

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

Question	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?	No statistical methods were used to predetermine sample sizes, and exact group numbers were determined by animal availability. However we did ensure that our sample sizes were similar to those generally employed in the field.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Exact group number were determined by animal availability.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Regarding the CNV experiments, all animals that showed signs of subretinal bleeding or confluent lesions were excluded from the study. Cx3cr1CreER:Rosa26-YFP mice that showed a recombination rate of less than 70% in microglia were excluded from the study.
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.	For scRNA Seq experiments C57BL/6 mice were purchased from Charles River and randomly divided into control, CNV d3 and d7. Whole litters of Cx3cr1CreER mice were treated together with tamoxifen at the age of 6 weeks to ensure consistency between mice.
For animal studies, include a statement about randomization even if no randomization was used.	See point 3.
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, the investigator was blinded during semi-automatic (Fig. 4d,e) or manual quantification (Fig. 3e). After establishing the experimental flow cytometry setup, all samples from the independent experiments were consistently analyzed with the same device settings and gating strategy template (Fig. 3b,c, i-m, S5f,g).
4.b. For animal studies, include a statement about blinding even if no blinding was done	See point 4a.
5. For every figure, are statistical tests justified as appropriate?	In the material and method section we clearly describe our statistical analyses (see below). The statistical test applied is always indicated in the figure legends.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Statistical analysis was performed using GraphPad Prism (GraphPad Software, Version 6.0, La Jolla, USA). Data were tested for normality applying the Kolmogorov–Smirnov test. If normality was given, an unpaired t test was applied or One-way ANOVA, if not indicated otherwise. If the data did not meet the criteria of normality, the Mann–Whitney or Kruskal-Wallis test was applied. Differences were considered significant when P value < 0.05.
Is there an estimate of variation within each group of data?	The variation of each group is shown with the standard error of mean (S.E.M).

Is the variance similar between the groups that are being statistically compared?	The within-group variance only accounts for one data set were we applied the One-way ANOVA (Fig. 3b, c) and we didn't notice a discrepancy between the groups.

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).	All antibodies used in this study can be found the respective parts of the methods and materials sections including clone and/or catalogue number, company and eventually labeled fluorophores.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	No cell lines were in this study.

* 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.	In this study, C57BL/6J mice were used as wildtype (WT) mice. The mice used in this study were of mixed gender and sacrificed between 8-12 weeks of age if not stated differently. Female C57BL/6J mice for scRNA-Seq experiments were purchased from Charles River. All transgenic lines (Acta1GFP/+, Ccr2RFP/+, Cx3cr1GFP/+, HexbtdT/tdT, Cx3cr1CreERT2, Flt3Cre, Rosa26-fl-stop-fl-EYFP (Rosa26-YFP) and Rosa26-fl-stop-fl-tdTomato (Rosa26-tdTomato) were bred on a C57BL/6J background under specific pathogen-free conditions and devoid of Crb1 (RD8) mutations. Itgax-DTR/EGFP (CD11c-GFP) mice were purchased from Jackson Laboratory (Stock No. 004509, C57BL/6J background). Flt3Cre mice were crossed to Rosa26-YFP and only male mice were used due to the localization of the Flt3-Cre transgene on the Y chromosome. Cx3cr1CreERT2 were crossed to Rosa26-YFP or Rosa26-tdTomato and treated with tamoxifen at the age of 6 weeks. For pulse labeling experiments, pregnant mice were treated with tamoxifen at E9.0 and the offsping was sacrificed at postnatal day 0. The timepoint of analysis of Cx3cr1CreERT2 Rosa26-YFP mice is indicated in the respective figure legends. Cx3cr1CreERT2 Rosa26-tdTomato mice underwent laser treatment for CNV experiments 8 weeks after tamoxifen treatment at the age of 14 weeks and were analyzed at the indicated time points in the respective figure legends.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal experiments were approved by local administration and were performed in accordance to the respective national, federal and institutional regulations. The local animal permissions were reviewed and approved by the Regierungspräsidium Freiburg.
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.	

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Ethics approval was granted from the local Ethics Committees Freiburg.
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.	Written informed consent was obtained from each patient and the experiments are conform to the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.
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.	The local Ethics Commitee Freiburg approved the study under the registration number 1/17.
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	The human Sequencing data (Fig. 7c) have been deposited in the Gene Expression Omnibus database (https://www.ncbi.nlm. nih.gov/geo; accession number GSE146887).
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).	NA
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