Reviewer’s report

Title: Eosinophils in anti-neutrophil cytoplasmic antibody associated vasculitis

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Reviewer: Adrian Schreiber

Reviewer's report:

The observation of disturbed eosinophil functions in patients with ANCA-vasculitis is of particular interest as the contribution of eosinophils in AAV remains largely unknown. However, we think that some corrections and additional data should be included in the manuscript before being process for publication.

- Point 1: Fig.1 and additional fig.1 present the different cell subsets as % of either leukocytes or PMN but the authors comment this figure in the manuscript using the term "number". Percentage does not always reflect cell number. For instance, the increase % of PMN in AAV patients might not be associated with an increased number of PMN but rather a consequence of a decreased number of other immune cells such as lymphocytes. Therefore, we recommend that the authors add the number of cells/ml or μl blood for all subsets in the figure 1 and additional figure 1.

- Point 2: In Fig.1 and additional figure 1, neutrophils and eosinophils are expressed as % of PMN but basophils as % of leukocytes. For consistency, basophils should also be expressed as % of PMN.

- Point 3: In Fig.2 and Fig.3, several surface markers are used to study the activation status of eosinophil from AAV patients and healthy controls by flow cytometry. CD16, CD64 and CD193 were found increased whereas CD35, CD88, CD11b, CD11c and Siglec-8 were found decreased in AAV patient compared to healthy controls. CD62L, usually decreased following eosinophil activation remains similar in both groups. We agree with the authors about the term "altered surface marker expression" used in the manuscript. However, on page 14 row 2 the authors write "...they showed altered surface marker expression and function indicating that they are activated" suggesting that this altered pattern is linked to eosinophil activation. Are these different surface markers associated with eosinophil activation? Are they all regulated in a way which reflects eosinophil activation? What is the activation status in comparison to other inflammatory conditions or autoimmune disease such as EGPA (see also point 4)?

- Point 4: The manuscript describes eosinophils in patients with granulomatosis with polyangiitis (GPA) and microscopic polyangiitis (MPA). Eosinophilic granulomatosis with polyangiitis (EGPA) is here not studied. EGPA is a combination of allergy, tissue/blood eosinophilia and necrotizing vasculitis with eosinophil infiltration. Circulating eosinophils are activated in EGPA and express high level of CD69 and CD11b (Diny et al. (2017), Front.Immunol. 8:484; Khoury et al. (2014), Nat.Rev.Rheumatol. 10(8):474)

Therefore, it would be interesting to have some information about eosinophils in EGPA in the
manuscript to compare with the current study on MPA and GPA.

-Point 5: Fig.4 describes intracellular ROS production in eosinophils. Interestingly, unstimulated and PMA- or E.Coli-stimulated eosinophils from AAV patients produced less intracellular ROS compared to healthy controls. The following gating strategy is described in the Method section: "At least 15,000 PMN were collected based on forward and side scatter properties. Eosinophils were defined as CD16negSiglec-8pos granulocytes". The authors should include this gating strategy as facs plot in fig.4. Also, a representative ROS fluorescence curve for each of the group should be included. Finally, the authors should stimulate eosinophils with ANCA-IgG (or isotype) to see if there is still a difference in an ANCA context.

-Point 6: Fig.5A describes the release of extracellular DNA trap (ETs) from stimulated neutrophils and eosinophils from healthy controls. Eosinophils stimulated with PBS, TNF and C5a (but not PMA) released more ETs compared to stimulated neutrophils. The authors conclude that eosinophils are more prone to release ETs than neutrophils. Eosinophils were negatively selected with the MACS eosinophil isolation kit but no clear information are given for neutrophils. From the method section, we assume that neutrophils were collected in the positive fraction after eosinophil negative sort. This is a crucial aspect as positive selection/purification can activate cells. Were neutrophils in the positive fraction? What was the purity of eosinophil and neutrophil sort? The authors should include a giemsa staining to illustrate this aspect.

Such technical aspect might directly influence the release of ETs from eosinophils and neutrophils. The authors themselves wrote in the Method section "In the first experiments (fig.5a) they were isolated using Histopaque 1119 (Sigma) and Percoll (GE Healthcare) gradient following the manufacturers protocols. The eosinophils were separated from the granulocytes using MACS Eosinophil Isolation Kit (Miltenyi Biotech) according to manufacturer's instruction. Due to a high activation state of the purified eosinophils we later used the MACSXpress Eosinophil Isolation kit". The author should comment these points and might reconsider the conclusion that eosinophils are more prone to release ETs than neutrophils.

-Point 7: Fig.5A describes the release of extracellular DNA trap (ETs) from stimulated neutrophils and eosinophils from healthy controls. Cells were stimulated with PMA, TNF and C5a. Why stimulation with TNF+ANCA IgG (or isotype) was not tested? ETs are only presented as statistics. The authors should include picture of ETs immunostaining in the Fig.5A (neutrophil and eosinophil).

-Point 8: Fig.5B describes the release of extracellular DNA trap (ETs) from stimulated eosinophils isolated from AAV patients or healthy controls. Here again, it would have been interesting to test TNF+ANCA IgG (or isotype). ETs are only presented as statistics. The authors should include picture of ETs immunostaining in the Fig.5B for the most relevant condition (C5a+Iso and C5a+ANCA).

Are the methods appropriate and well described?
If not, please specify what is required in your comments to the authors.

Yes

Does the work include the necessary controls?
If not, please specify which controls are required in your comments to the authors.
Yes

**Are the conclusions drawn adequately supported by the data shown?**  
If not, please explain in your comments to the authors.

Yes

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I am able to assess the statistics

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