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LETTER TO THE EDITOR

Reply to Letter to the Editor

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Dear Dr. Gendelman,

This reply is in response to the Letter to the Editor, which questions the data and conclusions of our recently published paper (Ko et al. 2018) in the Journal of Neuroimmune Pharmacology entitled "Macrophages but not Astrocytes Harbor HIV DNA in the Brains of HIV-1-Infected Aviremic Individuals on Suppressive Antiretroviral Therapy". Our paper, in part, investigated whether astrocytes are productively or latently infected by HIV-1 in vivo and if astrocytes can function as a latent HIV-1 reservoir. We acknowledge that there are parties with different beliefs on this important topic, but to date, little hard data. We studied CNS infection by HIV-1 using the state-of-the-art DNAscope/RNAscope in situ hybridization technology with single-copy sensitivity, and detected HIV-1 DNA (vDNA) and RNA (vRNA) in the brains of untreated and virally suppressed HIV individuals. In our analysis we found HIV, both vDNA and vRNA, in myeloid-lineage cells, but no evidence of productive or latent infection of astrocytes in the same human brain tissues. To the best of our knowledge, there do not exist published studies presenting in vivo evidence of HIV infection for astrocytes by either the authors of the letter or others, using this novel and powerful technology. The authors of the letter (commenters) raised several issues regarding our scientific approach, images and data interpretation. Below we address their criticism and concerns.

There are several issues raised in the letter, some not well supported, and in some cases erroneous. Overall, a major issue is related to Fig. 6b in our paper. Simply stated, this is a misinterpretation of the figure. The commenters point to a minor overlap between green (GFAP) and red (HIV vRNA) fluorescence at the few spots in the published low-magnification picture, and claim that this is "evidence of HIV RNA in astrocytes". We strongly advise against such an approach. In order to perform colocalization analysis appropriately, it is necessary to acquire and analyze single-channel fluorescence images and a series of high-magnification Z-stack images to rule out what appears to be double positive as autofluorescence (aging pigments) or epifluorescence events. We performed such robust image analysis, and did not find any true colocalization events. In addition, Fig. 6a clearly demonstrates that HIV vDNA is not localized to the nucleus in any GFAP+ cells.

In addition to concerns with Fig. 6b raised above, the commenters take issue with other figures stating that "*some 'co-localized' CD68 and HIV signal does not appear to exactly overlap (fig. 5 and fig. S2)*". We respond that the CD68 is found in the cytoplasmic part of the cells and HIV-DNA puncta is in the nucleus. This is simple biology. They are not expected to exactly overlap because of this, when found in the same cell. We note that, when criticizing our data, the commenters use HIV DNA and RNA interchangeably. That is confusing with reference to the figures they are taking issue with.

Another issue raised in the letter is the criticism of our assessment of tissues areas evaluated. The commenters are mistaken in falsely claiming that "evaluation of ONLY ~40 spots/image/patient" was done in our study. What we stated in our paper is that "we individually examined a MINIMUM of 40 vDNA+ cells", in order to indicate more than 40 spots were examined from each case. This is a misrepresentation of our study by the commenters.

The commenters criticize us for not showing negative control images. As we described in the paper, we used negative controls (uninfected tissue and infected tissue stained with RNAscope® Negative Control Probe DapB) in each run and these had minimal background. We opted not to show negative control images and were not asked to include them in the manuscript during the peer review process involving two revisions. We believe this is not something that could be used to

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"question" the data and conclusion of any published papers. We can provide negative control images upon request.

Lastly, the commenters take issue with using only GFAP as a marker for astrocytes, claiming that "*only a subpopulation of astrocytes immunostain for GFAP*". We are not clear how to respond to this assertion but do point out that we found localization of HIV in the brain exclusively to cells of the myeloid lineage, consistent with our conclusion. We do know that while rodent astrocytes might not consistently express GFAP, GFAP labels the majority of astrocytes in humans and is thus a conventional and prototypical marker for astrocytes.

The commenters are correct that we miscited a paper (Yuan et al. 2017a) published in Journal of Virus Eradication. It should be a paper by Yuan et al. (2017b) that was published in ACS Synthetic Biology. The mistake has been corrected in an Erratum.

In conclusion, we think that our responses above satisfactorily address all the concerns raised by the commenters. We stand firmly by the data and conclusions of our study. We believe that science is a process and that our approach is the first in addressing the issue of HIV-1 reservoirs in the CNS of virally suppressed patients with effective antiretroviral therapy. While very much welcoming feedback and interest from the field, we suggest that the best criticism of our paper be evidence of astrocyte infection published by the commenters or others.

Respectfully,

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