Author’s response to reviews

Title: CD14+ macrophages that accumulate in the colon of African AIDS patients express pro-inflammatory cytokines and are responsive to lipopolysaccharide

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Author's response to reviews: see over
Dear Editor,

Thank you for considering our paper for publication in BMC Infectious Diseases. We appreciate the thorough review of our manuscript and have carefully taken the reviewers’ comments into consideration in preparing our revision. We believe that the changes have improved and strengthened our manuscript.

The following summarizes our responses to the reviewers’ comments and questions. The changes are highlighted in yellow.

Thank you for considering our revised manuscript, which we hope is now suitable for publication in BMC Infectious Diseases.

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Point-by-point reply to the reviewer’s concerns

Reviewer 2
Minor Essential Revisions

Critique # 1
It’s known that during HIV infection there is an altered interplay between GUT mucosa and microbiota; could be possible analyze the functional gene content of GUT microbiota and define the metabolic pathways? In addition do you have data about the HIV patients treated with antiretroviral therapy and with low levels of HIV-RNA?

Response to Critique #1
The reviewer makes an important point and we agree that it would be very interesting to analyze the functional gene content of the intestinal microbiota from these late stage HIV patients with chronic diarrhea (both prior to and following the initiation of antiretroviral therapy). Unfortunately, we did not have enough tissue to perform microbiome 16s rRNA sequencing of adherent bacterial species. We were also unable to collect fecal samples from these subjects. Given the importance of these interactions, we hope that future studies will pursue this avenue of investigation and have added this point to the discussion (page 20, lines 14-17).

Critique # 2
Do you have found a correlations between the levels of HIV-RNA and the count of CD14 macrophages?

Response to Critique #2
No we did not find any correlations between the levels of HIV-RNA and CD14+ macrophage levels. We have added this point to the manuscript (page 15, lines 18-20).

Critique # 3
Do you have found any correlation between the aging and the levels of CD14 macrophages?

Response to Critique #3
The reviewer makes an important point as the aging process has been linked to increased inflammation and changes in the gastrointestinal microenvironment. In the current study, we did not find any correlations between the levels of CD14+ macrophages and age. This may be due to the fact that the majority of patients were under the age of 50 (only 3 patients were over 50 years old). We have added this point to the manuscript (page 15, lines 18-20).

Critique # 4
Have the control group of patients with Crohn high blood levels of CD14 inflammatory markers?

Response to Critique #4
In the current study, intestinal samples were obtained from HIV-negative patients with IBD undergoing biopsy as part of their routine clinical management and we were unable to obtain matched blood samples from these subjects. Several studies have shown that patients with Crohn’s disease have increased levels of systemic immune activation as well as increased levels of markers of monocyte activation and bacterial translocation (i.e., LPS, LBP and sCD14) [1-3]. This point has been added to the manuscript (page 18, lines 18-20).

Critique # 5
Can speculate the Author in the discussion about the interplay between the virus and the immunoactivation in HIV patients?

Response to Critique #5
As requested, we have added a short discussion on the interplay between virus levels and immune activation in the
gut of HIV-infected patients (page 17, lines 18-25 and page 18, lines 1-2).

**Reviewer 3**

**Major Compulsory Revisions**

**Critique # 1**

In figures 2 and 3, it appears that essentially all CD68+ cells co-stain with IL-b and TNF-a. This is somewhat surprising and there is some concern that this may be an artifact of the staining process. What controls have been performed to assure the specificity of these stains? Were there isotype control stains performed or secondary antibodies used alone? Some controls should be described or shown.

**Response to Critique # 1**

We appreciate the reviewer’s comments and feel that we can easily address his concerns. If you enlarge Figures 2 and 3 to 300%, you can clearly see that: 1) anti-CD68 stains of a subset of DAPI positive cells with a macrophage-like morphology; 2) many of these cells are making large amounts of intracellular TNF-α and anti-IL-1β as shown by their intense red staining; 3) there is variation in the intensity of TNF-α and IL-1β staining (ie. some of CD68+ cells are weakly stained and making only small amounts of TNF-α and anti-IL-1β), and, as would be expected, 4) a few cells, other than CD68+ macrophages, are also producing small amounts of TNF-α and anti-IL-1β (ie. the red cells in the merged photo). Dually labeled TNF-α and IL-1β producing CD68+ macrophages are shown in yellow in the merged photos. This data was interpreted by an experienced pathologist with expertise in IHC and was based on the intensity, location and nature of the positivity.

All of the cytokine stains shown in Figures 2 and 3 were obtained using positive and negative controls and well-established staining methods [4]. Double labeling was performed using secondary antibodies linked to fluorescent dyes. The analyses involved sequential staining with two sets of primary and secondary antibodies: the first set was directed against IL1β or TNF-α; the second against CD68. Slides were incubated with mouse anti-human IL1β or TNF-α, washed and incubated with Alexa Fluor-488-conjugated goat anti-mouse secondary antibody. After boiling and treatment with 3% H₂O₂/methanol to block the antigenicity of the first set of antibodies, the staining procedure was repeated a second time using anti-human CD68 and Alexa Fluor-568-conjugated secondary antibody. The optimal concentration of each primary antibody (either mouse anti-IL1β or anti-TNF-α, or in the case of the second primer set, mouse anti-CD68) was determined using positive (tissue sections from patients with acute intestinal inflammation) and negative (reagents lacking primary antibody) controls and a dilution series of the primary antibody of interest. We have added these additional details to the manuscript (page 10, lines 10-11 and lines 16-21).

**Critique # 2**

Representative flow cytometry histograms along with gating strategy should be provided for the analyses of colonic macrophages (table 3).

**Response to Critique # 2**

We agree with the reviewer and appreciate the comment. We have added representative flow cytometry histograms to the manuscript (Figure 4B). We have also clarified the gating strategy used for the study in the methods section (page 11, lines 12-16).

**Critique # 3**

56% of AIDS subjects experienced diarrhea and 44% weight loss (Table 1). What is the rationale for analyzing these subjects as a single group rather than as independent groups? Is it possible that inflammatory processes may be different in persons with chronic diarrhea compared to those with weight loss?
Response to Critique # 3
The reviewer makes a valid point. Diarrhea and weight loss continue to be responsible for a significant amount of morbidity in African patients with advanced HIV/AIDS. Enteric co-infections are common in HIV-infected patients in Africa and are likely to be an important contributor to these clinical disorders. We have observed that AIDS-associated diarrhea often disappears or becomes less severe following the introduction of antiretroviral therapy and that many patients experience significant weight gains. These findings suggest that HIV, on its own, is an important driver of diarrhea and weight loss in African AIDS patients with severely compromised immune systems. The current study was designed to investigate the contribution of HIV to intestinal enteropathy in the absence of enteric co-infections rather than to compare the inflammatory processes in patients with chronic diarrhea vs. weight loss. As a result the study is unlikely to provide insight into inflammatory processes leading to these different clinical disorders. A larger comparative study is needed if this issue is to be addressed in an informative manner.

Minor Essential Revisions

Critique # 4
The age and gender of control groups should be described for comparison to subjects with AIDS.

Response to Critique # 4
As suggested by Reviewer 2 we have better described the age and gender of the control groups for comparison to subjects with AIDS (page 8, lines 19-21 and Table 1)

Critique # 5
Please correct fig 2 legend which refers to Figure 3a

Response to Critique # 5
We appreciate the reviewer’s thorough review of our manuscript and have corrected the figure legend accordingly (page 27, lines 14-15).
References