First we would like to thank the editor, Dr. Guy Boeckxstaens, and the reviewers for their constructive advices that helped us improving our manuscript.

Editorial Committee Comments to Authors:

1. Clodronate has been previously shown to deplete or reduce the number of resident Cx3CR1 high macrophages, thereby reducing influx of monocytes and reducing POI (Wehner et al., Gut 2007). In the present study, the authors have evaluated the effect of clodronate on the population of immune cells 24 hours after manipulation. Clearly, in line with the study by Wehner, one would expect less influx of Ly6C+ cells (CCR2 dependent) as a result of the depletion of resident MF (activated by IM) known to attract these cells upon activation, and not as a result of clodronate on Ly6C+ cells. So the whole conclusion that clodronate depletes Ly6C+ monocytes/MF is incorrect and undermines many of the conclusions drawn throughout the paper (including the role of iNOS). The authors should provide data on the effect of clodronate on the resident MF and study its effect on the immune cell population before and early after IM.

The editor argues that clodronate might deplete also resident CX3CR1^{high} Ly6C^{-} macrophages, so that the reduced influx of monocytes may be due to the absence of macrophages rather than to the depletion of Ly6C+ monocytes/macrophages as we claimed. He asks us to show how clodronate affects resident macrophages. Perhaps the editor failed to notice that we had shown in Figure 3D of our previous manuscript version already that Ly6C^{-} macrophages were not significantly depleted by the clodronate regime.

The editor cites Wehner et al. 2007 to support his claim. It is true that these authors used clodronate to deplete resident macrophages. However, this was done in rats and not in mice. Moreover, the authors used a customized protocol and also injected gadolinium chloride (GdCl3), a selective inhibitor of stretch-activated ion channels (details on page 176 and in the Methods section, page 177, of Wehner et al., Gut, 56:176–185, (2007) doi: 10.1136/gut.2005.089615). Furthermore, Wehner et al did not discriminate between CX3CR1^{high} Ly6C^{-} macrophages (the true resident macrophages) and CX3CR1^{high} Ly6C^{+} monocytes. Hence, there are gating-, interspecies and agent-dependent differences between the studies by Wehner et al. and ours. These studies are not comparable.

As requested by the editor, we performed additional experiments to confirm that Ly6C– macrophages are not efficiently depleted by clodronate liposomes also at earlier time points. Again, we failed to detect a significant reduction of Ly6C– macrophages at the time point of IM (0h) and 3 hours as well as 24 hours after IM in clodronate-treated animals (see figure below). In contrast, the number of Ly6C+ monocytes were much reduced at all these time points. The presence of Ly6C– macrophages and the absence of Ly6C+ monocytes strongly supports our (and of many other labs') conclusion that Ly6C+ monocytes are directly depleted by clodronate. These findings are now shown as the new Figures 3C and D and in the new Suppl Fig 4B and C (see next page):

Abundance of Ly6C+ monocytes (left panel) and Ly6C– macrophages (right panel) in the small intestine:

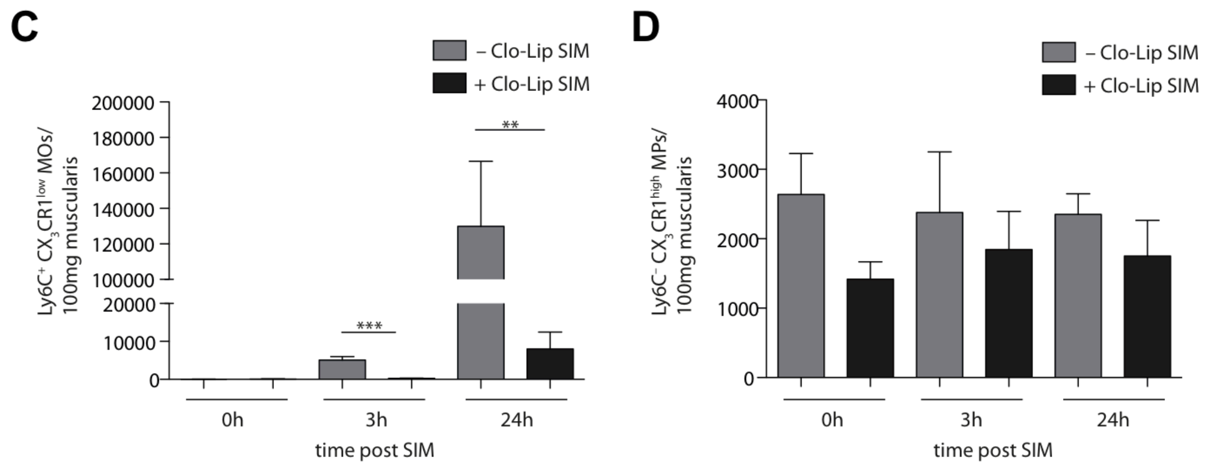
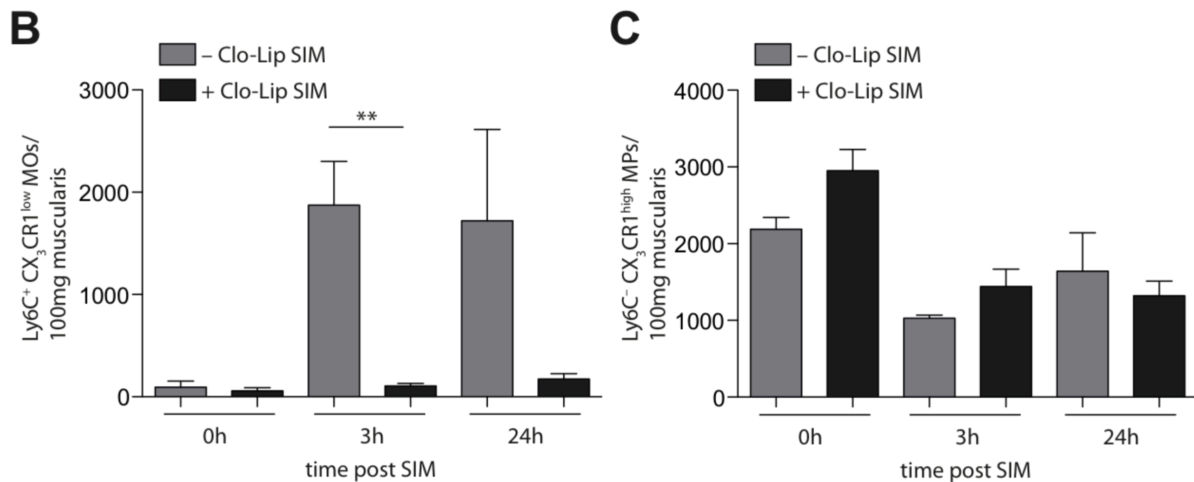


*Figure 3 (**C, D**) Flow cytometric analysis of $Ly6C^{+}CX_{3}CR1^{low}$ monocytes (MOs) (**C**) or $Ly6C^{-}CX_{3}CR1^{high}$ macrophages (MPs) (**D**), pregated on $Hoechst^{-}CD45^{+}F4/80^{+}$ cells, in the small intestine of $Cx_{3}cr1^{GFP/+}$ mice. Mice were treated with Clodronate Liposome (CloLip) 12h before SIM and the number of cells were analyzed 3 and 24 hours after SIM and in unmanipulated mice (0h) (Representative data of n=3-6).*

Abundance of Ly6C+ MO (left panel) and Ly6C– MPs (right panel) in the colon:



*Supplementary Figure 4 (**B, C**) Flow cytometric analysis of $Ly6C^{+}CX_{3}CR1^{low}$ monocytes (MOs) (**B**) or $Ly6C^{-}CX_{3}CR1^{high}$ macrophages (MPs) (**C**), gated on $Hoechst^{-}CD45^{+}F4/80^{+}$ cells, in the colon of $Cx_{3}cr1^{GFP/+}$ mice. Mice were treated with Clodronate Liposome (CloLip) 12h before SIM and the number of cells were analysed 3 and 24 hours after SIM and in unmanipulated mice (0h) (Representative data of n=3-6).*

2. It should also be emphasized that resident MF are not completely depleted by diphtheria toxin in the iDTRFlox mice, leaving possibly enough MF to trigger influx of Ly6C+ MF. The same may apply for clodronate treatment, requiring the combination of both treatments to prevent POI and obtaining the other data presented by the authors. Again, the interpretation of the data would be completely different from that proposed by the authors. Experiments excluding these possibilities should be provided before the proposed hypothesis can be accepted.

We showed in Figure 4A that the number of Ly6C– macrophages was strongly (**) reduced in iDTR animals. It is highly unlikely that such a low number of Ly6C– macrophages exert a similar immune response as all macrophages. And frankly speaking, this concern of the editor would challenge most studies in which inhibitors or depletion techniques were applied, not only in the intestine but also in other organs. One example is the recent report by Muller et al. (Cell, 158, 300-313, (2014) doi:10.1016/j.cell.2014.04.050): In Figure 2C and in Figure 3, a depletion efficacy of 75% of muscularis macrophages by application of an antibody against the Csf1R was sufficient to conclude that macrophages regulate intestinal peristalsis. We feel that our results are consistent with the state of the art and supported by a highly significant reduction of Ly6C– macrophages in iDTR animals.

In summary, we did not observe a strong reduction of Ly6C– macrophages after clodronate treatment, refuting the editors´ concern that further depletion of Ly6C– macrophages after iDTR and clodronate treatment reduces POI. Our experiments show that not only Ly6C– macrophages initiated POI, but also Ly6C+ macrophages were necessary.

Reviewer(s)' Comments to Author:

Reviewer: 1

Comments to the Author
The main novel conclusion of this manuscript suggesting that CD103+CD11b+ dendritic cells are the primary sensors of microbiota in the small intestine after abdominal surgery, is still not formally proven and not even addressed.

We agree with the reviewer that we have not formally discriminated whether CD103+CD11b+ DCs directly or whether another cell senses the microbiota and reports it to CD103+CD11b+ DCs, which then initiate POI. Clarifying this question is not possible with the techniques currently available. One would first have to identify the sensors by which the microbiome is recognized, and then generate mice in which only CD103+CD11b+ DCs lack all sensing mechanisms for the microbiome. A promoter that selectively drives gene expression in CD103+CD11b+ DCs is unknown (Irf4 is not specific for DCs, as it is also active in T cells). Accomplishing these tasks would require years and is clearly beyond the scope of our study.

We therefore thank the reviewer for making this point and modified the title, the manuscript text and our conclusions accordingly.

*We also discuss ways to address this question in the future: We showed previously that these cells initiate POI by IL-12 and that POI was strongly reduced after DC depletion or Il-12 blockade (Engel, D. R. et al. (2010) Nat Med **16**, 1407-1413, doi:10.1038/nm.2255). In our current study, we report reduced IL-12 levels in the absence of the microbiota (Figure 6A). Several publications showed that IL-12 depends on MyD88 signaling in DCs (Arnold-Schrauf, C. et al, Eur J Immunol **45**, 32-39, (2015) doi:10.1002/eji.201444747 and Hou, B. et al, Proc Natl Acad Sci, **108**, 278-283, (2011) doi:10.1073/pnas.1011549108), suggesting that sensing might be transmitted through the adapter MyD88. Importantly, POI was attenuated in MyD88 KO mice (Stoffels et al, Gastroenterology, 146:176–187 (2014)). If a promoter for CD103+CD11b+ DCs is discovered, one could generate cell-specific MyD88-deficient mice to investigate the sensing of the microbiota by CD103+CD11b+ DCs. These ideas have been added to the discussion.*

Moreover, how these dendritic cells communicate with monocytes and macrophages inducing iNOS expression is again not experimentally tested. In my opinion what is completely neglected in this study is the time scale of immunological events taking place in the muscularis externa after intestinal manipulation. Literature both on patients and murine POI suggest that activation of an inflammatory response in the muscularis externa during surgery is a rapid event, taking places in minutes with upregulation of chemokines and recruitment of myeloid cells. Thus, to finally test that microbiota sensing by dendritic cells represent the first in the pathogenesis of POI authors should test their hypothesis on much early time points, and identify the mediators involved in the cross talk between dendritic cells and macrophages in the muscularis externa.

The means of communication between monocytes and macrophages have been described previously by us (Engel et al., (2010) Nat Med, 16, 1407-1413, doi: 10.1038/nm.2255). We reported that DC-derived IL-12 induced the release of IFNg by Th1 memory cells, which activated macrophages to express iNOS. We also found that IL-12 and IFNg was already secreted 30 minutes after IM (Figure 4e) and activated T cells were also found 30 minutes after operation in patients (Figure 3f). The analysis of early time points after disease induction has already been investigated in a previous study.

We had briefly commented on Th1 cells in the discussion. Now, we also cover this important mechanism in the introduction.

Reviewer: 2

Comments to the Author
I thank the authors for their consideration of my comments and for addressing some of the issues raised. It has to be noted that the evidence for an interaction between the CD11b+CD103+ DC and monocytes/macrophages remains indirect and based on correlative results and it would probably be appropriate to acknowledge this in the Discussion at least. My other remaining comment is that the authors should change the naming of the MHCII+Ly6C+ cells as monocytes/macrophages as they suggested.

We thank the reviewer for their suggestions. We have acknowledged the fact that the interaction of DCs with macrophages and monocytes is indirect: "We found that iNOS production by monocytes and macrophages was severely reduced in the absence of $CD103^+CD11b^+$ DCs. This correlative finding does not indicate a direct interaction between these cells and as described previously, other leukocytes are involved in this signaling cascade[4]." We also agree that the infiltrating $Ly6C^+F4/80^+MHCII^{low}CD64^+CX3CR1^+$ cells are monocytes rather than macrophages. We have changed the manuscript accordingly.