EMBO reports

Autotaxin loss accelerates intestinal inflammation by suppressing TLR4-mediated immune responses

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1st Editorial Decision 21st Oct 2019

Dear Prof. Rhee,

Thank you for the transfer of your research manuscript to EMBO reports. We have now received the full set of referee reports that are copied below.

I am sorry to say that the decision on your manuscript is not a positive one. As you will see, none of the referees provides strong support for the publication of your work in EMBO reports. They indicate that the conclusions are not fully supported by the data, and that the data do not convincingly support major hypothesis. Further, the referees note technical shortcomings. As the reports are below, I will not detail them here.

Given the comments of the three referees, the amount of work required to address them, and the fact that EMBO reports can only invite revision of papers that receive enthusiastic support from the referees upon initial assessment, we cannot offer to publish your manuscript.

I am sorry to have to disappoint you this time. I nevertheless hope, that the referee comments will be helpful in your continued work in this area, and I thank you once more for your interest in our journal.

Yours sincerely

Achim Breiling
Editor
EMBO Reports

Referee #1:

In this manuscript, the authors have used LysM-Cre and ATXfl/fl, to delete the autotaxin gene selectively in macrophages. Autotaxin is a lipid metabolism enzyme that converts lysophosphatidylcholine (LPC) to lysophosphatidic acid (LPA, a signaling lipid that has GPCR receptors), as its most studied action. LPA generated by autotaxin may play a positive role in the development of some cancers and may be pro-inflammatory; it induces migration by some cell types.

In this study, the authors have studied ATX-deficient macrophages in vitro and also have studied how deletion of ATX in macrophages affects in vivo models of septic shock and inflammatory bowel disease. The analysis is broad and although the authors have tried to draw mechanistic conclusions for how autotaxin expression in macrophages may have the effects seen, these mechanistic conclusions are mostly not very well supported so they are more hypothesis than established conclusion. This represents a major limitation of this manuscript, namely there are well documented effects of deleting autotaxin in macrophages, but the mechanistic reason for these effects is claimed but is not well established. In the specific comments below, first are the more important issues, in the opinion of this reviewer. At the end of the review are listed under "minor criticisms" some issues that can probably be addressed in a simple fashion (revision of the text, etc.).

Major comments:
1. The authors clearly demonstrate that thioglycollate-elicited peritoneal macrophages from the

mice have defective responses in vitro to LPS (the main ligand for Toll-like receptor 4, TLR4). Surprisingly, at least in one assay, there are normal responses to two ligands for TLR2, as well as normal responses to IL-1beta (which signals via the same major adaptor, MyD88) and TNF. Moreover, in vivo injection of LPS, as a model of septic shock, indicates that in vivo there is a similar defect in macrophage response to LPS. The in vitro findings based on genetic ablation can largely be recapitulated by using a chemical inhibitor of autotaxin and treating either wild type macrophages or a commonly used murine macrophage-like cell line. This descriptive work (most of Figures 3-6) is reasonably compelling and would need only minor changes to be worthy of publication.

2. The defect in response to LPS seems to relate to an inability of CD14 (a lipid binding protein that is required for response to lower concentrations of LPS by binding LPS and transferring it to the TLR4/Md2 complex) to associate with TLR4 and the inability of TLR4 to associate with downstream signaling adaptors TIRAP and MyD88. This part of the ms. would be improved by directly accessing whether the macrophages express CD14 normally (it is a GPI-linked protein and can be shed from cells expressing it); whether CD14 binds normally to LPS; and whether it fails to transfer LPS to TLR4.

3. The authors have concluded that the defects in association between TLR4 and CD14 are the result of changes in the lipid composition of the macrophage plasma membrane resulting from reduced cleavage of LPC by autotaxin, which, the authors hypothesize has disrupted liquid ordered membrane microdomains ("lipid rafts") in the macrophage plasma membrane. Lipid rafts have been found to be important for TLR4 signaling and CD14 is a lipid raft resident protein (like other GPI-linked proteins). While the authors could be correct, this part of the manuscript lacks sufficient experimental evidence to derive a strong conclusion (as for example included in the current title). The main evidence in favor of this view is staining of the macrophages with fluorescent cholera toxin B subunit, which binds to the GM1 ganglioside, which is highly enriched in lipid rafts (like GPI-linked proteins) (Fig. 2D). The data shown are consistent with a defect in lipid raft formation, but are not convincing in this regard. Wild type macrophages do show the expected punctate staining pattern whereas mutant macrophages show a more homogeneous staining. The picture looks like it may be overexposed, but in any case, more data is needed. Higher resolution microscopy, and imaging of CD14 or other lipid raft-resident proteins may be helpful. Although somewhat controversial, cholesterol content of the plasma membrane can be manipulated rapidly by treatment of cells with beta-methyl cyclodextran (loaded with various concentrations of cholesterol). Does doing this restore the response to LPS once lipid raft staining patterns are achieved? Does the in vitro use of the autotaxin inhibitor also result in loss of lipid rafts at the same dose and time of treatment that substantially decrease the LPS responses? Are there changes in the lipid composition of the plasma membrane that can alter lipid raft formation? It is worth noting that the authors' hypothesis appears to be largely contrary to most work published with autotaxin, which interprets alterations resulting from deletion or inhibition of autotaxin as resulting from reduced LPA and the loss of effects mediated by its GPCRs (pro-migration, pro-inflammation, etc).

4. The connection of autotaxin expression in macrophages to prevention of inflammatory bowel disease and to immune defense against intestinal bacteria is intriguing but would benefit from additional analysis. In Fig 8E, the numbers of bacteria invading the mucosa should be quantitated and presented with error bars. Are the invading bacteria accompanied by immune inflammatory cells, as would be expected normally? (Neutrophils? Monocytes?) It is worth noting that the LysM-Cre used is reasonably good at deleting floxed genes in tissue macrophages (about 80%) but less good in monocytes (about 50%). Neutrophils do not delete appreciably with LysM-Cre. Lack of autotaxin might decrease inflammatory migration to sites of tissue invasion if LPA is part of this response, so some approach to addressing this conventional function of autotaxin and LPA would add to the analysis here. In addition, the IBD data from Fig. 1 uses mice that are completely deficient in IL-10 in addition to being deficient in macrophages for autotaxin, so what are the phenotypes of

bacterial invasion into the tissue and inflammation in this strain of mice?
5. The experiments addressing phagocytosis (Fig. 7) are unconvincing, perhaps due to lack of adequate explanation of what was done, but more likely due to inadequate analysis. LPS will enhance FcR-mediated phagocytosis, but this phagocytosis by elicited peritoneal macrophages should be pretty robust even in the unstimulated case, so all analysis needs to be done with and without LPS pre-treatment. The autotaxin negative cells may be less motile in vitro (due to lack of LPA-mediated migratory signaling), so it is critical to distinguish between decreased binding to the IgG-latex beads, vs. decreased internalization. With the assay used, the typical protocol is to use trypan blue to quench fluorescence from bound but not internalized beads, so the pictures should be taken first without trypan blue and then with it added and the number of bound IgG-latex beads quantitated and the % internalized determined. All of this needs to be presented with error bars. If the authors conclusions are correct, then FcR phagocytosis will be normal without pretreatment with LPS, although conceivably lipid rafts enhance FcR signaling.

Minor comments:
6. FRET analysis (Fig. 2GH): neither the text nor the figure legend says the cells were treated with LPS. So what is going on here, FRET without the ligand? How does this match what is in the literature? Is there an effect of adding LPS? Do the HEK293 cells express autotaxin and does addition of the inhibitor cause LPA levels to fall in the medium or changes in the lipid composition of the plasma membrane? The experiments here need further work.
7. In some experiments, the legend does not make clear how long the cells were pre-incubated with the autotaxin inhibitor. This needs to be made clear. Can added LPA overcome the effects seen by the autotaxin inhibitor?
8. Fig. 8A-D. These straining patterns and percentages are not believable, CD4 should not be directly proportional to the cytokine staining. The authors would have us believe that in the WT mice, many of the CD4 T cells stain for IFN-g, IL-17, IL-4 AND FoxP3 (or at least several of these), whereas the large majority of cells will stain for only one of these (there are sometimes some T cells positive for IFN-g and IL-17). Also, it should be made clear what is being gated on (total T cells? Total lymphocytes?)
9. I'm confused about the constructs used for FRET. For TLR4, which is a type 1 membrane protein, it looks like the fluorescent protein was inserted in place of the cytoplasmic TIR domain. What about CD14, which is a GPI linked protein. Where was the fluorescent protein inserted? Was the GPI-linkage sequence removed and a transmembrane added?? If so, why do we think this FRET is biologically meaningful? Was the inhibitor removed prior to doing FRET, if not do we know that it does not quench FRET in some way?

Referee #2:

The authors provide evidence that ATX, a secreted lysophospholipase D, regulates gut inflammation by affecting TLR4 complex formation and down stream signaling. Myeloid cell specific ATX deficient mice exhibited defective TLR4-induced signaling and TLR4-induced macrophage functions such as cytokine production and phagocytosis. This was associated with higher bacteria load in the intestines of ATX deficient mice. In a model of spontaneous colitis development using IL10 deficient mice, additional deficiency of ATX accelerated colitis development. This is an interesting study. However, there are some aspects that require further consideration.
Major:

1. Majority of the LPA in the circulation is produced by ATX. While the authors measure the

circulating levels of ATX in IBD patients, they have not measured LPA levels. This also needs to be measured.
2. ATX is a secreted protein and is responsible for producing majority of the LPA in the circulation. LPA is known to have pro-inflammatory effects on various cell types. Does the circulating levels of ATX and LPA change in the myeloid cell-specific ATX KO mice?
3. The authors suggest that the defective TLR4 signaling seen in ATX KO macrophages is due to disrupted lipid rafts caused by the absence of ATX function, in converting LPC to LPA. The lipid composition of the plasma membrane needs to be studied from ATX WT and ATX KO mice to prove this.
4. While the rationale for the altered membrane fluidity is clear, it is unclear how the authors think that ATX alters plasma membrane phospholipids? ATX being an extracellular protein would require its substrate to be available outside the cells. By measuring intracellular LPA levels do the authors claim that ATX functions intracellularly?
5. In several experiments (Fig 2B,8A-D) the authors have shown that they have a significant difference while performing a MannWhitney test with a n=3, this is surprising because it is highly unlikely to get a P value <0.05 with n=3 using this statistical test. Can the authors comment on this?
6. For the invitro experiments, the autotaxin inhibitor was used at a concentration of 50 µM. how was this concentration selected? Was it checked if this concentration of the inhibitor is toxic to the cells?
7. In Fig 6C-E, the authors show that ATX KO mactophages, after LPS stimulation, produce less TNFa, IL1b and IL6. However, after LPS injection in mice the authors only show serum levels of TNFa (Fig 6G). IL1b and IL6 serum levels also needs to be measured.
8. In the lamina propria of the intestine, the authors try to show that T cell activation is defective in ATX KO mice (Fig 8). But does the total T cell number change?
9. In Fig 8A-D, the facs plots show that the cell populations do not clearly separate but rather looks like the compensation for the two flurochromes was not performed properly. If the spectral overlap between two flurochromes is not corrected then the data obtained from these experiments are not reliable.

Minor:
1. The authors show that IL1R signaling does not require lipid rafts; because they show that IL1R signaling is not affected in ATX KO macrophages in which lipid rafts are distorted. However there is literature that shows the importance of lipid rafts in IL1R signaling. This needs to be discussed.
2. According to Fig 5E-H, the intracellular signalling activated by TLR2, IL1R and TNFR seems to be more in ATXKO cells as compared to ATX WT cells. Can the authors explain this?
3. The reduced LPA levels seen intracellularly could point to a defect in the triglyceride synthesis pathway, in which LPA is an intermediate. This needs to be discussed.

Referee #3:

This manuscript investigates the effect of Autotaxin deletion on IBD. The author showed that deletion of Atx in the myeloid cell of IL10-/- accelerates the development of spontaneous colitis, with the development of a Crohn's disease-like enteritis. Furthermore, they showed that Atx deficiency disrupts the plasma membrane lipid rafts in macrophage, that leads to the inhibition of the formation of the TLR4-CD14 receptor complex and, subsequently, to the inhibition of the entire TLR4-mediated pathway, resulting in compromised immune responses in the intestine and an increased bacterial infiltration into the intestinal mucosa. The authors conclude that ATX have an

important physiological relevance in onset and perpetuation of bacteria-associated chronic inflammation in the gut.

The authors use a range of experimental assays to show the effect of Atx deletion on TLR4-mediated pathway. These finding add helpful knowledge to understanding of Atx and IBD. However, some issues need to be addressed, including conflict results regarding the literature that are not discussed enough. Detailed comments are below:

Major concerns:
1. The authors report a decrease production of ATX in the serum of CD and UC patients. However, in the introduction they cite Hozumi et al., 2013 that reported an increase Atx mRNA levels in the inflamed mucosa of CD and UC patients. Even though these two studies investigate different cellular compartment, their results seem incompatible and this discrepancy is not discussed by the authors.

2. The authors report that in the literature previous studies have shown that Atx depletion or use of Atx inhibitor reduce the severity of colitis in DSS-induced mice and CD4+;CD25- T-cell transferred mice. These results are opposite to the one report by the authors, although in different colitis model, and the authors barely discussed that issues and don't offer any explanations.

Minor concerns:
3. The authors report a shift in the microbiota of the Atx deficient mice with an increase of specific Bacteroides species and a modification of mRNA expression but the authors do not discuss the potential impact of such modification.

4. In figure 5, different loading control have been used in the different panel. It would be more convincing if the same loading control was use for all the panel.

5. In figure 8, the authors reported that the deletion of Atx increased bacterial infiltration into the intestinal mucosa. Even if the FISH staining is convincing, it will be interesting to know if the mucus layer is also affected.

Our Responses to the Critiques from Referee #1

- Reviewer-1's Critique-1 (Major): The authors clearly demonstrate that Thioglycolate-elicited peritoneal macrophages from the mice have defective responses in vitro to LPS (the main ligand for Toll-like receptor 4, TLR4). Surprisingly, at least in one assay, there are normal responses to two ligands for TLR2, as well as normal responses to IL-1beta (which signals via the same major adaptor, MyD88) and TNF. Moreover, in vivo injection of LPS, as a model of septic shock, indicates that in vivo there is a similar defect in macrophage response to LPS. The in vitro findings based on genetic ablation can largely be recapitulated by using a chemical inhibitor of autotaxin and treating either wild type macrophages or a commonly used murine macrophage-like cell line. This descriptive work (most of Figures 3-6) is reasonably compelling and would need only minor changes to be worthy of publication.

- Our response to reviewer-1's critique-1 (Major)

This reviewer's comments appear to point out the strength of our study, and we appreciate these very kind words. In accordance with the reviewer's suggestions and critiques, we carried out many additional experiments which are featured in the revised manuscript. We hope the reviewer will consider this revision positively.

- Reviewer-1's Critique-2A (Major): The defect in response to LPS seems to relate to an inability of CD14 (a lipid binding protein that is required for response to lower concentrations of LPS by binding LPS and transferring it to the TLR4/Md2 complex) to associate with TLR4 and the inability of TLR4 to associate with downstream signaling adaptors TIRAP and MyD88. This part of the ms. would be improved by directly accessing whether the macrophages express CD14 normally (it is a GPI-linked protein and can be shed from cells expressing it).

- Our response to reviewer-1's critique-2A (Major)

We are grateful to the reviewer for this constructive suggestion. We confirmed that CD14 expression is preserved in the primary macrophages of $Atx^{\Delta ME/\Delta ME}$ mice and $Atx^{+/+}$ littermates, and this data has been included in the revised manuscript (Fig. 6B).

- Reviewer-1's Critique-2B (Major): Whether CD14 binds normally to LPS; and whether it fails to transfer LPS to TLR4.

- Our response to reviewer-1's critique-2B (Major)

When it comes to LPS sensing by CD14 and TLR4, LPS is initially recognized by LPS binding protein (LBP) in an extracellular region. LPS-LBP then binds to CD14, which proceeds to form a heteromeric complex with TLR4. The CD14-TLR4 complex is then quickly internalized to an endosome, in which the cargo molecules are rapidly degraded. Therefore, to our best knowledge, the direct-binding of LPS to CD14 protein should be addressed by structural biology studies such as X-ray crystallography or Cryo-crystallography. Accordingly, determining the direct binding of LPS to CD14 is beyond the scope of our manuscript.

However, to provide the best possible answer to the reviewer's question, we performed confocal microscopy of primary macrophages with a CD14 antibody and Alexa Fluor 594-labeled-LPS. In the peritoneal macrophages from $Atx^{+/+}$ littermates, we observed co-localization of CD14 and LPS at a punctate region of the plasma membrane (Reviewer-Only Fig. 1). Given the close proximity to the plasma membrane, co-localization of CD14 and LPS-double positive signals should indicate an endosome that harbors the LPS-sensing receptor complex. These data reflect the binding of LPS to CD14 at the plasma membrane.

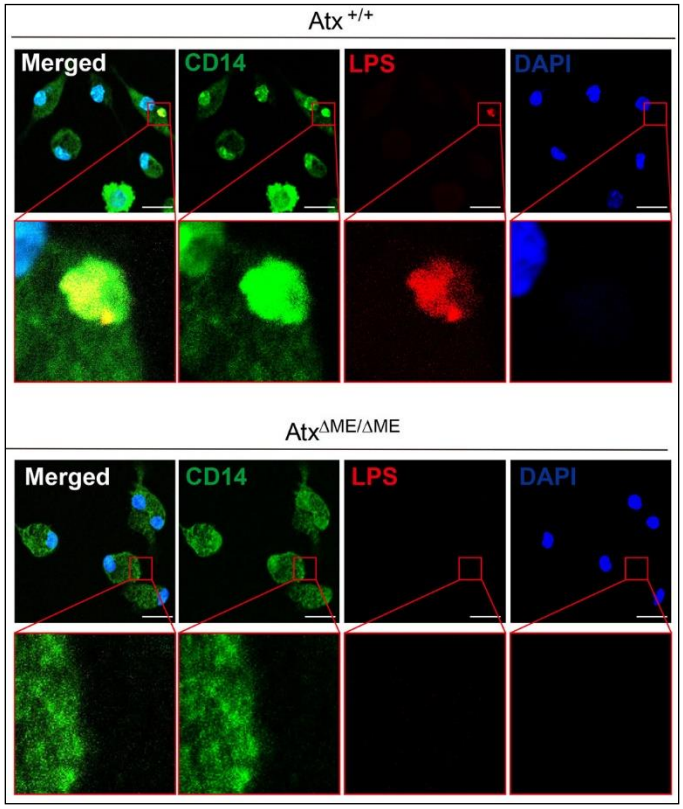
However, we could not see the co-localization of CD14 and LPS in macrophages from $Atx^{\Delta ME/\Delta ME}$ mice. Taken together, these results suggest that the binding of LPS to CD14 is inhibited in Atx deficient macrophages. Given the residence of CD14 in lipid rafts and the observed disruption of lipid rafts in Atx-ko macrophages, it is reasonable to believe that the ligation of LPS to CD14 could be slowed or inhibited in Atx-ko macrophages.

• Reviewer-1's Critique-3 (Major): The authors have concluded that the defects in association between TLR4 and CD14 are the result of changes in the lipid composition of the macrophage plasma membrane resulting from reduced cleavage of LPC by autotaxin, which, the authors hypothesize has disrupted liquid ordered membrane microdomains ("lipid rafts") in the macrophage plasma membrane.

Lipid rafts have been found to be important for TLR4 signaling and CD14 is a lipid raft resident protein (like other GPI-linked proteins).

While the authors could be correct, this part of the manuscript lacks sufficient experimental evidence to derive a strong conclusion (as for example included in the current title).

The main evidence in favor of this view is staining of the macrophages with fluorescent cholera toxin B subunit, which binds to the GM1 ganglioside, which is highly enriched in lipid rafts (like GPI-linked proteins) (Fig. 2D). The data shown are consistent with a defect in lipid raft formation, but are not convincing in this regard. Wild type macrophages do show the expected punctate staining pattern whereas mutant macrophages show a more homogeneous staining. The picture looks like it may be overexposed, **but in any case, more data is needed**. Higher resolution microscopy, and imaging of CD14 or **other lipid raft-resident proteins may be helpful**.



Reviewer-Only Fig. 1: Atx-ko macrophages did not exhibit co-localization of LPS and CD14. Peritoneal macrophages were labeled with Alexa Fluor 594-labeld-LPS (Red) and CD14 antibody and its secondary antibody labeled with FITC (Green). After washing three times, cells were counterstained by DAPI (Blue). Co-localization of CD14 and LPS was visualized by confocal microscopy. Representative images from three independent experiments were presented. Scale bar indicates 20 μm.

• Our response to reviewer-1's critique-3 (Major)

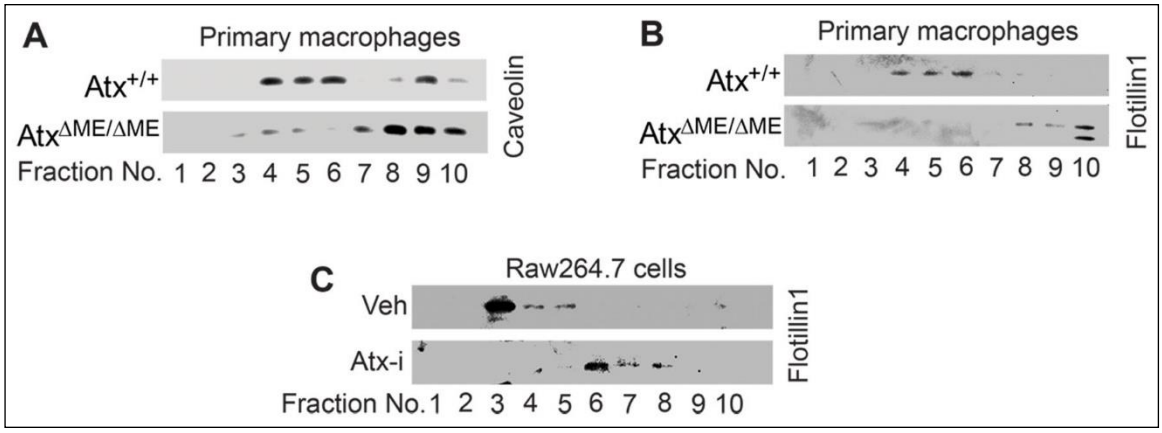
To address this critique, we carried out **"sucrose density-gradient ultracentrifugation,"** which is a powerful technique for fractionating lipid rafts. With this lipid raft fractionation assay, we examined whether Atx deficiency alters the integrity of lipid rafts.

We found that the distribution of lipid raft marker proteins (Flotillin-1 and Caveolin-1)[1] was changed in peritoneal macrophages from $Atx^{\Delta ME/\Delta ME}$ mice compared to that of cells from $Atx^{+/+}$ littermates. In Atx-wt cells, Flotillin-1 and Caveoliin-1 were observed mainly in the lipid raft fractions. However, in cells from $Atx^{+/+}$ mice the localization of these proteins was shifted into the non-lipid raft fractions (Reviewer-Only Fig. 2A and B).

Furthermore, we performed the lipid raft fraction assay with mouse macrophage cell line (Raw264.7 cells) treated with Atx inhibitor or vehicle. In vehicle-treated Raw264.7 cells, Flotillin-1 was identified in the lipid raft fraction. However, Atx inhibitor treatment shifted the distribution of Flotillin-1 into the non-lipid raft fractions in Raw264.7 cells. These data demonstrate that Atx deficiency disrupts the integrity of lipid rafts in macrophages.

In our first submission, we also included confocal microscopy data exhibiting disrupted lipid rafts in Atx-ko macrophages (Fig. 2H in the revised manuscript). Taken together, our data clearly show

that Atx deficiency disrupts the integrity of lipid rafts. **Please, be advised that this data (Reviewer-Only Fig. 2A to C) are included as Fig. 2E to G in the revised manuscript**.



Reviewer-Only Fig. 2: Atx deficiency disrupted lipid raft formation in macrophages. (**A** to **B**) Peritoneal macrophages from $Atx^{\Delta ME/\Delta ME}$ mice and $Atx^{+/+}$ littermates (A, B) and Raw264.7 cell treated with ATX inhibitor (Atx-i) or Vehicle (Veh., DMSO 0.1%) (C) were solubilized in 1% Triton X-100 buffer. Cell lysates were then subjected to sucrose density gradient (5-35%) ultra-centrifugation to separate raft and non-raft fractions. Each fraction was analyzed through Western blotting to examine the distribution of lipid raft marker proteins (Flotillin-1 and Caveolin). The presented blot is the representative from three independent experiments.

- Reviewer-1's Critique-4 (Major): Although somewhat controversial, cholesterol content of the plasma membrane can be manipulated rapidly by treatment of cells with beta-methyl cyclodextran (loaded with various concentrations of cholesterol).

Does doing this restore the response to LPS once lipid raft staining patterns are achieved?

Does the in vitro use of the autotaxin inhibitor also result in loss of lipid rafts at the same dose and time of treatment that substantially decrease the LPS responses?

- Our response to reviewer-1's critique-4 (Major)

As the reviewer indicated, methyl-β-cyclodextrin (MCD) has a strong affinity to cholesterol[2]. Through direct association, MCD rapidly depletes cholesterol and disrupts lipid rafts in the plasma membrane[3]. Accordingly, it is well-known that MCD treatment inhibits LPS/TLR4-induced responses in macrophages[4].

To address the reviewer's question, we did a lipid raft fraction assay with a mouse macrophage cell line (Raw264.7 cells) treated with Atx inhibitor or vehicle. Our data demonstrate that Atx inhibitor treatment disturbs the lipid raft integrity (Reviewer-Only Fig. 2C and Fig. 2G in the revised manuscript). Moreover, we demonstrated that Atx inhibitor treatment inhibited the recruitment of adaptors (MYD88, MAL/TIRAP) to TLR4 in LPS-treated murine macrophage Raw264.7 cells (Fig. 5B and D in the revised manuscript). Likewise, Atx inhibitor treatment suppressed the activation of NFκB and ERK1/2 in LPS-treated Raw264.7 cells (Fig. 6D in the revised manuscript).

In addition, we demonstrate that LPS-induced signaling pathways are inhibited in the primary macrophages from Atx-ko mice (Fig. 6, 7 and 8 in the revised manuscript).

Taken together, our in vitro, in vivo, and ex vivo studies clearly suggest that Atx deficiency inhibits LPS/TLR4-mediated responses, at least in macrophages.

- Reviewer-1's Critique-5 (Major): Are there changes in the lipid composition of the plasma membrane that can alter lipid raft formation?

- Our response to Reviewer-1's Critique-5 (Major)

We appreciate this reviewer's question. However, we respectfully feel that plasma membrane lipid analysis is not required to confirm our results that Atx-ko alters the lipid raft integrity and inhibits TLR4 responses.

Nevertheless, with the total lipid extract of Atx-ko and Atx-wt macrophages, we carried out LC-MS/MS analysis to measure endogenous lysophosphatidic acid (LPA), as the enzymatic activity of ATX converts LPC into LPA. We identified that the level of LPA was reduced in Atx-ko macrophages compared to that of Atx-wt cells (Fig. 2B in the revision manuscript). These data suggest that Atx-ko cells have lower levels of LPA compared to control cells.

Moreover, lysophosphatidylcholine (LPC, a lipid substrate lipid of ATX) is present in the plasma membrane[5]. We confirmed reduced levels of LPA in Atx-ko cells. We further confirmed disruption of lipid rafts using the lipid raft fractionation assay and confocal microscopy of Atx-ko macrophages and Atx inhibitor-treated Raw264.7 cells. These data represent compelling evidence that Atx deficiency disrupts the integrity of lipid rafts.

Therefore, analyzing lipid composition in the plasma membrane only is beyond the scope of our paper.

- Reviewer-1's Critique-6 (Major): It is worth noting that the authors' hypothesis appears to be largely contrary to most work published with autotaxin, which interprets alterations resulting from deletion or inhibition of autotaxin as resulting from reduced LPA and the loss of effects mediated by its GPCRs (pro-migration, pro-inflammation, etc).

- Our response to Reviewer-1's Critique-6 (Major)

We appreciate the preexisting research regarding ATX. In general, previous studies have suggested that LPA may stimulate specific LPA receptors (GPCRs) to mediate various cellular events, including cell proliferation and migration. It is worth noting that ATX not only converts lysophosphatidylcholine (LPC) into lysophosphatidic acid (LPA)[6], but also metabolizes sphingosyl-phosphorylcholine into sphingosine 1-phosphate (S1P)[7]. Thus, we did not limit our research scope to the axis of LPA and GPCRs. Rather, given the involvement of LPC and sphingosine lipids in lipid rafts at the plasma membrane, we investigated a novel effect of ATX in mainlining lipid raft integrity and its consequent role in TLR4-mediated immune and inflammatory responses.

In this study, we demonstrate a molecular mechanism by which Atx deficiency can inhibit LPS/TLR4-mediated signaling pathways and relevant inflammatory disorders in the intestine. To our best knowledge, these findings are novel; therefore, our study should make a sifnficant contribution to the understanding of the pathophysiology of microbe-associated intestinal inflammation. As the reviewer mentioned, our study does conceive a slightly different role for ATX than does the prior research. However, we believe that our study provides novel, paradigm shifting evidence accounting for the role of ATX in inflammatory disease.

- Reviewer-1's Critique-7 (Major): The connection of autotaxin expression in macrophages to prevention of inflammatory bowel disease and to immune defense against intestinal bacteria is intriguing but would benefit from additional analysis. In Fig 8E, the numbers of bacteria invading the mucosa should be quantitated and presented with error bars.

• Our response to Reviewer-1's Critique-7 (Major)

As kindly suggested, we have quantified the number of bacteria observed in the mucosa and presented this with error bars in the revised manuscript (Fig. 9E and F).

• Reviewer-1's Critique-8 (Major): Are the invading bacteria accompanied by immune inflammatory cells, as would be expected normally? (Neutrophils? Monocytes?)

• Our response to Reviewer-1's Critique-8 (Major)

We did not see any difference in neutrophil infiltration in the colon tissue sections of $Atx^{\Delta ME/\Delta ME}$ mice and $Atx^{+/+}$ littermates. However, neutrophil infiltration was greatly increased in colon tissue sections of $Atx^{\Delta ME/\Delta ME}$;$Il10^{-/-}$ mice compared to that of $Atx^{+/+}$;$Il10^{-/-}$ littermates. These results suggest that Atx deficiency accelerates the inflammatory response in an IL-10 deficient condition.

Upon microbial infection, macrophages in other parts of the body induce the production of inflammatory cytokines/chemokines that are responsible for neutrophil infiltration of the infected area. In contrast, macrophages in the intestine do not produce substantial levels of inflammatory mediators upon microbial stimuli, because IL-10 (a potent anti-inflammatory cytokine) is abundantly produced in the intestinal mucosa. Instead, intestinal macrophages are highly phagocytic to ingest and degrade any microbes that cross the epithelial barrier[8,9]. Therefore, it is reasonable to believe that neutrophils could not be recruited to the intestine of $Atx^{\Delta ME/\Delta ME}$ mice, even while bacterial prevalence of the intestinal mucosa was increased.

However, as seen in the colon of $Atx^{\Delta ME/\Delta ME}$;$Il10^{-/-}$ mice (Fig. 1D and F in the revised manuscript), Atx-ko does indeed cause increased neutrophil infiltration when the anti-inflammatory signaling provided by IL-10 is removed.

• Reviewer-1's Critique-9 (Major): It is worth noting that the LysM-Cre used is reasonably good at deleting floxed genes in tissue macrophages (about 80%) but less good in monocytes (about 50%). Neutrophils do not delete appreciably with LysM-Cre.

• Our response to Reviewer-1's Critique-9 (Major)

We agree with the reviewer about the deletion efficiency of the LyzM-Cre system. We are fully aware of the limited specificity when it comes to LyzM-Cre-mediated gene deletion approach. Please, be advised that in this manuscript, we do not indicate that LyzM-Cre-mediated Atx gene knockout (ko) represents a macrophage-specific gene ko. Instead, for the in vivo experiment, we refer to the ko mouse system as "myeloid cell lineage-restricted Atx-ko mice".

As the reviewer pointed out, the LyzM-Cre system is capable of deleting a floxed gene in approximately 83-98% of macrophages[10]. Gene deletion can be achieved in more than 95% of thioglycolate-elicited peritoneal macrophages, while approximately 16% of dendritic cells (DC) exhibit gene deletion[10]. In addition, the LyzM-Cre system can elicit approximately 50% gene deletion in neutrophils[11]. It does not induce a gene deletion in lymphocytes such as B or T cells[11,12].

With a general consensus from the majority of immunology research societies, it is accepted that the LyzM-Cre system is by far the best and most efficient approach to deleting a floxed gene in macrophages[11,12]. Given that monocytes/macrophages, DCs, and neutrophils are classified into the myeloid cell lineage, we described our LyzM-Cre-mediated gene ko mice as "myeloid cell lineage-restricted Atx-ko" mice in this manuscript.

• Reviewer-1's Critique-10 (Major): Lack of autotaxin might decrease inflammatory migration to sites of tissue invasion if LPA is part of this response, so some approach to addressing this conventional function of autotaxin and LPA would add to the analysis here.

• Our response to Reviewer-1's Critique-10 (Major)

It is worth noting that macrophages are not migratory cells, but instead reside in the tissues. As explained for "reviewer-1's critique-8 (Major)", we did not observe that Atx deficiency alters neutrophil infiltration in the mouse intestine. Moreover, intestinal macrophages are an excellent example of "resident macrophages," which are non-migratory. Given the fact that we utilized myeloid lineage cell-restricted Atx-ko mice ($Atx^{\Delta ME/\Delta ME}$) for in vivo experiments and primary macrophages for in vitro and ex vivo experiments, we respectfully do not agree with the reviewer regarding the possibility that Atx deficiency may change inflammatory migration to the tissue.

• Reviewer-1's Critique-11 (Major): In addition, the IBD data from Fig. 1 uses mice that are completely deficient in IL-10 in addition to being deficient in macrophages for autotaxin, so what are the phenotypes of bacterial invasion into the tissue and inflammation in this strain of mice?

• Our response to Reviewer-1's Critique-11 (Major)

In normal conditions, gut microbes are capable of translocating from the lumen into the intestinal mucosa. This bacterial invasion does not result in a pathologic phenotype because the invading bacteria are eliminated by an immune mechanism within the intestinal mucosa without eliciting any overt inflammatory response. However, these microbes can induce intestinal inflammation in a genetically susceptible subject such as one deficient in the Il10 gene[13].

In an IL-10 deficient condition (mouse), commensal bacteria translocate from the intestinal lumen into the mucosa, which in turn triggers inflammation. In fact, the loss of Il10 gene is a well-known genetic factor for IBD in humans[14,15]. Indeed, IL-10-ko mice housed in conventional conditions develop spontaneous colitis in response to bacterial invasion, while IL-10-ko mice maintained in a germ-free environment remain free of colitis[16]. Therefore, IL-10-ko mice are an appropriate model for studying chronic colitis induced by microbial invasion into the mucosa. This is the reason that we harnessed IL-10-ko mice to study the significance of Atx deficiency in the gut.

• Reviewer-1's Critique-12 (Major): The experiments addressing phagocytosis (Fig. 7) are unconvincing, perhaps due to lack of adequate explanation of what was done, but more likely due to inadequate analysis. LPS will enhance FcR-mediated phagocytosis, but this phagocytosis by elicited peritoneal macrophages should be pretty robust even in the unstimulated case, so all analysis needs to be done with and without LPS pre-treatment.

• Our response to Reviewer-1's Critique-12 (Major)

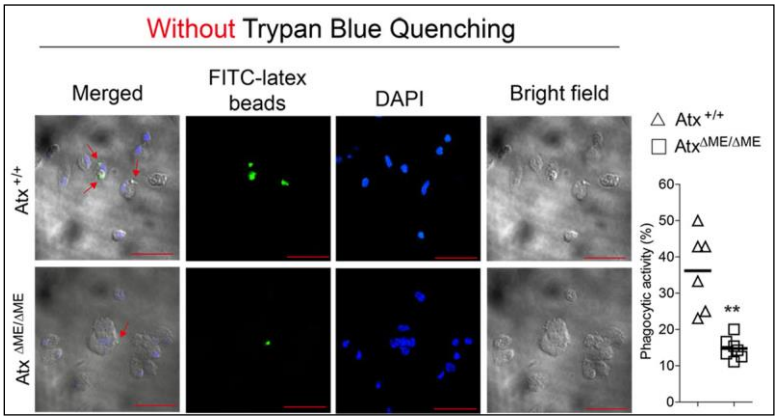
We examined the phagocytic activity of peritoneal macrophages from $Atx^{\Delta ME/\Delta ME}$ mice and $Atx^{+/+}$ littermates with and without LPS treatment. With these experiments, we confirmed that phagocytic activity is comparable between Atx-ko macrophages and Atx-wt macrophages in the absence of LPS stimulation (Fig. 8B in the revised manuscript). LPS treatment substantially enhances phagocytic activity in Atx-wt macrophages. However, the phagocytic activity was not enhanced in Atx-ko macrophages treated with LPS. Accordingly, these data demonstrate that Atx deficiency inhibits LPS/TLR4-mediated phagocytic activity of macrophages.

• Reviewer-1's Critique-13 (Major): The autotaxin negative cells may be less motile in vitro (due to lack of LPA-mediated migratory signaling), so it is critical to distinguish between decreased binding to the IgG-latex beads, vs. decreased internalization. With the assay used, the typical protocol is to use trypan blue to quench fluorescence from bound but not internalized beads, so the pictures should be taken first without trypan blue and then with it added and the number

of bound IgG-latex beads quantitated and the % internalized determined. All of this needs to be presented with error bars. If the authors conclusions are correct, then FcR phagocytosis will be normal without pretreatment with LPS, although conceivably lipid rafts enhance FcR signaling.

- Our response to Reviewer-1's Critique-13 (Major)

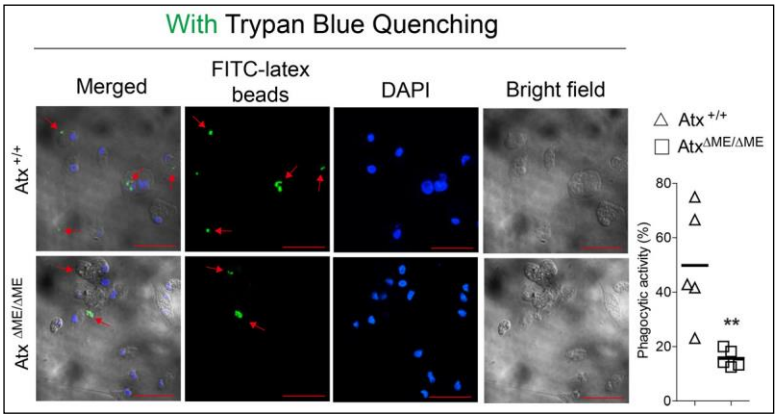
To evaluate the phagocytic activity, we presented the confocal micrographs of cells and further analyzed the fluorescent signal inside cells (Fig. 8A and B in the revised manuscript). With the confocal micrographs, we also quantified the phagocytic activity (%) by counting the intracellular positive signal of FITC-latex beads, which was divided by the total number of cells observed (the graph included in Fig. 8B in the revised manuscript).



Reviewer-Only Fig. 3: Atx-ko inhibited LPS-induced macrophage phagocytic activity. Without trypan blue quenching, we evaluated phagocytic activity. Peritoneal macrophages from $Atx^{\Delta ME/\Delta ME}$ mice and $Atx^{+/+}$ littermates were treated with LPS (20 ng/mL, 40 min), and the phagocytosis assay was then performed using FITC-latex beads without the quenching step. Through confocal microscopy, the fluorescent signal inside cells (red arrow) was examined. FITC-latex bead-positive cell numbers were then divided by the total number of DAPI-positive cells (n= 6 – 7 per group). **P < 0.01 (Mann-Whitney *U* test).

For the phagocytosis assay included in our manuscript, please, note that we carried out the experiment with the trypan blue quenching step, as the reviewer mentioned. This quenching step was needed to remove the fluorescent signal of FITC-latex beads remaining outside the cells.

Nevertheless, the reviewer asked us to examine the phagocytic activity with and without trypan blue quenching. To address this reviewer's comment, we first carried out the phagocytosis assay without trypan blue quenching. After being treated with LPS and FITC-latex beads, peritoneal macrophages were examined under confocal microscopy to evaluate the fluorescent signal within cells, which is indicative of phagocytosis. We found that Atx-ko macrophages exhibited substantially reduced phagocytic activity compared to that of control cells, which was determined in the absence of the quenching step (Reviewer-Only Fig. 3).



Reviewer-Only Fig. 4: Atx-ko inhibited LPS-induced macrophage phagocytic activity, determined "with the trypan blue quenching step". Peritoneal macrophages were treated with LPS (20 ng/mL, 40 min), followed by conducting the phagocytosis assay using FITC-latex beads with the quenching step. Through confocal microscopy, the fluorescent signal inside cells (red arrow) was examined. Then, FITC-latex bead-positive cell numbers were divided by the total number of DAPI-positive cells (n= 6 – 7 per group). **P < 0.01 (Mann-Whitney *U* test).

Although the phagocytosis data presented in the manuscript have been performed in the presence of the quenching step, we also independently carried out the phagocytosis assay with trypan blue quenching again. We confirmed again that LPS-

stimulated phagocytosis was suppressed in Atx-ko macrophages compared to Atx-wt cells (Reviewer-Only Fig. 4).

Taken together, these data substantiate our finding that Atx deficiency inhibits LPS-stimulated phagocytosis in macrophages.

- Reviewer-1's **Minor Critique**-1: FRET analysis (Fig. 2GH): neither the text nor the figure legend says the cells were treated with LPS. So what is going on here, FRET without the ligand? How does this match what is in the literature? Is there an effect of adding LPS?

- Our response to Reviewer-1's **Minor Critique**-1

For the FRET experiments, we generated mammalian expression constructs encoding TLR4-EYFP, TLR4-ECFP, or CD14-ECFP. A combination of TLR4-EYFP, TLR4-ECFP, and CD14-ECFP encoding constructs was transfected and overexpressed in HEK293 cells.

Please, be advised that the overexpression of TLR4 caused by the transfection of exogenous TLR4-encoding construct leads to the auto-activation of TLR4 in the absence of LPS stimulation in a variety of cell types including macrophage cells and HEK293 cells. This auto-activation is possible because the overexpression induces the formation of the receptor complex. This notion is well accepted and has been reproduced in multiple studies[17-19]. Therefore, we transfected the TLR-encoding construct into HEK293 cells, which induces the auto-activation of TLR4 in the absence of LPS stimulation.

- Reviewer-1's **Minor Critique**-2: Do the HEK293 cells express autotaxin?

- Our response to Reviewer-1's **Minor Critique**-2

Yes, it has been demonstrated that HEK293 cells express ATX[20].

- Reviewer-1's **Minor Critique**-3: Does addition of the inhibitor cause LPA levels to fall in the medium or changes in the lipid composition of the plasma membrane?

- Our response to Reviewer-1's **Minor Critique**-3

It has already been demonstrated that ATX inhibitor (PF8383) treatment efficiently inhibits the production of LPA from LPC in an in vitro or in vivo experimental setting[21]. Specifically, the ATX inhibitor is capable of inhibiting the enzymatic activity of ATX protein purified from HEK293 cells, which was tested with LPC as a substrate[21]. This effect of the ATX inhibitor was also tested in fibroblasts and human whole blood samples. Taken together, the ATX inhibitor should inhibit the enzymatic activity of ATX, resulting in reduced LPA production from LPC.

- Reviewer-1's **Minor Critique**-4: In some experiments, the legend does not make clear how long the cells were pre-incubated with the autotaxin inhibitor. This needs to be made clear.

- Our response to Reviewer-1's **Minor Critique**-4

As suggested, we revised the method section and figure legend to clarify ATX inhibitor (PF8380) treatment conditions.

- Reviewer-1's **Minor Critique**-5: Can added LPA overcome the effects seen by the autotaxin inhibitor?

• Our response to Reviewer-1's **Minor Critique**-5

As we described in the manuscript, while converting LPC into LPA[6], ATX is also capable of hydrolyzing sphingosyl-phosphorylcholine (SPC) to produce sphingosine 1-phosphate (S1P)[7]. Just as cholesterol interacts with LPC**[22],** sphingolipids are also important lipid components that are involved in maintaining the integrity of lipid rafts**[23]**.

Specifically, LPA is 1-acyl-glycerol-3-phosphate. As presented in our data (Fig. 2B), there are various species of LPA depending on the length of the acyl group and the double bond in the carbon skeleton (e.g. 14:0, 16:0, 16:1, 18:0, 18:1, 18:2, 20:2, 20:3 etc.)

Considering the specificity of ATX in generating LPA and S1P and the complexity of LPA isoforms, we believe it is very hard to anticipate that simply adding LPA would overcome the effects seen following ATX inhibitor treatment.

• Reviewer-1's **Minor Critique**-6: Fig. 8A-D. These straining patterns and percentages are not believable, CD4 should not be directly proportional to the cytokine staining.

• Our response to Reviewer-1's **Minor Critique**-6

We appreciate this important comment and apologize for the incomplete presentation of our data in the first submission. We found that the FACS data must be analyzed with fluorescence compensation to ensure that the fluorescence detected is from the fluorochrome that is being measured. Therefore, we performed fluorescence compensation using BD Accuri CFlow® Plus software (CFlow). We included the data with this compensation applied in Figure 9A to D (in the revised manuscript).

With these data, we found that the responses of Th1, Th17 and Treg cell activation were reduced in the lamina propria lymphocytes of $Atx^{\Delta ME/\Delta ME}$ mice compared to those of $Atx^{+/+}$ littermates. Given the fact that Th1 and Th17 responses participate in the immune response against invading microbes, and that Treg cells suppress inflammatory responses, these data suggest that reduced Th1, Th17, and Treg responses shift the environment of the intestinal mucosa toward a microbe-induced inflammatory condition

Regarding the degree of CD4 expression, the level of CD4 expression (or CD4 staining) can be varied even in a sample of CD4 enriched cells, in which FACS data exhibit a range of CD4+ staining[24,25]. Indeed, our FACS staining pattern appears to be similar to the data published by Uchiyama et al[25], in which the CD4+ staining pattern is proportional to the cytokine staining. Moreover, please be advised that we used CD4+ cell-enriched lymphocytes for the FACS analysis. Therefore, it is possible to see different degrees of CD4 expression in the sample.

• Reviewer-1's **Minor Critique**-7: The authors would have us believe that in the WT mice, many of the CD4 T cells stain for IFN-g, IL-17, IL-4 AND FoxP3 (or at least several of these), whereas the large majority of cells will stain for only one of these (there are sometimes some T cells positive for IFN-g and IL-17).

• Our response to Reviewer-1's **Minor Critique**-7

Many CD4+ T cells, especially those from the lamina propria, can be double positive and express a combination of IFN-g, IL-17, IL-4, or FOXP3. In that case, double positive cells can be classified as Th1/Th2, Th1/Th17, etc. We do not exclude such a possibility.

However, the aim of our study is to examine the expression of a single cytokine from mouse lamina propria CD4+ T cells. Therefore, we only investigated the expression of single cytokines

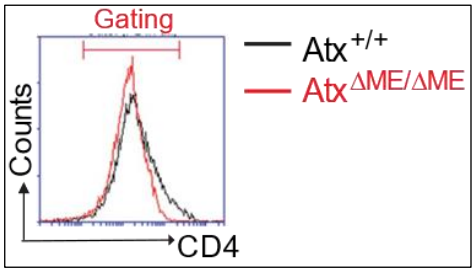
in CD4 enriched lymphocytes. Accordingly, we believe that addressing double positive T cells is outside of the scope in this study.

Given the complexity of lamina propria T cell responses, however, this is likely important enough to be further addressed in a future study by examining T cell populations harboring multiple types of effector T cells, and analyzing double-positive T cells.

• Reviewer-1's **Minor Critique**-8: Also, it should be made clear what is being gated on (total T cells? Total lymphocytes?)

• Our response to Reviewer-1's **Minor Critique**-8

For the data (Fig. 9A to D in the revised manuscript), we analyzed lamina propria CD4-enriched T cells. After color compensation, the overlapped CD4 positive range in each group was gated (Reviewer-Only Fig. 5), and 10,000 CD4 positive T cells in the gated range were analyzed for the expression of IFN-g (marker for T_h1 differentiation), IL-4 (marker for T_h2 differentiation), IL-17 (marker for T_h17 differentiation), and FoxP3 (marker for T_{reg} differentiation).



Reviewer-Only Fig. 5: The CD4 positive range in each group was gated for FACS analysis. From $Atx^{\Delta ME/\Delta ME}$ mice and $Atx^{+/+}$ littermates, the lamina propria lymphocytes were harvested. CD4+ T cells were then enriched. After FSC-SSC gating, CD4+ T cells were gated for the FACS analysis.

• Reviewer-1's **Minor Critique**-9: I'm confused about the constructs used for FRET. For TLR4, which is a type 1 membrane protein, it looks like the fluorescent protein was inserted in place of the cytoplasmic TIR domain.

• Our response to Reviewer-1's **Minor Critique**-9

As we described in detail in Materials and Methods, the TLR4-ECFP and EYFP construct were generated by modifying the TLR4 construct published in the PI's previous paper[26].

Previously, we were kindly provided pFlag-CMV1-TLR4 by Bruce Beutler (Southwestern Medical Center, Dallas, TX). This construct lacks DNA sequences for the first 20 amino acids representing the signal peptide. Instead, pFlag-CVM1 vector harbors alternative signaling sequences. Therefore, the TLR4 cDNA sequence was inserted in the pFlag-CVM1 vector, generating a pFlag-CMV1-TLR4 expression construct [27].

The corresponding author (Rhee SH) of this manuscript then sub-cloned a whole cDNA sequence of TLR4 (including the signaling sequence and TLR4 cDNA) from pFlag-CMV1-TLR4 to pcDNA3.1, generating a pcDNA3.1-TLR4 construct that has been published previously[26].

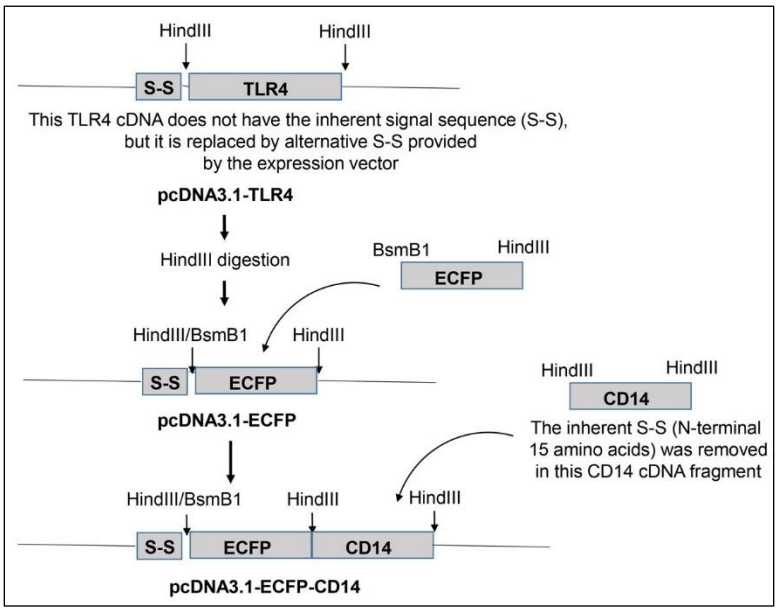
In this study, the cytoplasmic region of TLR4 has been replaced with ECFP or EYFP to generate TLR4-ECFP or TLR4-EYFP encoding constructs, respectively, by the sub-cloning strategy described in the Materials and Methods section. Note that the transmembrane region of TLR4 remains intact in these constructs. Therefore, the expression and plasma membrane localization of TLR4-EYFP or TLR4-ECFP was observed by confocal microscopy (Fig. 3C and D).

• Reviewer-1's **Minor Critique**-10: What about CD14, which is a GPI linked protein. Where was the fluorescent protein inserted? Was the GPI-linkage sequence removed and a transmembrane added?? If so, why do we think this FRET is biologically meaningful? Was the inhibitor removed prior to doing FRET, if not do we know that it does not quench FRET in some way?

• Our response to Reviewer-1's **Minor Critique**-10

As illustrated (Reviewer-Only Fig. 6), we generated an ECFP cDNA sequence fused with the signaling sequence (S-S). We then obtained a CD14 encoding cDNA fragment in which the intrinsic signal sequence (N-terminal 15 amino acid) was eliminated. This CD14 cDNA was then fused to the ECFP encoding construct, giving rise to the ECFP-CD14 encoding construct. Please, note that this ECFP-CD14 construct contains the signal sequence at the N-terminus, followed by ECFP and the subsequent CD14 encoding sequences.

Therefore, the GPI-linkage sequence is preserved in this ECFP-CD14 construct. Indeed, the expression and plasma membrane localization of ECFP-CD14 was verified by confocal microscopy (Fig. 3C). The cloning scheme (Reviewer-Only Fig. 6) has been included as Supplementary Figure 5 in the revised manuscript.



Reviewer-Only Fig. 6: Cloning scheme of ECFP-CD4 expression construct.

• Reviewer-1's **Minor Critique**-11: Was the inhibitor removed prior to doing FRET, if not do we know that it does not quench FRET in some way?

• Our response to Reviewer-1's **Minor Critique**-11

The cell culture medium containing the inhibitor has been removed, and the cells were washed with PBS, followed by fixation for FRET measurement. There should be no quenching due to the inhibitor.

Our Responses to the Critiques from Referee #2:

• Reviewer-2's Critique-1 (Major): Majority of the LPA in the circulation is produced by ATX. While the authors measure the circulating levels of ATX in IBD patients, they have not measured LPA levels. This also needs to be measured.

• Our response to Reviewer-2's Critique-1 (Major)

Atx is a secreted protein that catalyzes lysophosphatidylcholine (LPC) to lysophosphatidic acid (LPA)[28]. However, it is worth noting that **LPA in plasma and serum is highly unstable with increasing concentrations during storage**; therefore, ATX protein level in serum is a more reliable indicator than measuring LPA levels[29]. In this context, the serum level of ATX has previously been evaluated to study the correlation between LPA and human diseases[30]. Therefore, when analyzing human serum samples the stability of LPA must be considered. This is the reason that we evaluated the ATX protein level in the serum samples of IBD patients.

• Reviewer-2's Critique-2 (Major): ATX is a secreted protein and is responsible for producing majority of the LPA in the circulation. LPA is known to have pro-inflammatory effects on various cell types. Does the circulating levels of ATX and LPA change in the myeloid cell-specific ATX KO mice?

• Our response to Reviewer-2's Critique-2 (Major)

In this study, we utilize the myeloid cell lineage-restricted Atx-ko mice, in which primarily macrophages have Atx gene deletion and exhibit the inhibition of TLR4-mediated immune response. Indeed, we confirmed that Atx-ko mice had reduced LPA production in the primary macrophages (Fig. 2B).

Please be advised that ATX can be produced by a variety of cells or tissues. Therefore, it may not be possible to see changed levels of ATX protein or LPA in the circulation of myeloid cell lineage-restricted Atx-ko mice. Moreover, we respectfully feel that it is out of the scope of our study to examine the systemic effect of ATX/LPA, as our manuscript is dedicated toward studying the interplay between ATX and macrophage-mediated immune mechanism.

• Reviewer-2's Critique-3 (Major): The authors suggest that the defective TLR4 signaling seen in ATX KO macrophages is due to disrupted lipid rafts caused by the absence of ATX function, in converting LPC to LPA. The lipid composition of the plasma membrane needs to be studied from ATX WT and ATX KO mice to prove this.

• Our response to Reviewer-2's Critique-3 (Major)

We appreciate this great question. However, we believe that it would be difficult to quantify the lipid composition **only in the plasma membrane**, as the plasma membrane's lipid composition can be changed during the isolation of the plasma membrane. This is the reason that we analyzed the lipid composition of whole cell extracts through LC-MS/MS analysis, in which we confirmed reduced LPA levels in Atx-ko macrophages.

Nevertheless, we agree with the reviewer's concern about whether Atx deficiency genuinely alters the integrity of lipid rafts in macrophages. We think this question is a key issue in our study that should be addressed experimentally and succinctly. Therefore, **we carried out sucrose density-gradient ultracentrifugation**, which is a powerful technique for fractionating lipid rafts. With this lipid raft fractionation assay, we examined whether Atx deficiency could disturb the integrity of lipid rafts. We found that the distribution of lipid raft marker proteins (Flotillin-1 and Caveolin-1)[1] was changed in peritoneal macrophages from $Atx^{\Delta ME/\Delta ME}$ mice compared to that of control cells from $Atx^{+/+}$ littermates (Reviewer-Only Fig. 2A and B). This data has been included as Fig 2E and F in the revised manuscript).

Furthermore, we performed the lipid raft fractionation assay with a mouse macrophage cell line (Raw264.7 cells) treated with Atx inhibitor or vehicle. Compared to the fraction of vehicle-treated Raw264.7 cells, Atx inhibitor treatment changed the distribution of lipid raft marker protein in Raw264.7 cells (refer to Reviewer-Only Fig. 2C). This data has been included as Fig 2G in the revised manuscript).

Please, be advised that we have provided confocal microscopy images demonstrating disrupted lipid rafts in Atx-ko macrophages (Fig 2H in the revised manuscript).

Considering the results obtained from confocal microscopy and the sucrose density gradient fractionation assay, our data demonstrate that Atx deficiency disrupts the integrity of lipid rafts in macrophages.

• Reviewer-2's Critique-4 (Major): While the rationale for the altered membrane fluidity is clear, it is unclear how the authors think that ATX alters plasma membrane phospholipids? ATX being an extracellular protein would require its substrate to be available outside the cells. By measuring intracellular LPA levels do the authors claim that ATX functions intracellularly?

• Our response to Reviewer-2's Critique-4 (Major):

As the reviewer mentioned, ATX is an extracellular protein and converts LPC into LPA in extracellular regions and in the plasma membrane. LPC is present in the plasma membrane where it is regularly produced from phosphatidylcholine (PC)[31]. As an amphipathic lipid molecule, LPC can alter cell membrane fluidity[32]. Moreover, LPC interacts with cholesterol, which is a major constituent of lipid rafts[33]. Indeed, LPC regulates the distribution of receptor proteins at the plasma membrane[31]; therefore, treatment with an LPC analog can modify lipid raft integrity in the plasma membrane[34]. Together, these studies strongly support the hypothesis that altered LPC levels are associated with the disintegration of lipid rafts in the plasma membrane.

In addition, ATX converts sphingosyl-phosphorylcholine (SPC) into sphingosine 1-phosphate (S1P)[7]. SPC is also observed in the plasma membrane[35], and has previously been studied as a lipid raft regulator[36].

In agreement with these reports, our confocal microscopy data and the sucrose density-gradient fractionation data demonstrate that Atx-ko disrupts the integrity of lipid rafts.

• Reviewer-2's Critique-5 (Major): In several experiments (Fig 2B, 8A-D) the authors have shown that they have a significant difference while performing a MannWhitney test with a n=3, this is surprising because it is highly unlikely to get a P value <0.05 with n=3 using this statistical test. Can the authors comment on this?

• Our response to Reviewer-2's Critique-5 (Major)

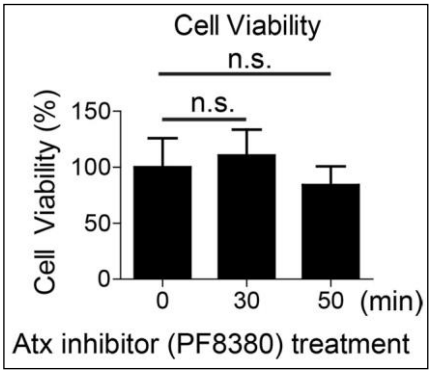
We apologize for these mistakes and are grateful to the reviewer for finding the error. It should be one-tailed unpaired t-test (Fig. 2B in the revised manuscript) and two-tailed unpaired t-test (Fig. 9A to D in the revised manuscript). The figure legends have been revised accordingly.

• Reviewer-2's Critique-6 (Major): For the in vitro experiments, the autotaxin inhibitor was used at a concentration of 50 µM. how was this concentration selected? Was it checked if this concentration of the inhibitor is toxic to cells?

• Our response to Reviewer-2's Critique-6 (Major)

Similar to other studies[21,37], we tested a range of ATX inhibitor concentrations (0 - 100 µM). To select an optimal concentration, we performed Western blot analysis using multiple inhibitor concentrations. As presented (Fig. 6D in the revised manuscript), LPS-induced activation of NFκB (p65) and ERK1/2 started to decrease at 10 µM of Atx inhibitor treatment, and were completely blocked at 100 µM. These data indicate that the Atx inhibitor inhibited LPS/TLR4-induced responses in a dose-dependent manner. Therefore, we chose the concentration of 50 µM for our experiments.

To examine the cell-toxicity at this concentration of Atx inhibitor, we carried out a cell viability assay. Raw264.7 cells were treated with 50 µM of ATX inhibitor (PF8380) for 30 min and 50 min. We confirmed that ATX inhibitor treatment was not toxic to cells at this concentration (50 µM) (Reviewer-Only Fig. 7).



Reviewer-Only Fig. 7: Cell viability assay to test the toxicity of Atx inhibitor (PF8380). Raw264.7 cells were treated with Atx inhibitor (50 µM) for the indicated period of time. Trypan Blue dye exclusion test was carried out to determine the number of viable cells. Time points were selected based on the time period of inhibitor treatment (pretreatment for 30 min and co-treatment for 20 min) employed to study the effect of Atx inhibitor in LPS-stimulated responses in Raw264.7 cells such as the data presented in data (Fig. 3, 5, and 6 in the revised manuscript).

• Reviewer-2's Critique-7 (Major): In Fig 6C-E, the authors show that ATX KO mactophages, after LPS stimulation, produce less TNFa, IL1b and IL6. However, after LPS injection in mice the authors only show serum levels of TNFa (Fig 6G). IL1b and IL6 serum levels also needs to be measured.

• Our response to Reviewer-2's Critique-7 (Major)

As the reviewer mentioned, TNFα, IL-6, and IL-1β are pro-inflammatory cytokines produced by LPS stimulation. In LPS-induced sepsis, however, the level of IL-1β may not be upregulated to the same degree as TNFα[38]. Moreover, measuring the level of TNFα alone from the mouse serum samples has been well accepted as an excellent, sufficient indicator of LPS-induced mouse sepsis[39]. Therefore, we respectfully do not feel that it is also necessary to measure IL-6 levels in the mouse sepsis experiment, and do not believe doing so would further confirm this study's finding.

• Reviewer-2's Critique-8 (Major): In the lamina propria of the intestine, the authors try to show that T cell activation is defective in ATX KO mice (Fig 8). But does the total T cell number change?

• Our response to Reviewer-2's Critique-8 (Major)

We have not tested whether the total T cell number of the mouse would be changed. For our data of the FACS analysis (Fig. 9A to D), we first harvested the lamina propria lymphocytes and carried out the enrichment of CD4+ T cells. As aforementioned (Reviewer-Only Fig. 5), CD4+ range in each group was gated, and 10,000 CD4+ T cells in gated range were analyzed for the expression of IFNγ (marker for T_{h1} differentiation), IL-4 (marker for T_{h2} differentiation), IL-17 (marker for T_{h17} differentiation), and FoxP3 (marker for T_{reg} differentiation).

• Reviewer-2's Critique-9 (Major): In Fig 8A-D, the facs plots show that the cell populations do not clearly separate but rather looks like the compensation for the two flurochromes was not performed properly. If the spectral overlap between two flurochromes is not corrected then the data obtained from these experiments are not reliable.

• Our response to Reviewer-2's Critique-9 (Major)

We thank the reviewer for this very constructive comment. To ensure that the fluorescence detected is from the fluorochrome that is being measured, we performed fluorescence compensation using BD Accuri CFlow® Plus software (CFlow). With this analysis, we found that Th1, Th17 and Treg cell responses were reduced in the lamina propria lymphocytes of $Atx^{\Delta ME/\Delta ME}$ mice compared to those of $Atx^{+/+}$ littermates.

Given the fact that Th1 and Th17 responses participate in the immune response against invading microbes, and that Treg cells suppress inflammatory responses, these data suggest that reduced Th1, Th17, and Treg responses shift the environment of the intestinal mucosa toward a microbe-induced inflammatory condition.

Please be advised that the data with applied compensation were included with the quantification in the revised manuscript (Figure 9A to D). In accordance with the revised data, the main text relevant to this data was also revised.

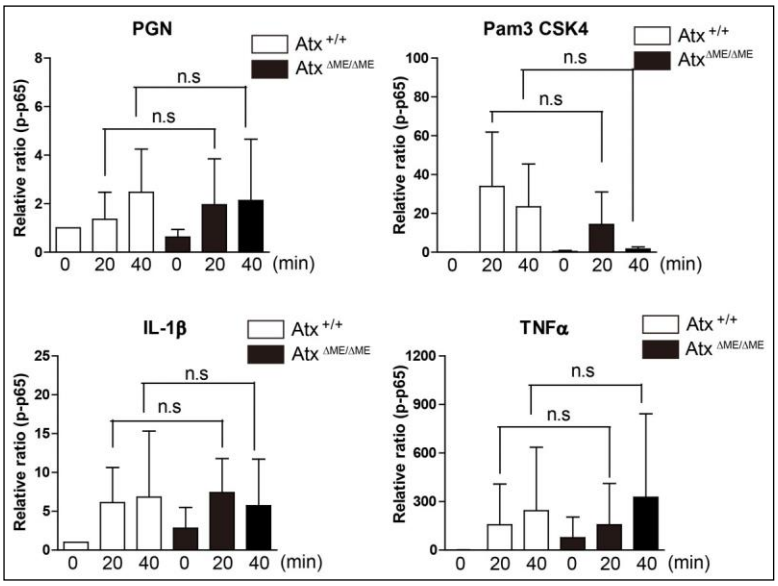
• Reviewer-2's **Minor Critique**-1: The authors show that IL1R signaling does not require lipid rafts; because they show that IL1R signaling is not affected in ATX KO macrophages in which lipid rafts are distorted. However there is literature that shows the importance of lipid rafts in IL1R signaling. This needs to be discussed.

• Our response to Reviewer-2's **Minor Critique**-1

It has been suggested that, in addition to TLR4, other membrane receptors such as IL-1R and TLR2 may reside in lipid rafts where they participate in receptor-mediated intracellular signaling pathways[40,41]. However, it is worth noting that the disruption of lipid rafts does not abort IL-1R-mediated signaling events because IL-1R can be activated in non-lipid raft regions of the plasma membrane[42]. Moreover, the lipid composition of lipid rafts does not affect TLR2 engagement[40]. In contrast, the activation of TLR4 by LPS is highly specific to the integrity of lipid rafts, because CD14, the co-receptor of TLR4, specifically resides in a lipid raft and first interacts with LPS. After initial interaction of CD14 with LPS, TLR4 moves to lipid rafts in order to make a CD14:TLR4 receptor complex in myeloid cells[3]. In this way, TLR4 makes a heteromeric complex with CD14 at the membrane lipid raft to elicit LPS-induced responses. Therefore, the integrity of lipid rafts is crucial for TLR4 activation at least in myeloid cells, including macrophages.

In this context, we believe that Atx deficiency alters TLR4-mediated signaling pathways, while TLR2-, IL-1R-, and TNF-R-mediated responses are preserved in Atx-ko cells.

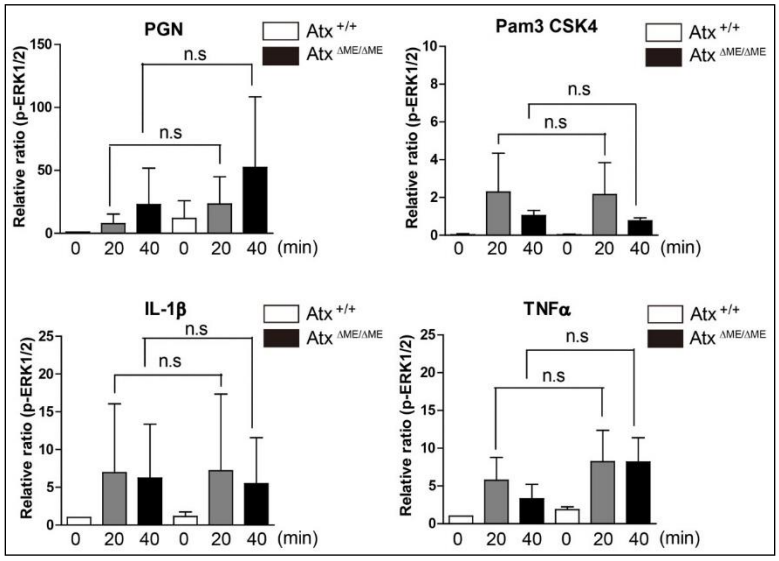
Reviewer-Only Fig. 8: TLR2-, IL-1R-, and TNF-R-induced NFκB activation (p-65) were not reduced in Atx-ko macrophages. We quantified the density of the phospho-p65 (p-p65) normalized by that of ERK1/2 from the immunoblots presented in Fig. 6E to H (in the revised manuscript). We confirmed that Atx-ko did not reduce these responses compared to those of controls. n=3 per group. n.s., not significant (t-test).



- Reviewer-2's **Minor Critique**-2: According to Fig 5E-H, the intracellular signaling activated by TLR2, IL1R and TNFR seems to be more in ATXKO cells as compared to ATX WT cells. Can the authors explain this?

- Our response to Reviewer-2's **Minor Critique**-2

The data presented (Fig. 6E–H in the revised manuscript) show that TLR2-, IL-1R-, and TNF-R-mediated responses were not reduced in Atx-ko macrophages compared to those of controls. Although the intensity of a couple of immunoblot bands appear to be slightly different in Atx-ko groups, that falls within the standard deviation of Western blot analysis.



Nevertheless, to address this critique, we quantified the density of each immunoblot band obtained from three independent experiments, and confirmed again that TLR2-, IL-1R-, and TNF-R-mediated responses (p-p65 and p-

ERK1/2) were not changed in Atx-ko macrophages compared to control cells (Reviewer-Only Fig. 8 and 9).

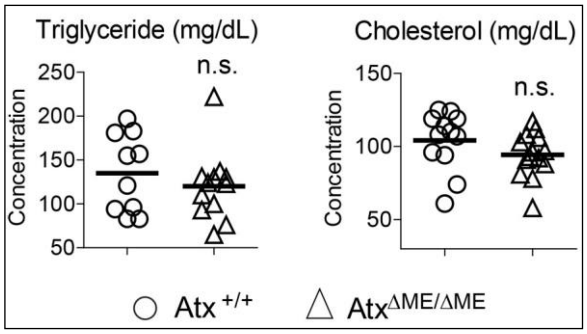
Reviewer-Only Fig. 9: TLR2-, IL-1R-, and TNF-R-induced ERK1/2 activation (p-ERK1/2) were not reduced in Atx-ko macrophages. We quantified the density of the phospho-ERK1/2 (p-ERK1/2) normalized by that of ERK1/2 from the immunoblots presented in Fig. 6E to H (in the revised manuscript). We confirmed that Atx-ko did not reduce these responses compared to those of controls. n=3 per group. n.s., not significant (t-test).

- Reviewer-2's **Minor Critique**-3: The reduced LPA levels seen intracellularly could point to a defect in the triglyceride synthesis pathway, in which LPA is an intermediate. This needs to be discussed.

- Our response to Reviewer-2's **Minor Critique**-3

We think it is of interest to assess whether the triglyceride synthesis pathway might be defective in Atx-ko mice in which altered triglyceride levels would consequently change LPA levels. However, ATX is a very specific enzyme that converts LPC into LPA. In this study, we utilized Atx-ko macrophages from myeloid cell-restricted Atx-ko mice. Therefore, examining the triglyceride synthesis pathway is outside of the scope of our study.

However, to provide an answer to the reviewer's question, we examined the levels of triglyceride and cholesterol in the serum samples of the mice used in this study ($Atx^{\Delta ME/\Delta ME}$ mice and $Atx^{+/+}$ littermates). We did not see significantly altered levels of triglyceride and cholesterol between Atx-ko and control mice.



Reviewer-Only Fig. 10: Triglyceride and cholesterol levels are comparable in the serum of $Atx^{\Delta ME/\Delta ME}$ mice and $Atx^{+/+}$ littermates. The serum samples of the mice were analyzed to measure Triglyceride and Cholesterol levels. n=10 - 13 per group. n.s., not significant (Mann-Whitney *U* test).

Taken together, we do not think that Atx deficiency could result in altered triglyceride levels.

Again, examining the triglyceride synthesis pathway in Atx-ko mice ($Atx^{\Delta ME/\Delta ME}$ mice) is out of the scope of our study. Therefore, we feel that including a discussion of triglyceride synthesis in our revised manuscript may distract from the main focus of our study without substantially enhancing the work's merit.

Our Responses to the Critiques from Referee #3:

- Reviewer-3's Critique-1 (Major): The authors report a decrease production of ATX in the serum of CD and UC patients. However, in the introduction they cite Hozumi et al., 2013 that reported an increase Atx mRNA levels in the inflamed mucosa of CD and UC patients. Even though these two studies investigate different cellular compartment, their results seem incompatible and this discrepancy is not discussed by the authors.

- Our response to Reviewer-3's Critique-1 (Major)

It is worth noting that the TNFα-NFκB pathway substantially upregulates the expression of ATX[43]. This study indicates that inflammatory factors, including TNFα, are capable of inducing Atx gene expression. Indeed, increased levels of ATX protein were observed in patients with chronic liver diseases such as chronic hepatitis C infection[44], primary biliary cholangitis[45], or non-alcoholic fatty liver disease[46].

Accordingly, in the inflamed intestine, it is reasonable to believe that a plethora of inflammatory cytokines therein are able to induce the expression of ATX; in this case, elevated ATX level should

be an outcome of the inflammation. Regarding Atx expression in IBD patients or in experimental mouse models of IBD, it should be underscored that the inflammatory response itself could elicit the expression of Atx.

However, in our study we utilized myeloid lineage cell-restricted Atx knockout mice to study the direct role of Atx in the onset and perpetuation of intestinal inflammation. We found that deletion of Atx accelerates the development of spontaneous colitis in an IL-10 deficient condition, which itself is one of the most clinically relevant genetic risk factors of human IBD. Therefore, our study firmly suggests that Atx deficiency can cause chronic inflammatory disease in the gut.

To address the reviewer's comment, we included this discussion in the Discussion section of the revised manuscript.

- Reviewer-3's Critique-2 (Major): The authors report that in the literature previous studies have shown that Atx depletion or use of Atx inhibitor reduce the severity of colitis in DSS-induced mice and CD4+;CD25- T-cell transferred mice. These results are opposite to the one report by the authors, although in different colitis model, and the authors barely discussed that issues and don't offer any explanations.

- Our response to Reviewer-3's Critique-2 (Major)

Previous studies have suggested that the inhibition of ATX may provide a protective effect in experimental mouse colitis. For example, increased Atx mRNA levels have been implicated in the inflamed colons from dextran sodium sulfate (DSS)-induced[47,48] or T cell-transferred colitis mice[47]. The ATX protein level was increased in the inflamed colons of DSS-treated Alkaline-SMase-ko mice[49]. ATX inhibitor treatment attenuated the severity of colitis in experimental mouse models such as DSS-induced[47,50,51], T-cell transferred[47], and SAMP1/Fc mouse colitis[52]. Similarly, Atx deletion reduced the severity of DSS-induced colitis in mice[48].

However, given the fact that residual DSS contamination in RNA samples from DSS-treated mice inhibits quantitative real-time PCR analysis[53,54], several observations made in the preexisting research ought to be tested by an alternative method. Moreover, the previous studies heavily rely on the model of DSS-induced colitis, which is primarily a model for epithelial injury; likewise, the T-cell transfer colitis model relies on T-cell migration in immunocompromised SCID mice, which also does not accurately reflect the etiopathology of human IBD. On the other hand, a recent study suggested that ATX can be produced as a pro-inflammatory factor, since the NFκB signaling pathway induces the expression of ATX. This may help to explain why an ATX blockade would be able to confer a protective effect in experimental mouse colitis.

Regarding the interaction between gut microbes and host immunity, it is of note that even in normal conditions, luminal gut bacteria translocate into the submucosa where they help to shape host immunity. In the submucosa, they are subsequently eliminated by innate and adaptive immune mechanisms. During this elimination process, the involvement of anti-inflammatory mediators such as IL-10 plays a critical role in suppressing the microbe-induced inflammatory response. Due to the potent expression of IL-10, therefore, intestinal macrophages can only produce limited levels of pro-inflammatory cytokines in response to microbes, while maintaining their highly phagocytic activity. In this way, intestinal homeostasis can be maintained even during the active immune responses occurring against invading microbes in the intestinal mucosa. Therefore, IL-10 deficient mice spontaneously develop colitis in a gut microbe-dependent manner. Indeed, genetic defects in IL-10 causes aggressive intestinal inflammation in humans, and therefore loss of the IL-10 encoding gene is a well-known genetic factor for IBD in humans[14,15]. Given that TLR4 activation initiates and galvanizes macrophage-immune mechanisms, TLR4 and IL-10 double knockout mice exhibit accelerated colitis development compared to IL-10-ko mice[55].

Considering the premise of the prior studies about Atx and IBD and the nature of gut mucosal immune responses, we harnessed $Atx^{\Delta ME/\Delta ME}$ mice and $Il10^{-/-}$ mice to investigate the role of Atx in intestinal inflammation. In this study, we demonstrate that Atx-ko suppresses TLR4-mediated responses in myeloid cells that are enriched in the gut submucosa. We further identified that $Atx^{\Delta ME/\Delta ME}$ mice had increased bacterial infiltration of the submucosa with reduced lamina propria T cell activation. Therefore, we theorized that Atx-ko could inhibit TLR4-mediated immune mechanisms, thereby accelerating gut inflammation in an IL-10 deficient condition. Indeed, we observed accelerated colitis development in $Atx^{\Delta ME/\Delta ME}$;$Il10^{-/-}$ mice compared to $Atx^{+/+}$;$Il10^{-/-}$ littermates.

Given that recent studies regard IBD as abnormal immune responses against commensal gut microbes in genetically susceptible individuals[56], our study suggests one component of the mechanism by which gut microbes can cause the onset and perpetuation of inflammatory disorders in the gut.

To address the reviewer's comment, we have included this discussion in the Discussion section of the revised manuscript.

- Reviewer-3's **Minor Critique**-1: The authors report a shift in the microbiota of the Atx deficient mice with an increase of specific Bacteroides species and a modification of mRNA expression but the authors do not discuss the potential impact of such modification.

- Our response to Reviewer-3's **Minor Critique**-1

An increased abundance of the *Bacteroides* genus is commonly observed in the feces of IL-10-ko mice that develop spontaneous colitis[15,57]. Therefore, the elevated level of *Bacteroides* is of significance because it indicates that the intestinal inflammation observed in $Atx^{\Delta ME/\Delta ME}$;$Il10^{-/-}$ mice is similar to the chronic colitis that occurs in IL-10 defective mice in a commensal microbe-dependent manner. Moreover, *Bacteroides* can adhere to and invade the intestinal epithelium whereby it fulfills its pathogenic role[58]. Indeed, commensal *Bacteroides* are capable of inducing colitis in a genetically susceptible mouse of colitis[59]. Accordingly, *Bacteroides* would be considered pathobionts at least in mice. In this context, we speculate that elevated levels of the *Bacteroides* may excel at invading the intestinal epithelium of Atx-ko mice in which Atx deficiency dampens mucosal immune mechanisms. Consequently, Bacteroides may accelerate the onset and perpetuation of gut inflammation in an IL-10 deficient condition.

To address the reviewer's comment, we included this discussion in the Discussion section of the revised manuscript.

- Reviewer-3's **Minor Critique**-2: In figure 5, different loading control have been used in the different panel. It would be more convincing if the same loading control was use for all the panel.

- Our response to Reviewer-3's **Minor Critique**-2

In the corresponding data (Fig. 6 in the revised manuscript), we showed the protein level of regular ERK1/2 as a loading control for Western blotting (Fig. 6A, E, F, G, H).

With the Western blotting data (Fig. 6B), we show that the protein levels of TLR-associated signaling molecules are not changed in primary macrophages from $Atx^{\Delta ME/\Delta ME}$ mice and $Atx^{+/+}$ littermates, and in fact are similar to one another. In the panel of Fig. 6B, the protein levels of TLR4, CD14, MYD88, MAL/TIRAP, TRIF, IRAK1, and AKT are all similar between the groups. Therefore, we do not feel that an additional blot for loading control needs to be included.

In Fig. 6C, we measured the protein level of RasGAP that is an adaptor protein. A number of manuscripts have employed RasGAP as a Western blotting loading control, and **moreover, the use of RasGAP as a loading control has been accepted in papers published in The EMBO Journal**[60,61].

In Fig. 6D, we used the bet-Actin protein level as a loading control for Western blotting, which is a house-keeping protein that is frequently used as a loading control.

In this way, our loading controls for Western blotting are based on a scientific rigor, which minimizes bias in interpreting the data. Therefore, we strongly believe that our loading controls are scientifically reasonable, and we would like to respectfully ask the reviewer to accept the data as presented.

- Reviewer-3's **Minor Critique**-3: In figure 8, the authors reported that the deletion of Atx increased bacterial infiltration into the intestinal mucosa. Even if the FISH staining is convincing, it will be interesting to know if the mucus layer is also affected.

- Our response to Reviewer-3's **Minor Critique**-3

To visualize the mucus layer, which is composed of mucins, the intestinal tissue should be stained using the Alcian blue staining method or the PAS technique, which are most widely used for this purpose. ATX is a lipid enzyme that converts LPC and SPC into LPA and S1P, respectively. It would be a very interesting research topic to study whether a myeloid cell lineage-restricted Atx gene knockout might alter mucin production, thereby causing changes to the mucus layer of the intestine.

However, please, be advised that the goblet cell (a form of intestinal epithelial cell) is the cell which directly produces mucins in the intestine. In this study, we examined myeloid cell lineage-restricted Atx gene knockout ($Atx^{\Delta ME/\Delta ME}$) mice and $Atx^{+/+}$ littermates. With these mice, our research aim is to demonstrate whether Atx deficiency disrupts the integrity of lipid rafts at the plasma membrane, thereby suppressing TLR4-mediated immune mechanisms. With this goal considered, we believe that studying the mucus layer of the mice is beyond the scope of this paper. Therefore, we respectfully hope that the reviewer will accept this response to their question.

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1st Revision - Editorial Decision 24th Mar 2020

Dear Prof. Rhee,

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the three referees that were asked to re-evaluate your study, you will find below. As you will see, the referees now support the publication of your study in EMBO reports. Nevertheless, referees #1 and #2 have remaining concerns, I ask you to address in a final revised version of the manuscript. Please also provide a point-by-point response that addresses these points of the referees.

Further, I have these editorial requests:

- Please provide the abstract written in present tense.

- Please provide individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures and EV figures. Please upload these as separate, individual files upon re-submission, which highest possible resolution/quality. We can accommodate up to 8 main figures!

The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf file labeled Appendix. The Appendix should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

For more details please refer to our guide to authors:
http://www.embopress.org/page/journal/14693178/authorguide#manuscriptpreparation

See also our guide for figure preparation:
http://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf

Presently, none of the figures have production quality! Many images over-contrasted (see also below), the resolution is too low (see e.g. Fig. 7C-E), or figures span more than one page, and some figures have landscape format (need portrait).

- Please format the references according to our journal style. See:
http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

- Please add a formal "Data Availability section" to the manuscript after the methods section. This is now mandatory (like the COI statement). If no primary datasets have been deposited in any database, please state this in this section (e.g. 'No primary datasets have been generated and deposited'). See also:
http://embor.embopress.org/authorguide#datadeposition

- Please add scale bars to all microscopic images. Presently, several images do not have scale bars.

Do not write on the scale bars. Please check that their length is defined in the respective figure legends.

- Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments (biological replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable, and also add a paragraph detailing this to the methods section. See:
http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

- Please show the Western blot data as unmodified as possible. All WB images are presently over-contrasted! Please show these as in the original source data (see below).

- As the Western blots are significantly cropped, we ask you to provide the source data for all the blots (main EV and Appendix figures). The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. Please submit the source data (scans of entire blots) together with the revised manuscript. Please include size markers for scans of entire blots, label the scans with figure and panel number, and send one PDF file per figure. Only for the Appendix (in case you provide one), please put together one source data file compiling all the WB source data for the Appendix figures.

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries, we ask you to address. Please provide your final manuscript file with track changes, in order that we can see the modifications done.

In addition I would need from you:
- a short, two-sentence summary of the manuscript
- two to three bullet points highlighting the key findings of your study
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

Yours sincerely,

Achim Breiling
Editor
EMBO Reports

Referee #1:

The authors have addressed many of the more important criticisms of the previous review. I now believe that the authors main conclusions are well supported by the data presented but two of the figures are still problematic and in need of additional corrections before all of the presented data is scientifically acceptable and worthy of publication. In addition, there are two minor editing changes needed.

1.) The data in Figure 9 remain problematic for the following reasons: 1A) Why does the percent of cells that are CD4+ (so adding Q2 and Q4 as shown in the summary bar graph to the right of panels A-D) vary so much between panels A to D? With this much variation, it seems likely that there is a substantial technical problem with these data and quality control is insufficient. Presumably, the authors are accumulating the type of data shown in all 4 panels from each mouse analyzed, so panels A-D of representative data either show data from different mice (Why?) or there is a substantial technical problem, or both. If from different mice, is the fraction of cells that are CD4+ really this variable between mice? Assuming these problems can be fixed or new, better data is generated: 1B) The summary bar graph to the right is essential and it should show two averaged numbers: % of CD4 T cells that are Th1, Th2, Th17 or CD4+FoxP3+ by presenting the ratio of Q2 to (Q2+Q4); Absolute number of Th1, Th2, Th17 or CD4+FoxP3+ (or if that cannot be reasonably determined, then % of these cell types of total lymphocytes, which is presumably the Q2 value). The current summary graph to the right of panels A-D shows "%Q1 +Q2", but the value of this summary is not clear as it adds together CD4+ and CD4- cells making the cytokine in question. Q1 could be ILC's, CD8's or gamma-delta T cells, so generating a composite Q1+Q2 number here has very limited value, compared to what I've suggested for the two numbers to include in the summary bar graph. I would have to see properly presented data for Figure 9 A-D to judge whether or not the authors present conclusions from these data are accurate or not.

2.) Legend to Fig. 8 and Methods section, "Phagocytosis measurement ..." The first and second paragraphs in the Methods subsection both have the same second half, so this seems to be a problem with the editing. I think the first paragraph should have an explanation of distinguishing bound vs. internalized latex beads, which requires use of trypan blue to quench fluorescence of beads that have not been fully internalized. Similarly the legend to Fig. 8 should make it clear what is going on here. How were latex beads that were internalized identified? Which panel does that and how? Also, as I mentioned previously, the authors should present data analyzing the number of bound IgG-coated latex beads vs. the number that are internalized, as they want to conclude that phagocytosis is impaired, but strictly speaking it could also be binding that is impaired, which would require a different conclusion on the part of the authors. In addition, in my experience, IgG-coated particles are phagocytosed quite well by macrophages without pre-treating with LPS, so some sort of kinetic analysis is probably necessary to really understand what is going on here (time vs. % internalization). It could be that the size of the particle and/or the IgG density on the particle used here is somewhat suboptimal and therefore phagocytosis is highly dependent on LPS-pretreatment. That would be OK, but it emphasizes that a more complete analysis is needed here, as the experimental setup may differ from what many people have used.

3.) "LPS" is used as an abbreviation for two different compounds, lipopolysaccharide and lysophosphatidylserine, clearly the authors need to change one of these abbreviations. I recommend keeping LPS as the abbreviation for lipopolysaccharide, given the central role of lipopolysaccharide in these studies and in the innate immunity literature.

4.) P, 15 (Discussion): "forks" is a typo, should be "forms"

5.) The methods are at least twice as long as in most publications these days. It reads more like a PhD thesis than a journal paper. When a method has been well described in a previous publication, it would be appropriate to just describe it in conceptual terms, cite the reference, and omit the detailed experimental parameters.

Referee #2:

1. For figure 9 (related to former comments 8 and 9): The authors say that they have gated 10,000 CD4+ T cells and analysed for the expression of IFNg, IL4, IL17 and Foxp3. If this is true then all or majority the cells in the facs plots (Figure 9A-D) should be CD4 positive (the population should only be in quadrant Q-4 or Q2 of the plot), which is not the case. Since there is no clear separation of different cell populations in these intracellular stainings, it is important to have proper control stainings (such as FMO controls) to identify the correct cut off to separate the positive and negative populations, but the authors do not state on what basis they selected the positive cells.

2. Related to comment#1: In humans, positive correlation between increased circulating levels of LPA and inflammatory diseases such as liver fibrosis and acute coronary syndrome has been shown by others and in the current study they show that ATX is reduced in patients with IBD so it would be essential to see if LPA is also reduced.

3. One inconsistency, for comment #5 in the response to reviewers the authors say they have used two-tailed unpaired t-test for fig 9A-D, but in the figure legend they state that they have used one-tailed.

Referee #3:

The authors have made great effort to change both the experimental plan and the discussion in order to respond to reviewers' comments and concerns. It is my opinion that the authors gave relevant answers to my different concerns, in detail:

Critique 1 and 2 (major):
The authors give a convincing discussion on the possible discrepancies that could appear between the results that they report and the literature they cited. My only concern is the emphasis the authors put on the unreliability of RNA samples studies in a DSS model, as this is a widely used and published method, and protocols are available to purify RNA sample to avoid any DSS interference in the q-PCR (see Viennois, E., et al. "Purification of Total RNA from DSS-treated Murine Tissue via Lithium Chloride Precipitation." Bio Protoc, 2018).

Critique 3 (minor):
The authors made an effort to give a proper discussion to their microbiota results.

Critique 4 (minor):
The authors made compiling scientific arguments regarding their use of different loading control that is acceptable.

Critique 5 (minor)
I understand the authors arguments that mucus study could be beyond the scope of this paper, but I hope they will keep this suggestion in mind for future research.

Our Resubmission Responses to the Critiques from Reviewer #1

• Reviewer-1's Critique-1 (Major): The data in Figure 9 remain problematic for the following reasons: 1A) Why does the percent of cells that are CD4+ (so adding Q2 and Q4 as shown in the summary bar graph to the right of panels A-D) vary so much between panels A to D? With this much variation, it seems likely that there is a substantial technical problem with these data and quality control is insufficient. Presumably, the authors are accumulating the type of data shown in all 4 panels from each mouse analyzed, so panels A-D of representative data either show data from different mice (Why?) or there is a substantial technical problem, or both. If from different mice, is the fraction of cells that are CD4+ really this variable between mice? Assuming these problems can be fixed or new, better data is generated: 1B) The summary bar graph to the right is essential and it should show two averaged numbers: % of CD4 T cells that are Th1, Th2, Th17 or CD4+FoxP3+ by presenting the ratio of Q2 to (Q2+Q4); Absolute number of Th1, Th2, Th17 or CD4+FoxP3+ (or if that cannot be reasonably determined, then % of these cell types of total lymphocytes, which is presumably the Q2 value). The current summary graph to the right of panels A-D shows "%Q1 +Q2", but the value of this summary is not clear as it adds together CD4+ and CD4- cells making the cytokine in question. Q1 could be ILC's, CD8's or gamma-delta T cells, so generating a composite Q1+Q2 number here has very limited value, compared to what I've suggested for the two numbers to include in the summary bar graph. I would have to see properly presented data for Figure 9 A-D to judge whether or not the authors present conclusions from these data are accurate or not.

• Our response to Reviewer-1's Major Critique-1:

We appreciate reviewer-1's insightful comments regarding the FACS data and, upon further examination of our data, believe this criticism to be entirely valid. Therefore, we carried out additional experiments with mouse lamina propria CD4+ samples which unfortunately did not yield any convincing data. With this in mind, we would like to remove the FACS data of lamina propria CD4+ T cells (which were Fig. 9A to D in the previous submission).

Nonetheless, we are certain that the removal of the FACS data does not fundamentally alter the main conclusions of our study, and preserves its rigor and quality. The following further explain our rationale in removing the FACS data:

(A) Even with the omission of the FACS data, all the remaining data still convincingly support the main scientific conclusions. Using myeloid cell lineage-restricted Atx knockout ($Atx^{\Delta ME/\Delta ME}$) mice and $Atx^{+/+}$ littermates, our research aim is to determine whether Atx deficiency disrupts the integrity of lipid rafts at the plasma membrane, thereby suppressing TLR4-mediated responses and immune mechanisms (e.g. phagocytosis). With this goal considered, the data presented in the revised manuscript substantiate the scientific conclusions succinctly.

Microbes which have translocated into the intestinal mucosa from the lumen must be eradicated by innate immune mechanisms in order to maintain gut homeostasis. In agreement with this notion, we found that myeloid cell-restricted Atx-ko ($Atx^{\Delta ME/\Delta ME}$) mice have higher bacterial loads in the intestinal mucosa compared to control littermates. Much in line with this finding, we also observed that in an Il-10-ko condition, $Atx^{\Delta ME/\Delta ME}$ mice exhibited accelerated development of spontaneous colitis.

Therefore, we strongly believe that our remaining data sufficiently demonstrate the role of Atx in the regulation of TLR4-mediated immune mechanisms in macrophages, and the immunological significance thereof.

(B) Myeloid cells, including macrophages and dendritic cells (DCs), are closely associated with T cells. DCs specialize in presenting antigen to naïve T-cells, which leads to T cell differentiation and proliferation to generate effector T cells (T_{h1}, T_{h2}, T_{h17}) and regulatory T cells (T_{reg}). Macrophages, however, do not directly present antigen to activate naïve T cells, and instead can assist in T-cell proliferation through direct contact with T cells.

In this study, we use the LysM-Cre system to generate a myeloid cell-restricted Atx knockout mouse line. It is worth noting that the LysM-Cre system works more effectively for macrophages than for DCs. Accordingly, the LysM-Cre system is generally accepted as a decent system to obtain a macrophage-targeted gene deletion. With this in mind, $Atx^{\Delta ME/\Delta ME}$ mice may not exhibit dramatic changes in T cell activation compared to $Atx^{+/+}$ littermates. We speculate that this may be the reason why we could not see consistent changes in the activation of lamina propria CD4+ T cells from $Atx^{\Delta ME/\Delta ME}$ mice compared to controls.

As such, we would like to remove the FACS data of lamina propria T cells from the manuscript. Even with this omission, the data remaining in the revised manuscript convincingly support the main scientific conclusions without compromising the scientific rigor or quality of this study. Therefore, we would greatly appreciate it if you take this as a scientifically acceptable justification and worthy of publication.

• Reviewer-1's Critique-2 (Major) (A): Legend to Fig. 8 and Methods section, "Phagocytosis measurement ..." The first and second paragraphs in the Methods subsection both have the same second half, so this seems to be a problem with the editing. I think the first paragraph should have an explanation of distinguishing bound vs. internalized latex beads, which requires use of trypan blue to quench fluorescence of beads that have not been fully internalized.

On the other hand, I'd like to explain again that we had provided the data that the use/not use of trypan blue in the phagocytosis measurement assay did not alter the results (Refer to the Reviewer-Only Fig. 3 and 4 in our previous response letter)

• Our response to Reviewer-1's Major Critique-2A:

In the Methods section titled "Phagocytosis measurement and visualization of phagocytic cup formation", the first paragraph describes the method of phagocytosis measurement. As described in the Methods section, phagocytosis was examined with a Phagocytosis Assay Kit (IgG FITC) (Cat. No. 500290, Cayman Chemical, Ann Arbor, MI) in accordance with the manufacturer's instructions, in which the use of trypan blue is included to quench fluorescence of beads that have not been internalized.

The second paragraph describes the method for visualization of phagocytic cup formation. While both methods are similar, the treatment time differs between the two. Furthermore, trypan blue was not used for the visualization of phagocytic cup formation.

• Reviewer-1's Critique-2 (Major) (B): Similarly the legend to Fig. 8 should make it clear what is going on here. How were latex beads that were internalized identified? Which panel does that and how? Also, as I mentioned previously, the authors should present data analyzing the number of bound IgG-coated latex beads vs. the number that are internalized, as they want to conclude that phagocytosis is impaired, but strictly speaking it could also be binding that is impaired, which would require a different conclusion on the part of the authors. In addition, in my experience, IgG-coated particles are phagocytosed quite well by macrophages without pre-treating with LPS, so some sort of kinetic analysis is probably necessary to really understand what is going on here (time vs. % internalization). It could be that the size of the particle and/or the IgG density on the particle used here is somewhat suboptimal and therefore phagocytosis is highly dependent on LPS-pretreatment. **That would be OK**, but it emphasizes that a more complete analysis is needed here, as the experimental setup may differ from what many people have used.

• Our response to Reviewer-1's Major Critique-2B:

In Figure 8 we examined FITC beads that are internalized in macrophages. Therefore, as the reviewer suggested, the Figure 8 legend has been updated to clarify this.

As the reviewer suggests, analyzing the number of membrane-bound beads vs. the number of internalized beads may be an important question that merits further study in regard to the impact of Atx on macrophage phagocytosis. However, we are somewhat concerned that this may distract the research scope of the present manuscript toward Fc Receptor-guided phagocytosis.

Again, in this study we aim to determine the role of Atx in TLR4-mediated responses in macrophages and its immunological significance. Accordingly, our data clearly suggest that Atx deficiency disrupts the integrity of the membrane lipid rafts, thereby suppressing TLR4-mediated signaling pathways and innate immune mechanisms. Phagocytic activity measurement is one of the approaches used to investigate the effect of Atx deficiency on TLR4-mediated immune mechanisms. This approach successfully demonstrated that Atx deficiency inhibits phagocytosis in macrophages.

We agree with the reviewer that one remaining question is whether the changes in phagocytic activity are due to changes in membrane binding activity or altered kinetics of phagocytosis. However, as the principal investigator of the study, it is respectfully hard to agree that it is necessary to examine the kinetics of phagocytosis or to quantify the number of membrane bound beads vs. the number of internalized beads in our study conditions. Nonetheless, we are grateful to the reviewer for this insightful comment, which will be an excellent research question to address in a subsequent project studying the immunological impact of Atx.

• Reviewer-1's Critique-3 (Minor) "LPS" is used as an abbreviation for two different compounds, lipopolysaccharide and lysophosphatidylserine, clearly the authors need to change one of these abbreviations. I recommend keeping LPS as the abbreviation for lipopolysaccharide, given the central role of lipopolysaccharide in these studies and in the innate immunity literature.

• Our response to Reviewer-1's Minor Critique-3:

We apologize for this error and have updated the manuscript accordingly. As suggested, we now use "LPS" as an abbreviation only for lipopolysaccharide and no longer use an abbreviation for lysophosphatidylserine.

• Reviewer-1's Critique-4 (Minor) P, 15 (Discussion): "forks" is a typo, should be "forms"

• Our response to Reviewer-1's Minor Critique-4:

We apologize for the typo. It is corrected in the revised manuscript.

• Reviewer-1's Critique-5 (Minor): The methods are at least twice as long as in most publications these days. It reads more like a PhD thesis than a journal paper. When a method has been well described in a previous publication, it would be appropriate to just describe it in conceptual terms, cite the reference, and omit the detailed experimental parameters.

• Our response to Reviewer-1's Minor Critique-5:

Although we wanted to provide a very detailed, comprehensive description of the methods used in this study, this unfortunately resulted in a lengthy methods section. As suggested, we cited the references for the methods that have been well described in previous publications. With this change, we hope to provide an appropriate description of the experimental protocols.

Our Resubmission Responses to the Critiques from Reviewer #2

• Reviewer-2's Critique-1 (Major): For figure 9 (related to former comments 8 and 9): The authors say that they have gated 10,000 CD4+ T cells and analysed for the expression of IFNg, IL4, IL17 and Foxp3. If this is true then all or majority the cells in the facs plots (Figure 9A-D) should be CD4 positive (the population should only be in quadrant Q-4 or Q2 of the plot), which is not the case. Since there is no clear separation of different cell populations in these intracellular stainings, it is important to have proper control stainings (such as FMO controls) to identify the correct cut off to separate the positive and negative populations, but the authors do not state on what basis they selected the positive cells.

• Our response to Reviewer-2's Major Critique-1:

"We appreciate reviewer-2's insightful comments regarding the FACS experiment and believe they make a valid criticism. This was similarly brought up in to Reviewer-1's Major Critique-1; **please see our response to their comment abovementioned**."

• Reviewer-2's Critique-2 (Major): Related to comment#1: In humans, positive correlation between increased circulating levels of LPA and inflammatory diseases such as liver fibrosis and acute coronary syndrome has been shown by others and in the current study they show that ATX is reduced in patients with IBD so it would be essential to see if LPA is also reduced.

• Our response to Reviewer-2's Major Critique-2:

As we had explained in the previous response letter in regards to this issue, Atx is a secreted protein that catalyzes the conversion of lysophosphatidylcholine (LPC) to lysophosphatidic acid (LPA) (Nakanaga *et al*, 2010). However, LPA in plasma and serum is highly unstable with increasing concentrations during storage; therefore, the serum ATX protein level is a more reliable indicator than direct measurement of LPA levels (Beuers *et al*, 2014). Moreover, serum LPA can be elevated as a result of blood coagulation

due to the generation of LPA by platelets (Aoki, 2004). Therefore, when analyzing human serum samples, the stability of LPA and the mechanism (source) of LPA generation must be taken into account as potential sources of error. This is the reason that we evaluated the ATX protein level in the serum samples of IBD patients. Considering the multitude of factors which can lead to alterations in the serum LPA level, we strongly believe that measuring serum ATX protein levels in lieu of LPA is a scientifically rigorous approach to investigate correlation with human disease. Nevertheless, we fully appreciate that a correlation between increased circulating levels of LPA and human diseases has been suggested, as the reviewer mentioned (Kremer *et al*, 2010).

Therefore, when considering the potential inaccuracy introduced by the instability of LPA and the various sources of LPA generation in the serum, we respectfully do not feel that measuring LPA levels in human blood samples would enhance the scientific rigor and quality of this manuscript. However, I do agree with the reviewer when it comes to the potential utility of the LPA serum level as a diagnostic tool for certain human diseases.

Nevertheless, in an effort to address this reviewer's request to evaluate the level of LPA in serum samples of IBD patients, we attempted to collect samples from human subjects through our collaboration with the UCLA IBD Center. Unfortunately, due to the COVID-19 pandemic we have found that many research institutions in the US have faced shutdowns to prevent further spread of the virus. Under these unprecedented restrictions, we have found it almost impossible to recruit patients to collect the human blood samples needed to obtain the data which would address the reviewer's question.

Considering the biochemical nature of LPA and the current restrictions imposed by the pandemic, we would greatly appreciate it if the reviewer accepts our justifications as scientifically reasonable and deems our current evidence acceptable for publication.

- Reviewer-2's Critique-3 (Minor): One inconsistency, for comment #5 in the response to reviewers the authors say they have used two-tailed unpaired t-test for fig 9A-D, but in the figure legend they state that they have used one-tailed.

- Our response to Reviewer-2's Minor Critique-3:

As mentioned above, Figure 9A-D have been removed from the revised manuscript, which resolves this issue.

Our Resubmission Responses to the Critiques from Reviewer #3

Reviewer-3's Critique-1 and 2 (Major): The authors have made great effort to change both the experimental plan and the discussion in order to respond to reviewers' comments and concerns. It is my opinion that the authors gave relevant answers to my different concerns, in detail:

The authors give a convincing discussion on the possible discrepancies that could appear between the results that they report and the literature they cited. My only concern is the emphasis the authors put on the unreliability of RNA samples studies in a DSS model, as this is a widely used and published method, and protocols are available to purify RNA sample to avoid any DSS interference in the q-PCR (see Viennois, E., et al. "Purification of Total RNA from DSS-treated Murine Tissue via Lithium Chloride Precipitation." BioProtoc, 2018).

• Our response to Reviewer-3's Major Critique-1 and 2:

We are enormously grateful to reviewer-3 for his/her professional comments about our manuscript. We appreciate that RNA purification protocols to avoid DSS contamination are available for qPCR. However, to the best of our knowledge it is not clear whether the RNA purification protocols necessary to avoid DSS contamination were used for the studies that we have cited to discuss the possible discrepancies. Therefore, examining the level of Atx mRNA in samples obtained from DSS-induced colitis mice using the DSS-removing RNA purification protocol would make for an excellent small study. However, because our study is based on $Atx^{\Delta ME/\Delta ME}$; $Il10^{-/-}$ mice in which the translocation of communal bacteria into the intestinal mucosa from the lumen initiates spontaneous colitis development, we are concerned that combining any DSS-induced colitis experiment with the current study may distract from the main focus. Nonetheless, the reviewer's comment regarding the RNA purification protocol will be kept in mind for a follow-up study.

Reviewer-3's Critique-3 (Minor): The authors made an effort to give a proper discussion to their microbiota results.

Reviewer-3's Critique-4 (Minor): The authors made compiling scientific arguments regarding their use of different loading control that is acceptable.

Reviewer-3's Critique-5 (Minor): I understand the authors arguments that mucus study could be beyond the scope of this paper, but I hope they will keep this suggestion in mind for future research.

• Our response to Reviewer-3's Minor Critique-3 to 5:

Thank you for your thoughtful comments about our study. The comments and suggestions provided by the reviewer are excellent advice not only for this project, but also for other ongoing projects in our lab. We will keep these suggestions in mind for future research. Thank you again!

References Cited in the Response Letter for Resubmission

Aoki J (2004) Mechanisms of lysophosphatidic acid production. *Semin Cell Dev Biol* 15: 477-489

Beuers U, Kremer AE, Bolier R, Elferink RP (2014) Pruritus in cholestasis: facts and fiction. *Hepatology* 60: 399-407

Kremer AE, Martens JJ, Kulik W, Rueff F, Kuiper EM, van Buuren HR, van Erpecum KJ, Kondrackiene J, Prieto J, Rust C *et al* (2010) Lysophosphatidic acid is a potential mediator of cholestatic pruritus. *Gastroenterology* 139: 1008-1018, 1018 e1001

Nakanaga K, Hama K, Aoki J (2010) Autotaxin--an LPA producing enzyme with diverse functions. *J Biochem* 148: 13-24

2nd Revision - Editorial Decision 10th Aug 2020

Prof. Sang Rhee
Oakland University
330 Dodge Hall
118 Library Drive
Rochester, MI 48309
United States

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Corresponding Author Name: Sang Hoon Rhee
Journal Submitted to: EMBO Report
Manuscript Number: EMBOR-2019-49332V2

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 iustified
- ➔ 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 $\chi 2$ 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.

B- Statistics and general methods

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?	We choose the sample sizes based on what is generally implemented in our research field (e.g. IL-
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	The sample size of animal studies was determined based on our experimenced in the research area of IBD and TLR4 biology. For example, in the animal study of spontaneous colitis with Atx and IL-10 double knockout mice, we choose the sample size based on our previous experience published (Im E et al, Gastroenterology 2014). We did not perform a statisical analysis to descide
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Samples or animals involved the experiemnts were not excluded when we analyzed the final data.
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.	We used sex-matched, age-matched mice to minimize the effects of sujective bias when allocating animals. Moreover, all mice used in this study are C57BL/6 background.
For animal studies, include a statement about randomization even if no randomization was used.	There were no randomization procedures in this study because the aim of an experiment was to compare the data between WT and KO mosue group. For in vitro experiemnt, we used a pool of cells from a group of mice (WT or KO).
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.	For image analysis, the researcher who carried out an image analysis was blinded to the mouse information. For in vivo animal experiments, no blinding was applied. Instead, sex-matched and age-matched mice (littermates) were used to minimize the effect of subjective bias. In vitro experiments such as qPCR, ELISA, Western blot, flow cytometry were conducted in a non-blind manner because the experimental readouts were quantitative and not influenced by the subjective
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding experimentation was conducted for animal experiments because all experimental data were quantitative and not influenced by a subjetive bias.
5. For every figure, are statistical tests justified as appropriate?	The statistical analysis, the sample size, and number of independent experiments performed were specified in each figure legend
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We assessed normal distribution using Graph Pad Prism, and choose the statistical test accordingly. The result of normality test was included in figure legends.
Is there an estimate of variation within each group of data?	Yes. We presented data as mean +/- SEM or mean +/- SD. Error bars were shown to indicate variation within each group of data.

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http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor

http://grants.nih.gov/grants/olaw/olaw.htm
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http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo

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http://biomodels.net/miriam/
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http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	No F-test was run to statistically compare the variance.

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).	Catalog numbers for all antibodies used in immunoblot, immunoprecipitation and immunofluorescence staining and sequence for primers were specified in the Materials and Methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Mouse macrophage cell line Raw264.7 and human embryonic kidney cell line HEK293 cells used in this study were tested for mycoplasma-free status. Raw264.7 and HEK293 were purchased from ATCC.

* 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.	Animal information was described in the Materials and Methods section. Atx-floxed mice and Atx-heterozygous knockout mice were kindly provided by Dr. Moolenaar (The Netherlands Cancer Institute, Amsterdam, the Netherlands). Their genotypes were determined by a genotyping PCR protocol. Macrophage-specific Cre-expressing LysM-Cre mice and Il10-/- mice on a C57BL/6 background were purchased from the Jackson laboratory. Genotyping PCR was performed in accordance with the protocol provided by the Jackson laboratory. Atx-floxed mice were crossed with LysM-Cre mice to generate macrophage specific Atx-ko and littermate control mice (Atx+/+) mice. An Atx-ko mouse was crossed with an Il10-/- mouse to generate Atx-ko;Il10-/- mice. Atx-ko mice and Atx-ko;Il10-/- mice were backcrossed into a C57BL/6 background for at least 8 generations prior to performing the experiments.
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 the Institutional Animal Care and Use Committees of Oakland University and Pusan National University. Mice were bred and maintained in a specific pathogen free condition with normal drinking water ad libitum at the AAALAC accredited animal facility of the Biomedical Research Support Facility, Oakland University (IACUC no. 16122), and Pusan National University (IACUC No. PNU-2018-1843) under the approval of the IACUC.
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.	Animal experiments were carried out in compliance with the ARRIVE reporting guidelines. All the relevant aspects of mouse experimentation are detailed in the Material and Methods section

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	All human blood samples were collected and analyzed with the approval of the UCLA Institutional Review Board (IRB number: 12-000420). All participants were provided with complete information about the study and gave written informed consent to the study protocol. The patients examined in this study have never been included in any of the previous studies. This statement has been included in the Materials and Methods section.
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.	All participants were provided with complete information about the study and gave written informed consent to the study protocol.
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15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	Not applicable.
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.	Not applicable.
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.	Not applicable.

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	Not applicable.
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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).	Not applicable.
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.	Not applicable.

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.	The study in this paper does not fall under dual research restriction.