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Dr. Lian-Sheng Ma, MD. Ph.D,Editorial Office Director, Company Editor-in-Chief, Editorial Office
World Journal of Gastroenterology
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Dear Dr. Lian-Sheng Ma,

Thank you very much for the opportunity to revise and resubmit our manuscript "*ox-LDL stimulates M2 polarization of macrophages to upregulate CD44 expression in colorectal cancer associated with a high-fat diet*". (Submission ID: 76311) to the *World Journal of Gastroenterology*. We greatly appreciate the editors' and reviewers' constructive comments and critiques, which we believe will help us to improve the quality of our manuscript. We have carefully considered the comments and have substantially revised the manuscript to address the comments and questions. We have prepared a point-by-point response to these comments and highlight the changes in the revised manuscript.

Once again, thank you for your letter, the insightful review and the opportunity for revision and resubmission.

Please do not hesitate to contact me, should you have any additional question.

Sincerely yours,
(On behalf of the authors)
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A point-by-point response to the comments from Editors and reviewers:

General comments from Editor:

The manuscript describes that the increased expression of ox LDL and the increased number of M2 macrophages were detected in colorectal tissues of patients with colorectal cancer and HFD mice. Ox LDL can lead to the increased expression of cd206 and CD44 in monocytes, so as to prove the important role of ox LDL in colorectal cancer Interesting articles, but the depth of research is not so deep. The typing of macrophages is too hasty, but using cd206 positive is not enough. The flow of the experiment should be illustrated by a small figure.

Response:

Thank you for the comment. In order to explain our conclusion more strictly, we have changed "M2 macrophage" to "CD206-positive macrophage" or "CD206+ macrophage". In addition, to confirm the phenotypic changes of macrophages, we detected both the expression of CD206 in macrophages and the cytokines associated with CD206-positive cells to explain the function of the cells (Figure 4F). In the manuscript, we added a flow chart to illustrate our experimental design clearly (Figure 5A, B).

General comments from Company editor-in-chief:

I have reviewed the Peer-Review Report, the full text of the manuscript, and the relevant ethics documents, all of which have met the basic publishing requirements of the World Journal of Gastroenterology, and the manuscript is conditionally accepted. I have sent the manuscript to the author(s) for its revision according to the Peer-Review Report, Editorial Office's comments and the Criteria for Manuscript Revision by Authors. Before final acceptance, uniform presentation should be used for figures showing the same or similar contents; for example, "Figure 1Pathological changes of atrophic gastritis after treatment. A: ...; B: ...; C: ...; D: ...; E: ...; F: ...; G: ...". Please provide decomposable Figures (in which all components are movable and editable), organize them into a single PowerPoint file. In order to respect and protect the author's intellectual property rights and prevent others from misappropriating figures without the author's authorization or abusing figures without indicating the source, we will indicate the author's copyright for figures originally generated by the author, and if the author has used a figure published elsewhere or that is copyrighted, the author needs to be authorized by the previous publisher or the copyright holder and/or indicate the reference source and copyrights. Please check and confirm whether the figures are original (i.e. generated de novo by the author(s) for this paper). If the picture is 'original', the author needs to add the following copyright information to the bottom right-hand side of the picture in PowerPoint (PPT): Copyright ©The Author(s) 2022.

Response:

Thank you for the comment. We have unified the format of the figure legends and organized the figures into a PPT. The attachment has been uploaded to the *World Journal of Gastroenterology* website.

Reviewer #1:

1. Comments:

The clinical part lacks the analysis of the correlation between ox-LDL and patient progression and survival; in vitro experiments, it is necessary to knock

out the ox-LDL gene in macrophages to observe whether it can inhibit the growth and metastasis of colorectal cancer. These data serve to demonstrate that ox-LDL is a predictor and prognostic biomarker in CRC.

Response:

Thank you very much for these constructive comments which have helped us to improve the quality of our manuscript markedly. In this study, we tried our best to knock out the ox-LDL gene, but ox-LDL is produced by the oxidation of LDL in the blood so that it's difficult to knock out ox-LDL in macrophages. To resolve this problem, we searched the previous studies and found that LOX-1 is a specific receptor of ox-LDL, which can be knocked out as an alternative. We transfected LOX-1siRNA into macrophages to obtain macrophages with weak expression of ox-LDL receptor. When we inhibited the expression of LOX-1, the CD206+ macrophages stimulated by ox-LDL were significantly inhibited (Figure 4D, E). The up-regulation of CD44 and CD133 were also inhibited in LoVo cell co-cultured with the transfected macrophages (Figure 5B, E-F).

As to the second question, it is a pity that we failed to make a convincing analysis the correlation between ox-LDL and progression and survival of the enrolled patients because of our tiny specimens. But we plan to collect more and bigger samples and look forward to having positive data in the near future.

2. Comments:

Results 2 and 4 only show the expression of CD206 (marker of M2-type polarized macrophages), which should be compared with M1-type macrophage polarization markers at the same time.

Response:

Thank you very much for the comment. This is a good suggestion, and we have modified it in the results. In order to clarify the expression levels of M1 and M2 macrophages in human colorectal cancer and hyper-lipidaemia mice, we detected the expression of F4/80(macrophage polarization marker) and

iNOS (M1 macrophage polarization marker), and compared its expression between CD206+ macrophages and iNOS+ macrophages. These results further confirmed the up-regulation of the M2-type macrophages in human colorectal cancerous tissues (Figure 1E, F, K, L) and colorectal tissues of hyperlipidemic mice (Figure 3A-D).

3. Comments:

To understand the effects of ox-LDL on M2 polarization, specific cellular and molecular pathways associated with polarized cells need to be linked to their specific functions, rather than purely quantitative. M2 macrophages are closely related to Th2 cytokines, such as IL-10, IL-4 and IL-13 or transforming growth factor-β, so the expression of related cytokines or chemokines should be detected.

Response:

Thank you very much for the comment. This is a good suggestion, and we have detected the levels of related cytokines including IL-10, IL-4, IL-13 and TGF-β in THP-1 cells, and compared the functional changes of THP-1 cells treated with ox-LDL. The experiment to determine the function of CD206+ macrophages showed that after ox-LDL stimulation for 72h, the levels of CD206+ macrophage-related cytokines such as IL-4, IL-10 and TNF-β increased significantly in THP-1 cells (vs control, $P<0.05$) except IL-13 (Figure 4F).

4. Comments:

The conclusion of the study mentioned that ox-LDL induces M2 polarization to promote the increase of CD44 levels in colorectal cancer cells. Whether the verification process is too simple, it is necessary to further explore the signaling pathway regulated by M2 macrophages. Is there a causal relationship between the two? In addition to CD44, does it also affect other analysis expressions?

Response:

Thank you very much for the comment. This is a good suggestion. A lot of evidence proves that LOX-1 was the specific surface receptor of ox-LDL. In order to verify the correlation between ox-LDL and M2-type macrophage, we transfected LOX-1siRNA into macrophage to inhibit the expression of LOX-1, and then stimulated the macrophage by ox-LDL. Our results suggested that after ox-LDL stimulation for 72 hours, the regulation like inhibition of iNOS expression and the promotion of CD206 expression, was significantly weakened in transfected THP-1 cells (Figure 4D, E). As for the mechanism, unfortunately we haven't identified the signal pathway related to ox-LDL promoting M2-type macrophages. But it's our next project. We plan to use the technology of single cell sequencing and/or RNA-Seq assay to future explore the potential signal pathways. We are confident that there will be exciting data in the near future.

To explain the second question, we used the other tumor stem cell marker CD133 as well as CD44 which is recognized as a typical tumor stem cell marker. We are happy to obtain exciting results similar to CD44. We found that both CD44 and CD133 were up-regulated in LoVo cells co-cultured with THP-1 cells stimulated by ox-LDL (Figure 5A, C-D). Meanwhile, the expression of both CD44 and CD133 were inhibited following LOX-1 knocked down in THP-1cell (Figure 5B, E-F). Our results confirmed the function of ox-LDL on colorectal cancer mediated by macrophages.

Reviewer #2:

1. Comments:

Ox-LDL staining is not specific, no interstitial cell staining is seen in the picture used in the figure 1, only a non-specific brown shading that is usually given by secondary antibody staining. The authors should show a specific staining of interstitial cells where brown staining is seen surrounding a nucleus, differentiating the staining of the interstitium from the cell cytoplasm. Photos should be taken at a minimum of 40x. Authors should choose the same type of

cut in the tissues shown in figure 1, in the control the crypts are cut transversely and the CA longitudinally.

Response:

Thank you very much for the comment. This is a good suggestion, and we have chosen immunofluorescence staining (IHC) to detect the location of ox-LDL and its specific receptor LOX-1. Our results showed that the expression levels of ox-LDL and LOX-1 were both significantly increased in colorectal tissues of human colorectal cancer (Figure 1A, C, G, I). To ensure the consistency of colorectal tissues between two groups, we keep choosing the same type of incision as well as transverse recess incision.

2. Comments:

A co-localization of CD206/oxLDL or some kind of correlation in CA and HFD mice is mandatory.

Response:

Thanks for your comments. To show the strong correlation between ox-LDL and CD206, we used immunofluorescence-staining of ox-LDL and CD206 in human and mouse colorectal tissues. Our results showed that the number of CD206+/ox-LDL+ cells increased abundantly in colorectal tissues of human colorectal cancer (Figure 1B, D, H, J) and hyperlipidemic mice (Figure 3E-H).

3. Comments:

The objective in the abstract is not specific. The objective does not contain the word macrophage which is an important part of the whole work.

Response:

Thank you very much for the comment. We have revised the part of abstract and added some description of macrophages to the objective section.

4. Comments:

The authors should specify the origin of the healthy tissue, i.e. the pathology of the patients undergoing colonoscopy.

Response:

Thank you very much for the comment. We added the origin of the healthy tissues in the "1 Materials & methods1.1 Patient samples" in the manuscript.

5. Comments:

Grammatical errors: 3μm-thick, 10ug/ml, 1h, 10μg, 1%BSA, 50μg/ mL, 800rpm, 3min, 50μg/ mL, cell line(RAW 264.7) … go separately; interstitium, in vitro are in italics; GADPH is GAPDH.

Response:

Thank you very much for the comment. We have revised the grammatical errors mentioned in the third point.

6. Comments:

There is no table with primary antibodies used, dilutions used for HI, IF and WB. . Molecular weight in WB of CD44 and GAPDH

Response:

Thank you very much for the comment. We added two tables to the supplementary materials, including "Supplementary Table 1. Antibody dilution ratio for Immunofluorescence and Western Blot", and "Supplementary Table 2. Molecular weight in Western Blot of CD44, CD133 and GAPDH".

Reviewer #3:

1. Comments:

The method for enumeration of ox-LDL expression in colorectal cancer tissues is unclear. Due to the pronounced background staining in Figure 1, it is necessary to clarify the tissue localization of ox-LDL, either within cells or in the intercellular substance.

Response:

Thanks for your comments. We choose immunofluorescence staining to detect the location of ox-LDL and its specific receptor LOX-1. We found that the expression of both ox-LDL and LOX-1 were significantly up-regulated in human colorectal cancerous tissues (Figure 1A, C, G, I).

2. Comments:

The authors used the CD206 marker to detect M2 macrophages. In this regard, there are several comments. The authors show an increase in the number of CD206. Is this a consequence of an increase in the total number of macrophages in the tumor, or is it the result of a change in the macrophage phenotype. In this regard, usually along with the markers of the functional state of macrophages, some general macrophage marker is used, for example, F4/80.

Response:

Thank you very much for your comments. In order to clarify the expression levels of M1 and M2 macrophages in human colorectal cancer and hyperlipidemic mice, we detected the expression of F4/80 (macrophage polarization marker) and iNOS (M1 macrophage polarization marker). We found that both the expression of F4/80 and CD206 were increased, then we used co-staining and compared the expression between CD206+ macrophages and iNOS+ macrophages. These results further confirmed the increased proportion of the CD206+ macrophages in macrophages in human colorectal cancerous tissues (Figure 1E, F, K, L) and hyperlipidemic mice (Figure 3A-D). Furthermore, we detected the levels of cytokines including IL-10, IL-4, IL-13 and TGF-β in THP-1 cells, while we compared the functional changes of THP-1 cells stimulated with ox-LDL. Our experiments about the function of CD206+ macrophages showed that after ox-LDL stimulation for 72h, the levels of CD206+ macrophage-related cytokines such as IL-4, IL-10 and TNF-β increased significantly in THP-1 cells but not IL-13 (Figure 4F). We found that the

increased CD206 is resulted from not only the total number of macrophages increased but also the phenotype of macrophages changed.

3. Comments:

M1/M2 nomenclature is convenient for describing the obtained data. However, the M1/M2 paradigm has now been revised (https://pubmed.ncbi.nlm.nih.gov/25035950/). It is believed that clearly distinguishable M1 and M2 macrophages are absent, and between them there is a continuous series of transitional forms. In this regard, the markers of the corresponding states of macrophages have also been revised. Surface markers, including CD 206, have been almost completely removed from the list of nomenclature. In this regard, I think it is necessary to abandon the term M2-macrophages in the article, since only the marker CD 206 is used, and macrophages are still called CD 206-positive macrophages.

Response:

Thanks for your nice advice. We are sorry for this mistake. In order to explain our conclusion more strictly, we used "CD206 positive macrophage" or "CD206+ macrophage" to replace total 51 of "M2 macrophage" in the manuscript.

Re-reviewer:

Comments: I consider that all the answers received are satisfactory; therefore, the revised manuscript can be accepted for publication in general priority.

Response:

Thanks for your comments.

Once again, we fully appreciate the insightful and constructive comment and questions from the Editors and Reviewers.