Author's response to reviews

Title: Atherosclerosis, inflammation and lipoprotein glomerulopathy in kidneys of apoE-/-/LDL-/- double knockout mice

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Version: 3  Date: 1 July 2010

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

This is a re-submission of our revised manuscript entitled

“Atherosclerosis, Inflammation and Lipoprotein Glomerulopathy in Kidneys of ApoE-/-/LDL-/- double knockout mice”.

The authors thank you and the reviewers for the comments and recommendations. We answered concerns on a point-by-point basis and have indicated all changes made in the manuscript.

We hope that the present version of the manuscript is now acceptable for publication in your journal.

Yours sincerely,

Alexander C. Langheinrich
Reviewer's report
Title: Atherosclerosis, inflammation and lipoprotein glomerulopathy in kidneys of apoE-/-/LDL-/- double knockout mice
Version: 2 Date: 10 June 2010
Reviewer: Georgette M Buga

Reviewer's report:
Reviewer's Report for the Revised Manuscript #4349194973581801
The authors answered reviewer’s comments or questions on a point-by-point basis. Many of the suggested changes have been applied or explained. Additional comments concerning some of the minor suggested revisions that have not been applied are included for the revised version of the manuscript (in blue bold font). These are incorporated below some of the author’s replies.

Title: Atherosclerosis, Inflammation and Lipoprotein Glomerulopathy in Kidneys of ApoE-/-/LDL-/- double knockout mice
Version: 1 Date: 5 April 2010
Reviewer: Georgette M Buga
Reviewer's report:
Ms #4349194973581801:
The authors propose to test the potential for quantitative imaging the renal vasculature as a marker of kidney function in a mouse model of advanced atherosclerosis using high resolution micro-CT and histology to correlate plaque formation and inflammation with alterations in kidney vasculature and morphology.
Major Compulsory Revisions are listed page by page:
1. Page 6:
Quantification of vasa vasorum in the two groups of mice would make a more compelling argument for the proposed hypothesis that histopathological changes occur in the experimental group. Please include in the resubmission.
AUTHORS: The authors agree that VV neovascularization of the renal artery is of great interest and might enhance our hypothesis. Indeed, VV neovascularization has been described in detail in different vascular beds in our mouse model of atherosclerosis (Langheinrich AC et al. ATVB 2006; Atherosclerosis 2007, Invest Radiol 2008). Our findings of VV neovascularization in the adventitia of the renal artery support our previous findings concerning neovascularization of the aorta and branching neck arteries. Moreover, VV were not present in the renal artery in C57/BL mice. The present study was not designed to quantitate VV neovascularization of the renal artery.
OK
2. To support the authors claims that inflammatory cells are present in the renal artery and in the kidney, data is required showing inflammation. It is not clear that specific cell markers were used to identify the two types of inflammatory cells mentioned in the manuscript? I strongly recommend using the methods described in the reference 12 (Ishimura et al., 2009).
AUTHORS: Inflammatory cells in the kidney were detected at light microscopy of hematoxylin and eosin stained slides. Lymphocytes and plasma cells could be clearly detected without any additional immunostaining. It was beyond the scope of the manuscript to evaluate the inflammatory cells in detail. OK
3. Page 7-First paragraph:
Although Atherosclerosis and Lipoprotein Glomerulopathy (part of the manuscript title) represent important factors contributing to the vascular modifications described in this manuscript, there is no experimental evidence that these mice have increased/altered serum lipoproteins. Total cholesterol, LDL-C, HDL-C (which is markedly decreased in apoE null mice and contributes to the pro-oxidant environment in these mice) and triglycerides, are important parameters that need to be presented in the manuscript. It would give a more clear indication of the mechanisms involved in the vascular modifications described.

AUTHORS: Cholesterol, triglycerides, LDL, HDL levels have been described in detail in the apoE-LDL double knockout mouse. Examples of such publications are:
Considering the title of this manuscript, including information and references regarding the altered serum lipoproteins in this mouse model of atherosclerosis would add support to the data presented.
We use the same commercial available mouse model as described by several publications, therefore, re-measurements of serum lipoproteins will not contribute to the mechanism(s) involved in the vascular modifications as described in our manuscript.

AUTHORS:
Genetic blocks in the catabolism of cholesterol-rich lipoproteins are important causes of hypercholesterolemia and atherosclerosis in humans. The prototypic diseases are familial hypercholesterolemia, caused by a defect in the low density lipoprotein receptor (LDLR) and familial type III hyperlipoproteinemia, caused by a defect in one of the ligands for this receptor, apolipoprotein E (apoE).
The apoE/LDL receptor– double knockout mice (a genetic model of hyperlipidemia), develop advanced atherosclerotic lesions with high similarity to human disease. Those murine models that lack the gene encoding apoE or a combined apoE and LDL receptor knockout develop spontaneous hypercholesterolemia/hyperlipoproteinemia (1).


This reference has been added to the reference list.

4. It is surprising that serum creatinine levels were not different from control mice as indicated at the end of the first paragraph on page 7 that refers to Table 1, although Table 1 does not have any entry on creatinine. Please include it or modify the text.
AUTHORS: The serum creatinine levels have been deleted in the revised version of the manuscript as suggested by the reviewer. OK
5. Also, it is highly possible that these mice are diabetic. Fasting blood glucose levels should be measured as well to be able to include or exclude Type 2 diabetes mellitus as an additional risk factor contributing to the vascular and renal pathological changes in the experimental mice.

AUTHORS: Our findings of reno-vascular alterations and glomerulopathy in this mouse model of atherosclerosis might be affected by additional risk factors such as diabetes. Up to now, there is no evidence that our mice are diabetic. Diabetes is a well known risk factor contributing to atherogenesis. The authors agree that there is a possibility that our mice are diabetic. Our well-established mouse model of atherosclerosis is based on lipoprotein disturbance. Additional investigations concerning metabolic disturbances are the focus of ongoing studies. OK

6. Discussion Section Page 7:
It is not clear how the lipoproteins were identified in the renal vasculature described as "factor 8 containing lipoprotein emboly", factor 8/vWF being an endothelial not a lipid marker? Oil Red O or Sudan III for lipids need to be used. I strongly recommend using the methods described in the reference 12 (Ishimura et al., 2009).

AUTHORS: Factor 8 related antigen is certainly an antigen present in/on endothelial cells. Beyond it is well known that immunostaining allows detection of Factor 8 in the cytoplasms of megakaryocytes and it allows the detection in platelets as well as Factor 8 protein within the blood plasma. Thus factor 8 immunopositivity of emboli within the renal vasculature was interpreted as a marker of plasma and thrombocyte aggregates. From the literature we drew the conclusion that the emboli will probably contain lipoproteins. Thus, we changed the term "factor 8 containing lipoprotein emboli" to "factor 8 containing emboli". There are still some left, please use the Find/Replace Microsoft Word feature to change all.

AUTHORS: Thank you, the text has been re-checked.

7. The second paragraph contains the references that were missing in the Background section on pages 4-5. Please either include them in that section or modify the information in Discussion, to have the references listed appropriately.

AUTHORS: Please specify the references that should be listed appropriately.

NOTE: The Reference List should contain the references in the order presented in the text, which is not the case in this revised version.

Page 3, Background section contains references 1 to 14. On page 4 the next reference is # 19 in the last paragraph. Therefore, references # 15-18 listed on page 11 in the Reference List are missing in the text on page 4. The references # 15-18 appear in text only on page 7, in Discussion section, paragraph 2. I suggest changing the order in the Reference List to reflect the information listed in the text. It appears that the authors spent very little time carefully reading the manuscript and making some of the suggested changes. Inconsistencies are still present throughout the revised manuscript and at times, although they have indicated that changes have been implemented, that is not apparent in the revised manuscript.

AUTHORS: Thank you, the references have been re-checked.

8. Figure Legends:
Fig. 2:
Usually magnification results from multiplying the Ocular magnification (10x) with the Objective magnification (5, 10, 20 or 40x). Please indicate what is the total
magnification (50x?) in each of the two images which appear to be of different magnification.
In addition, Fig 2 B exhibits the cortico-medular junction, where the glomerular size is usually increased, whereas Fig 2A represents the cortical area of the kidney. Please include similar areas of the kidney to be shown at the same magnification for both experimental groups.
Alternatively, sections from both cortical and medular areas could be compared between the groups.
AUTHORS: The total magnification in figure 2 has been implemented as suggested by the reviewer. We added additional images (Figure 5) showing the cortex and the cortico-medullar junction. OK

9. Please indicate the type of inflammatory cells shown, the staining method used and magnification.
Fig. 3B:
Fig 3 B appears to be of a different magnification than the others. Please select images from the same area and of the same magnification.
AUTHORS: Inflammatory cells in the kidney were detected at light microscopy of hematoxylin and eosin stained slides. Lymphocytes and plasma cells could be clearly detected without any additional immunostaining. We added the used staining and magnification in Figure 3 as suggested by the reviewer. OK

10. Figures 5-8:
Redundant. They duplicate the information presented in the Table 1. I suggest eliminating them.
AUTHORS: Figures 5-8 have been deleted in the revised version of the manuscript as suggested by the reviewer. OK

Minor Essential Revisions:
11. Page 4:
Please indicate the type of diet used and the age of controls.
AUTHORS: We used a standard chow diet and used age-matched controls. This has been implemented in the revised version of the manuscript (Methods, experimental design). OK

12. References 15-18 are missing and appear only on Page 7 in the 2nd paragraph of the Discussion Reference List and Methods section need to be coordinated.
AUTHORS: The references are now implemented in the revised version of the manuscript. No changes were made. Please see point #7 above.

13. Page 5, paragraph 2 in Histology Section
The Immuno Histo Chemistry portion is not clear. Please explain: (a) the reason for using factor VIII/vWF antibody which is a marker for endothelial cells and for not using any markers for the inflammatory cells present in the atherosclerotic plaques of the renal artery and in the periglomerular areas? (b) the reason that the link antibody and APAAP were used in the absence of a mouse monoclonal antibody or a dual staining. Were there any immune complexes that needed to be identified? Please elaborate and include in this section.
AUTHORS: a) Please refer to question # 2 & 7. b) the link antibody and APAAP method served as a negativ control for the specificity of the immunoreactivity of factor VIII/vWF staining. Dual staining was not performed.

14. Page 8, Paragraph 3:
Regarding the use of V V or vasa vasorum :It is also important to maintain consistency in the use of abbreviations throughout the text.

AUTHORS: Thank you. We changed the wording to consistently to „Vasa vasorum“ There are still some left, please use the Find/Replace Microsoft Word feature to change all.

**AUTHORS: Thank you, the text has been re-checked.**

15. Page 8 paragraph 4:
AUTHORS: The reference (12) has been implemented to clarify the relationship. **OK**

16. Page 8 paragraph 4:
Diabetes and advanced age should be considered as risk factors for LPG in these mice.
AUTHORS: Thank you. Diabetes and age has been implemented in the revised version of the manuscript (page 8, last para). **OK**

17. Are micro-CT features different in younger double ko mice versus the 80 day olds?
AUTHORS: The present study was not designed to investigate the time-course of renal alterations in this mouse model. We agree that this might be of great interest to characterize the renal alterations over the time. **OK**

18. Page 11:
References 15-18 are not in the same order as in text. Please revise.
AUTHORS: The reference list has been changed as suggested by the reviewer. No changes observed. Please see Questions #7 and 12 above.

Level of interest: An article of importance in its field
Quality of written English: Acceptable
Statistical review: No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests:
I declare that I have no competing interests.

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