Author's response to reviews

Title: Influence of NADPH oxidase on inflammatory response in primary intestinal epithelial cells in patients with ulcerative colitis

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Author's response to reviews: see over
Point-by-Point Response for MS 1536046019881024: „Influence of NADPH oxidase on inflammatory response in the primary intestinal epithelial cells from patients with ulcerative colitis“

Reviewer 1
Fabian Schnitzler

1. More information about the activity of the IBD would have been helpful: Endoscopic scoring, e.g. Mayo score and histologic scoring of the biopsies in the 19 patients with UC should be given in the paper.

The patients have been graded for mucosal inflammation according to the Mayo UC Endoscopic Score. All 19 patients had Mayo UC Endoscopic Score 1 to 2 points (mild and moderate activity of UC). Histologically, these patients had active chronic UC. This explanation has been included in the paper.

The statistical analysis did not reveal any significant differences in the TNF-α and ROS concentrations or cell viability between patients with mild and moderate activity of UC.

2. The authors mentioned that patients receiving immunosuppressive therapy or treated surgically were excluded from the study. Was there also steroid treatment included? This should clarify in the patients and methods section of the paper.

The patients included in the study did not use steroids for at least 3 months before the biopsies have been obtained. This explanation has been included in the paper.

3. Did the patients receive 5-ASA treatment? 5-ASA seems to reduce the oxidative DNA damage in animal models. This should be addressed in the paper.

Only five patients using 5-ASA preparations as maintenance therapy (≤ 1.5g/d) were included in the study. This explanation has been included in the paper.

The statistical analysis did not reveal any significant differences between 5-ASA treated and untreated cohorts.

4. Furthermore no information was provided with respect to iron supplementation. Therapeutic iron can increase iron-mediated oxidative stress in ulcerative colitis. This information would be helpful.

In this study, all patients with UC did not use any iron supplementation. This explanation has been included in the paper.

5. Stimulation of colonic epithelial cells with bacterial endotoxin LPS significantly increased the level of TNF-α and generation of ROS related to NADPH oxidase activity in the colonic epithelial cells of UC patients. NOD2/CARD15 seems to play a minor role in UC patients as seen in several genetic studies because of low incidence of NOD2/CARD15 mutations in UC patients. However, in this study information about the NOD2/CARD15 status of the patients would have been interesting, as mutations in the NOD2/CARD15 gene have an influence on the LPS induced immune response.
In our study we did not aim to analyse the influence of NOD2/CARD15 mutations on LPS induced inflammation signalling pathway. However, NOD2 genotypic information was available for eight patients. These subjects were part of the IBD Immunochip project conducted by International Inflammatory Bowel Disease Genetics Consortium (Jostins L, et al. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. Nature. 2012; 491(7422):119-24). Eight UC patients, who had available genotyping information, did not have risk alleles of for two NOD2 SNPs (rs2066845 - disease associated allele: C and rs2066847 - disease associated allele: insC). Two patients had heterozygous genotype TC of NOD2 rs2066844 (disease associated allele: T), however, due to a small sample size statistical analysis for this SNP comparing individuals with different genotypes was not feasible. Although genetic information was available for less than half of the UC patients, we think, that genetic alterations of NOD2 due to low incidence of risk alleles could have only a minor effect on the results of our study.

6. Also information about TLR4 mutations would have been interesting. Toll-like receptors (TLRs) are also linked to the recognition of pathogen-derived products, including LPS and therefore mutations in the TLR4 receptor might have an influence on the results of the presented study.

In our study we did not aim to analyse the influence of TLR4 mutations on LPS induced inflammation signalling pathway. However, TLR4 genotypic information was available for 8 patients. All of the patients had a common AA genotype of rs4986790 (disease associated allele: G) Genetic information was retrieved from the IBD Immunochip project (mentioned in comment no.5).

Reviewer 2
Priyanka P Trivedi

1. Few aspects of the present study are very flimsy.

We clarified the aspects included in the reviewers’ comments below (2 to 9 remarks).

2. Keywords: Instead of writing IBD-basic, the authors should write ulcerative colitis.

The note has been changed as proposed (“IBD-basic” has been corrected to “ulcerative colitis”).

3. Materials and methods: Patients: Line 9. The authors have mentioned that “The control group consisted of patients with irritable bowel disease or functional constipation.” Please clarify whether the control group consisted of healthy individuals or individuals with mild colitis.

Patients with irritable bowel syndrome or functional constipation were included in the study. They had a normal colonoscopy and un-inflamed mucosa on histopathological examination. This explanation has been included in the paper.

4. Statistical analysis: In case ANOVA showed significant differences, which post-hoc analysis was performed?

The post-hoc analysis has been performed using the least significant difference (LSD) test. This explanation has been included in the paper.
5. What is the significance of adding LPS to the colonic epithelial cells? In UC, already due to increased gut permeability, the gut bacteria invade the colonic mucosa; and Gram negative bacteria possess LPS. How do the authors differentiate the effects produced by gut bacterial LPS and the LPS added externally?

LPS is widely used for the studies of inflammation pathways. This endotoxin is an important pathological factor involved in etiopathogenesis of chronic inflammatory diseases such as UC. Interaction of LPS with colonic epithelial cells induced inflammation, activation of innate immune system and stimulated the synthesis of large spectrum of inflammatory mediators. Different studies have shown that LPS induced inflammation pathway associated with NADPH oxidase activity and ROS generation (Roda G et al.: Intestinal epithelial cells in inflammatory bowel diseases. World J Gastroenterol 2010, 16(34):4264-4271; Raetz CR, Whitfield C: Lipopolysaccharide endotoxins. Annu Rev Biochem 2002, 71:635-700; Powers KA et al.: Oxidative stress generated by hemorrhagic shock recruits Toll-like receptor 4 to the plasma membrane in macrophages. J Exp Med. 2006, 203 (8):1951-1961; Bochkov VN et al.: Protective role of phospholipid oxidation products in endotoxin-induced tissue damage. Nature 2002, 419(6902):77-81). Total concentrations of endogenous LPS in the gut is thought to be low, therefore, we used exogenous LPS as inflammation mediator, which should reinforce inflammatory response in the colonic epithelial cells. The design of our study did not allow us to differentiate between the effects of endogenous and exogenous LPS.

6. Instead of writing approx., the authors should write approximately.

The note has been changed as proposed (“approx.” has been corrected to “approximately”).

7. Why apocynin interferes with Amplex Red assay?

Apocynin can interfere with the detection of ROS in assay systems selective for hydrogen peroxide or hydroxyl radicals. A potential explanation could be that apocynin acts as a radical scavenger, which increases the ROS level in these detection systems. Thus ROS are not measured accurately and cannot reflect the effect of apocynin on the ROS level related to the NADPH oxidase activity. (Heumüller S et al.: Apocynin is not an inhibitor of vascular NADPH oxidases but an antioxidant. Hypertension 2008, 51(2):211-217). Therefore, we applied another inhibitor of NADPH oxidase – DPI - to test whether hydrogen peroxide was produced by NADPH oxidase. This explanation has been added in the paper.

8. How NADPH oxidase level can be measured?

In this study, level of NADPH oxidase was not measured directly. However, we can assess the levels of NADPH indirectly according to ROS measurement data. It is known, that ROS generation activity correlates with NADPH oxidase activity (Cariello M, et al.: Coagulation activation is associated with nicotinamide adenine dinucleotide phosphate oxidase-dependent reactive oxygen species generation in hemodialysis patients. Antioxid Redox Signal. 2012, 16(5):428-439).

9. Discussion section is largely descriptive.

The discussion section has been updated. The interpretation of our results in the light of the results from other studies has been incorporated according to the reviewer remarks.

10. Language is poor, there are several grammatical errors throughout the manuscript.

Language style and grammatical errors have been corrected throughout the paper.