Author’s response to reviews

Title: A redundant role for dectin-1 in experimental colitis models.

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

We would like to thank the reviewers for their constructive comments.

We would like to submit our revised manuscript entitled “Genetic deletion of dectin-1 does not affect the course of murine experimental colitis.” for publication in BMC gastroenterology.

Below we give a point-by-point response to the concerns made by the reviewers.

Changes in the text are marked in an extra PDF of the current resubmission.

Looking forward to hearing from you.

Yours sincerely,

Sigrid Heinsbroek

Reviewer's report 1

1: In figure 1A and 1B the expression of dectin-1 in colon of normal WT and DSS colitic mice is shown. The pictures are of very bad quality and there is a lot of tissue damage after the cryosection that make it impossible to see what kind of cells is expressing the dectin-1. Double staining on this tissue to answer whether we are looking at macrophages, neutrophils or other cell is needed to support the use of macrophages in the in vitro experiments.

Au response

*In the current submission, new fluorescent double staining immunohistochemistry is included as figure 1B showing that the large majority of dectin-1 expressing cells also express CD11b suggesting that dectin-1 is expressed by macrophages, dendritic cells and neutrophils in the intestine. We also included isotype control staining.*

2: In figure 1C and D the cytokine production by macrophages isolated from wt and dectin-1 deficient mice is shown. From the material and methods section I
understand that the isolated macrophages were first cultured ON and then plated out and stimulated. This process could greatly affect the expression of various PRR and thereby affect their response to the various ligands. Since the hypothesis for the mouse colitis experiments seems based on these observations the changes in PRR expression between the freshly isolated macrophages and those stimulated in vitro should be presented. Also the effect on cell death by the various compounds, especially that of the cecum preparation should be reported.

**Au response**

This was a mistake in the text. Cells were isolated and plated immediately in the 24 well plates. We allowed the macrophages to adhere for 2 hours and then stimulated the cells with ligands. So the cells were freshly isolated for the experiments. No death seemed to occur after 24 hour with the ligands and cecum preparations (microscopically we did not observe unusual numbers of floating cells).

We corrected the text accordingly.

**Text was adjusted as follows.**

Thioglycollate-elicited peritoneal Mf were isolated 4 days after intraperitoneal injection of 1 ml 4% (w/v) Brewer’s thioglycollate medium (BD). 2.5x10^5 primary Mf were plated in 24 well tissue culture plates with RPMI (Invitrogen) containing 50 IU/ml Penicillin, 50 ul/ml streptomycin and 2mM L-glutamine at 37ºC in 5% CO_2_. After 2 hours, non-adherent cells were removed by washing three times with medium. Diluted cecum content, LPS or zymosan (Molecular Probes) were added and incubated with the cells for 24 hours. Diluted cecum content was produced by suspending the contents of a mouse cecum in 50 ml PBS which was subsequently filtered over a 40um filter and frozen at -20ºC and used in a 1:1000 dilution. After 24 hours incubation no growth of micro-organisms was found in the wells and no cell death was observed by microscopic check for floating cells. Cytokine levels were determined using ELISA kits (R&D) according to manufacturer’s protocol.

3: In comparison to zymosan or the LPS stimulation the macrophages make very little TNF-alpha in response to the cecum preparation. An explanation should be provided.

**Au response**
We do not know exactly why this difference occurred. However, the IL-10 and TNFα responses depend on the combination of pattern recognition receptors that are triggered. It has been shown that different bacteria are able to induce completely different cytokine profiles and can even work against each other (Lammers et al. FEMS Immunology & Medical Microbiology (2003) vol. 38 no.2; 165–172; Christensen et al. The Journal of Immunology (2002) vol. 168 no. 1;171-178)

Faeces contains a whole array of various stimuli and our mouse faeces seems to contain components that can induce a high IL-10 response but does not stimulate TNF-α production that much. The difference in magnitude of IL-10 and TNFα induced by faeces compared to zymosan is very likely due to different cell activation by the components, however a role for dectin-1 in the responses is clear in both stimuli.

4: Based on the in vitro observation the authors hypothesize that the dectin-1 deficient mice will have a less severe colitis. Their data showed a slight decrease in IL-10 and a stronger effect on TNF-alpha. Based on this observation it might be even more likely that the mice will have a milder colitis because there will be relatively more IL-10 then TNF-alpha. This alternative hypothesis should be addressed.

**Au response**

We do not think the in vitro data can be directly projected to the in vivo situation in that context. IL-10 may not always be that anti-inflammatory, but irrespective thereof, we interpret that the in vitro data merely point to a more selective reduction of TNF if compared to other cytokines such as IL-10. However, we now note the effect in IL-10 production in the current text as well.

5: The role of mannan binding lectin (MBL) has previously been studied in DSS colitis. Mice deficient for MBL did not show any difference with regard to DSS colitis, but had enhanced disease severity when DSS was combined with C. albicans infection in MBL-deficient mice. Does the fungal part of microbiota present in mice represent those in humans? Is the contribution of dectin-1 simply not picked up because the right fungus is not present in the current models? How does infection with C. albicans influence the DSS colitis in dectin-1 deficient
mice?

**Au response**

As we describe in the current manuscript, indeed, the fungal part of the microbiota in mice is certainly different from that of humans. However, between humans there are also big differences in fungal microbiota. Candida albicans is a good example, this fungus does not naturally colonise mice but can be present in humans. But not all humans have candida as part of their intestinal microbiota.

*C. albicans does not naturally infect mice and C. albicans infection of dectin-1 deficient mice leads to obstruction of the gastrointestinal tract and subsequently death of these mice (see figure below). Since this strong phenotype already exists before even inducing DSS inflammation we don't think inducing DSS colitis will be more informative.*

6: Is there a difference between cecum preparations from WT vs dectin-1 deficient mice and control vs DSS colitis?

*There is no difference in response towards WT or KO cecum preparations (see figure below). We did not save cecal content from colitic mice and are unable to do this experiment unless we induce colitis just for the collection of the cecal content. We expect that since there was no difference at the start of colitis seven days later after DSS feeding there will not be much of a difference either especially since there is no difference in inflammation between the two strains.*

*In the text we wrote the following:*

*The results shown are the response towards WT faeces, the same results were found when using dectin-1/- faeces which suggests there are no differences in the intestinal microbiota between these mice.*

7: The authors discuss that other PRR may compensate for the loss of dectin-1.

This is in line with the idea that there are other PRR involved in the recognition and response to fungi and food components. Does anti-fungal treatment affect DSS colitis?

**Au response**
This is a nice suggestion but we feel this experiment will no further strengthen our hypothesis. As published by Muller et al (Gut 2010), infection of WT mice with C albicans does not affect the course of DSS colitis. We do not anticipate that anti-fungal treatment will affect the outcome in our models used nor will it change the conclusion that Dectin-1 expression is redundant for the course of colitis in the two models used.

8: The rationale for using the H. hepaticus model is not clear. Especially since these mice are treated with anti-IL-10 as cytokine that seems reduced in dectin-1 deficient macrophages exposed to faeces.

Au response

In the H. hepaticus model mice are treated with antibodies against the receptor for IL-10 not IL-10 itself. The antibody does not influence IL-10 levels. To clarify the choice, we added this model to the study for two reasons:

1. we aimed to show the role of dectin-1 in experimental colitis in more than one model of experimental colitis, and the H.hepaticus model is an microbial driven colitis model,
2. because dectin-1 has been suggested to interact with the adaptive immune system and this model depends on both the innate and adaptive immune system.

Minor essential revisions:

1: In their discussion the authors state that although PPR are important in human IBD, their data suggest that dectin-1 is redundant in intestinal inflammation. The last part should be extended with…..experimental colonic inflammation induced by either DSS or H. hepaticus in mice. Because based on this study we cannot draw conclusions for the human situation.

Au response

This was changed in the text.
2: The role of dectin-1 in TLR-2/6 signaling should be discussed

**Au response**

*We adjusted the following sentence in the discussion*

*Various PRR have been shown to play an important role in human IBD and dectin-1 has been shown to co-signal with TLR2 and TLR6 for the production of various pro-inflammatory cytokines [19,20,22]. Clearly, although our data suggest dectin-1 signalling is redundant in intestinal inflammation TLR 2 and/or TLR6 deficiency does affect experimental colitis via several mechanisms indicating that dectin-1 deficiency does not seem to affect TLR signalling. This was also indicated by our observation of normal responses to TLR ligands other than dectin-1 in deficient cells.*

3: The current study only addressed the role of dectin-1 in the innate component of intestinal inflammation. We cannot draw any conclusions on its potential effect on the adaptive immune system. Since this latter plays a very important role in established IBD, this should be discussed.

**Au response**

*We do not fully agree; mice were complete deficient in T and B cell compartments. Moreover, the reason for inducing colitis with H. hepaticus infection, next to the DSS induced colitis, was to study the effect in a model that is more dependent on the adaptive immune system.*

Discretionary revisions

1: Some data on ROS production by the macrophages in vitro would be nice.

**Au response**

*Such data would indeed be informative. However, we do not have these assays running in our lab and given the outcome of these studies it does not seem opportune to extend the studies with these assays.*

**Reviewer's report 2**

**Title and abstract:**

The term “redundant role” implies that there is some effect that is possibly compensated for. At the moment, however, this is speculative. An alternative title that describes the main findings might be: “Genetic deletion of dectin-1 does not
affect the course of murine experimental colitis”

Au response

*We agree and have adapted the title.*

Results:

Controls for immunohistochemistry, such as isotype-matched antibody, and 
dectin-1 +/- should be shown and mentioned in legend.

Au response

*We included the isotype-matched control in figure 1A.*

The TNF response by isolated macrophages to fecal extracts is much lower than 
then IL-10 response, and this difference is not seen with zymozan. This merits 
some discussion.

Au response

*See point 3 reviewer 1.*

In figure 3, the levels of IL-10 are much lower in dectin1 +/- than wild-type, 
although not statistically significant. In two instances, the levels are zero. This 
seems like an experiment to repeat to see if indeed there is some biological 
effect.

Au response

*The numbers of mice chosen for this experiments should reach enough power to disclose significant changes along the parameters chosen. Given the in vitro data indeed dectin deficiency could lead to lower IL-10, but this did not reach significance. There is one mouse in the WT group that produced more IL-10, but even with this outlier there is no difference between WT and KO groups in this, or the other parameters, rendering it of little use to repeat the experiment based on the outlier.*

Discussion:
The antigens that elicit ASCA reactivity are not definitively known, although Candida albicans is suspected/assumed, see, for example: Gastroenterology. 2006 May;130(6):1764-75. Candida albicans is an immunogen for anti-Saccharomyces cerevisiae antibody markers of Crohn's disease, and Am J Gastroenterol. 2009 Jul;104(7):1745-53. Candida albicans colonization and ASCA in familial Crohn's disease.

**Au response**

*Text has been adjusted as following:*

_Crohn's disease patients have been found to produce antibodies against fungal glycocarbohydrates including b-glucans and mannans [16,17]. C. albicans is a suspected immunogen for these antibodies [38,39] and as a major receptor for C. albicans [28,40], dectin-1 is likely to be important in immuneresponses involving patients with an intestinal C. albicans infection._

The summary statement “we showed that dectin-1 is able to induce a cytokine response towards mouse faeces” is based on indirect evidence, i.e. that the genetic absence results in change in the response of macrophages in vitro, rather than a formal demonstration, for instance by blocking dectin-1 signalling, and probably should be modified. For the rest, the conclusion that this does not substantially affect at least two models of intestinal inflammation seems fair, and is an important contribution to the literature on the subject.

**Au response**

*The concluding remark was changed into:*

_Our in-vitro data suggest that dectin-1 is able to induce a cytokine response towards mouse faeces.*