Author's response to reviews

Title: Long-term culture of cholangiocytes from liver fibro-granulomatous lesions

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Author's response to reviews: see over
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Dear Sir or Madam,

We have the pleasure to submit a revised version of our manuscript entitled “Long-term culture of cholangiocytes from liver fibro-granulomatous lesions”. Please find below a point-by-point explanation of how we addressed the reviewer’s comments. We would like to thank the reviewer’s comments that resulted in a much improved manuscript. We are confident that we have satisfactorily addressed all the reviewer’s concerns and the paper is now suitable for publication. We have added Dr. Christina M. Takiya as an author to reflect her contribution during the revision process. Please do not hesitate to contact me if you need any further clarification.

Sincerely,

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RESPONSE TO REVIEWERS

Reviewer 1

We should see a photomicrograph of the typical lesions that develop at the time points mentioned.

Response: We have included 6 images of the typical lesions stained with hematoxylin-eosin or Masson’s trichrome. They are now shown as Fig. 1A-F.

We need to see controls for the immunofluorescent immunohistochemistry.

Response: We performed two negative controls as follows: a) No primary antibody was used. Incubation was done with 0.1% BSA in PBS for 1h at 37°C. b) An irrelevant primary antibody was used (mouse α-rat IgG) also incubated in 0.1% BSA in PBS for 1h at 37°C. Both presented with similar negative results. A picture of cells incubated with the irrelevant antibody is now included in Figure 5. Please note that the photograph was overexposed (which can be seen by the grey, instead of black, background) to show that there were no nonspecific reactivity. These
antibodies were extensively characterized. We added this information to the Material and Methods section.

Have monoclonal cell cultures been produced (from a single cell)?

Response: Establishment of clonal cultures was not attempted.

Reviewer 2

The author should improve the description of the optical microscopical methods.

Response: We added more details about the stains that were used in the Methods section.

Did the authors use a method to block unspecific binding of the antibodies in the immunofluorescence stainings? And a method to permeabilize the cells (for protein expressed intracellularly)?

Response: Blocking was done by incubating cell for 15 min in 0.1% BSA in PBS. In addition, the incubation with the primary and secondary antibodies was also done in the presence of 0.1% BSA in PBS. Cells were fixed with Methanol which allows for permeabilization. We did not use any additional permeabilization reagent.

In the immunofluorescence methods the authors should indicate the description of primary antibody and secondary antibodies (species reactivity sources and if possible, company that provided the primary antibody), the dilution used and the time of incubation with secondary antibodies.

Response: This is now described in the Methods section.

Figure 3A: the H&E staining should be changed with a better section (the nuclei are not well displayed) or/and a section with Masson’s trichrome stain.

Response: We have attempted several sections and stains but have noted that sections from collagen gels always present a problem with definition, which we judge to be due to shrinking and expansion during fixing and staining as well as limited accessibility of the staining solution in the gel matrix. In fact, this was what led us to perform impregnation for electron microscopy under vacuum.

In the discussion the authors do not explain why they have not been able to isolate cholangiocytes from dissociated chronic phase granulomas or dissociated liver fragments. This aspect should be better elucidated in the discussion.

Response: Although we do not know the reason why we have not been able to isolate cholangiocytes from dissociated chronic phase granulomas or dissociated liver fragments we can speculate. Judging by observations made of granulomas in these different phases we and others have noted that in the acute phase there is intense ductal proliferation with small cells. In the chronic phase ducts with mucinous generation (hypertrophic cholangiocytes with pathological aspects filled with PAS-positive substance). We suspect that cells derived from acute phase granulomas are more viable and proliferative than the ones derived from chronic phase. Regarding the inability to derive cells from liver fragments we believe it may be due to
intense connective tissue proliferation that may interfere with epithelial proliferation. This is now included in the Discussion section.

In the discussion some sentences are not supported by references

Response: References have been added.

The cell line should be tested for epithelial markers (CK-19 at least) at least after 16 passages and at one year and eight months of culture.

Response: We thawed cells that had been frozen at 40 passages but they displayed very low viability and we could not perform the staining for the markers. We are now thawing cells that were frozen at 19 passages but they will take a considerable amount of time to reach the time point suggested by the reviewer. We modified the claim in our text to state that cells did retain differentiated characteristic and high proliferative capacity at 9 months (16 passages).

Legend figure 3, line 13: microvilli instead of villi

Response: This was corrected.

Legend figure 5, line 2: the label B is missing
Response: This was corrected.

Figure 1. Chronic phase is described as 45 days after infection instead of 90 days.

Response: This was corrected.