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Reply to the four Reviewers' comments for submission 21166 "The Interaction between Castanospermine an Oligosaccharide Processing Inhibitor and Cyclosporin A in Rat Cardiac Transplantation "

Reviewer 504335

Comment 1. The survival curves for individual groups should be shown – not only to show the number of surviving animals at 100 days after grafting.

We have added Figure 1 showing the transplant survival for groups with CsA, castanospermine or the combination of the two drugs. To highlight the synergism from the combination of drugs we have thickened these curves in contrast to the survival for single drugs alone. Please note that the number of transplants surviving to 100 days is already contained in brackets in Table 1a.

Comment2. In Materials and Methods the authors correctly wrote that MLR was determined according to the 3H-thymidine incorporation and that data are expressed as cpm. However, the results from MLR (Fig. 1 and Fig. 2) are expressed as Lymphocyte counts (Mean number of lymphocytes).

Thank you for pointing these errors: we have changed the y axes of these two figures (now figures 2 and 3).

Comment3. Immunohistochemistry shows only isograft, rejected allograft and allograft treated with Castanospermine. To show synergism between Castanospermine and CsA, there must be included a sample of allograft treated with CsA and sample of allograft treated with CsA and Castanospermine.

The purpose of this Figure (originally 3 and now 4) is to suggest a mechanism of action of castanospermine, namely, to impair the transit of alloreactive cells through the basement membrane of the endothelium. Hence the figure shows marked clustering of cells near the graft venules when treated with castanospermine in contrast to the diffuse infiltration of cells when the allograft is untreated. The purpose is not to show synergism which depends totally upon statistical analyses that are displayed in Tables 1 and 2. Hence we have not changed the illustration but as requested have provided a lot more explanation of the figure in the legend. We thank the reviewer for ensuring that the purpose of this

illustration is clarified.

Comment4. Expression Hazard Ratio should be explained.

This has been explained in the section on statistical analyses by our statistician. Again the Reviewer's comments have strengthened our paper.

Comment5. Figure legends are not sufficiently describing the figures.

These legends have been expanded to improve the meaning of the illustrations. We believe the illustrations are now better integrated into the text and their meaning is improved. We thank the reviewer for pointing out the need to change.

Comment6. Abbreviation CI is in the text as IC.

The use of IC in the text (experimental design/ mixed lymphocyte reaction) is an abbreviation of "inhibitory concentration" not CI which is an abbreviation for "confidence interval". We have added IC to this list of abbreviations to clarify. We thank the reviewer for pointing out the need for clarification.

Comment 7 References must be checked and corrected by the authors. For example reference 19 – full title of the journal, other journals are in abbreviated form, etc.

These have been checked using PMID or doi

Reviewer 00225232

Comment1. How do you explain the synergistic interaction between CAST and CsA specifically at the dose of 2mg/kg?

We favour the following explanation for the synergistic interaction at this CsA dose: Castanospermine acts at a site in the allograft response that differs completely from that of CsA i.e. it does not act to change either the secretion of Il-2 or its gene regulator (CsA predominant action). But it does act to impair the transit of alloreactive cells through the basement membrane of the vascular endothelium into the graft by impairing heparanase production in intragraft alloreactive cells. We have unpublished evidence to support this view. Heparanase present in T cells acts to break down heparan sulphate that abounds in the BM and so the cells can transit through. Impairment of heparanase then leads to clustering of cells about the endothelium.

Comment2. The data showed that CAST reduces the CsA blood level in one of the 3 doses studied. What are your hypotheses concerning the two other tested doses? This should be discussed.

Table 3 b shows that if CAST was stopped the blood level of CsA significantly increased for the Group receiving CsA at 2mg/kg but not significantly for the Groups receiving CsA at 4mg/ kg or 6mg/ kg. The implication is that CAST reduced the level of CsA when using 2mk/ kg.

Our hypothesis for these findings is that CAST may impair the mechanism used for the absorption of CsA in the small bowel known to depend upon a glycoprotein transporter. This mechanism may be competitively inhibited at CsA 2mg/ kg but at higher CsA doses the inhibition is less effective.

We have revised the discussion to this effect.

We thank the reviewer for this comment.

Comment 3 Tables 1a and 1b: there are errors on CsA dose.

We thank the reviewer for pointing out our error in these two tables. We have corrected them.

Comment 4 Figure 3: Please clarify the pictures. Clearly indicate which is a, b, or c.

This has been done.

Comment 5 Please add colors to enhance your tables and your figure.

The tables have been coloured up as requested.

Reviewer 00503243

Comment1. The study on interactions between Castanospermine and Cyclosporin A is interesting, new and well written in the section concerning the basic science study. In my opinion the study weaknesses arise in the section of the study aimed to transfer the data to the clinic. Indeed, it should be rewritten in the sections concerning this point. My main concerns are: a) the study is limited to rats; b) the study is limited to cardiac allograft;

The aim of our study was to find a way of reducing the dose of cyclosporin A and thus its nephrotoxicity. We maintain that this theme should remain in our manuscript i.e. our work is finally directed towards a clinical outcome. However we admit that there are many unanswered steps before castanospermine can be considered for the clinic. Hence we have altered the abstract, the core tip and the discussion where we make this simple point.

We thank the reviewer for this comment and believe that the manuscript by including a realistic view about the pathway needed to get castanospermine or its derivative into the clinic.

Comment 2 c) nothing is said about antibody mediated rejection. Do we have any data or slides to answer this question. I just remember the authors that years ago an important study on reducing CsA dose by FTY720 (fingolimod) failed because an excess of ABMR. While CAST inhibits the leukocyte adhesion to the endothelial cells, FTY inhibited Lymphocyte exit from Lymphonodes. Unfortunately antibodies were not blocked and caused severe rejections. In addition, looking at the CAST action on ER, please add note of caution linked to CAST action

We have not studied antibody mediated rejection in this work as our first step was to

establish synergism. Further study is needed to answer the reviewer's question which is important but needs further work to answer it.

Comment 3 Please, dear authors revise your manuscript and avoid any clinical implication.

We have answered this point under comment 1 above.

Reviewer 504150

Comment1. There are a few major questions that the authors should elaborate on to strengthen the manuscript as well as several minor points that should be addressed. 1. I did read the some of the papers listed above (I do not have access to some old papers). The authors should explain detail and stress what is new finding in this study comparing to the studies.

- A. Our data on the mixed lymphocyte reaction for castanospermine, for cyclosporin A and for their interaction is new.*
- B. Our data on the effect of castanospermine on the blood level of cyclosporin A is new; this includes the addition of castanospermine and its removal to baseline cyclosporin A usage at three doses.*
- C. Our data on the synergism of castanospermine and cyclosporin adds significantly to the study published in the Polish Journal Immunology in 1995 which is preliminary only. This published study is really an extended abstract and not a full paper, in our view. We have extended the published data in the following ways:*
 - 1. We have 7 combinations of castanospermine with cyclosporin A compared with 2 in the published study (see table 1a of our submitted paper)*
 - 2. We have used a total 36 rats in the 7 groups treated with castanospermine combined with cyclosporin A compared with 18 rats in the 2 groups published (see table 1a of our submitted paper)*
 - 3. We have 5 different doses of castanospermine compared with 4 in the published study*
 - 4. We have a total of 33 rats in the 5 groups treated with castanospermine compared with 25 in the published study*
 - 5. We have 29 rats in the 4 groups treated with cyclosporin A compared with 27 in the 4 groups in the published study*
 - 6. We have entered the data on the survival of the syngeneic control in our submission but it is not in the published paper (Table 1a). This control reinforces the inbred genetic status of this rat strain and confirms the reproducibility of our technique of microvascular heterotopic cardiac transplantation in rats.*

Our view is that the published study has insufficient statistical power both in group number and size to adequately establish the synergistic interaction between castanospermine and cyclosporin A. Hence a thorough, statistically adequate study of the interaction of castanospermine and cyclosporin A has not been published, in our view. Further it needs to be published because it implies that there may be a further target for

immunosuppression in transplant recipients separate from the T cell (the target for Cyclosporin A). Hence it may have potential benefit for transplant recipients. We believe that our study presented here is definitive in that respect for a rat model but there are many other "harder" assessments that are necessary before use in the clinic.

We are also concerned that this study was published in a Journal that published for 1 year only and could not therefore be considered to have substantial scientific impact.

We also note that the other data listed in our submission reinforces the basic point that the synergistic interaction between castanospermine and cyclosporin A is not due to its effect on the T cell.

Further I was not given the opportunity to review this manuscript before submission and publication in the Polish Journal of Immunology nor did I sign off on it as adequate before its submission.

D. Finally we have not previously published the hypothesis that castanospermine may act by impairing passage of alloreactive cells through the vascular basement membrane.

In our paper the new findings are summarized in the first paragraph of the discussion and in the conclusions.

Comment2. The authors should declare that there are no duplications in data between the submitted study and the previous published study, including control syngeneic transplant data.

As listed in comment 1 above, there is duplication of data in table 1 but extensive new data has been added enabling a satisfactory power to the study. Therefore the validity of the conclusion is strengthened.

The control syngeneic transplant data is new.

Comment 3 For Immunohistological study, more information is required. The authors did use isograft model without mentioning which combination (PVG to PVG or DA to DA).

It is DA in to DA; the legend to Figure 4 has been updated.

On which day after transplantation the graft removal was performed?

It was day 5; the legend to Figure 4 has been updated.

How many transplants were done for histological analysis purpose in each group?

In each group 3 grafts were analysed for histology; the legend to Figure 4 has been updated.

In Figure 3c, what treatment was done (castanospermine only?) and which dose?

The rats were all treated with castanospermine only at 200mg/ kg; the legend to Figure 4 has been updated.

Comment 4 Minor points 1. There is no need for capital “C” for castanospermine.

Once you start use abbreviation (CAST) in the text, please continue to use the abbreviation thereafter throughout the text.

The text, tables and figures have been changed using this instruction

Comment5. In Table 1a, data for combination of 50 mg/kg castanospermine and 3.0 mg/kg cyclosporine is missing.

This error has been corrected (see comment 3 above under reviewer 225232).

Comment6. Similarly, data for combination of 50 mg/kg castanospermine and 0.5 mg/kg cyclosporine is missing in Table 1b.

This error has been corrected (see comment 3 above under reviewer 225232).

Adrian Hibberd 3

November 2015