Author's response to reviews

Title: Lymph node removal enhances corneal graft survival in mice at high risk of rejection

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PDF covering letter
Dear Editor,

Thank you very much for your reply. Please, see the replies to the reviewers' comments on the separate page.

Yours sincerely

Jarka Plskova
Reviewer: Aize Kijlstra

1) Mention the numbers of mice used in the experiments in the materials and methods section.

The numbers of mice are now included in the Methods section.

2) Details should be provided concerning the number of spleen cells used for adoptive transfer. No positive controls were included. Is the amount of cells transferred sufficient to generate a response in other mouse models? Please discuss this issue in the discussion section.

Number of spleen cells used for adoptive transfer is now included in the Materials and Methods section. The amount of cells transferred is sufficient to generate a response in other mouse models as now discussed in the Discussion section.

3) Statistical analysis was not performed on the data as represented in the figures but on the day when rejection started. Please include these data in a table.

We respectfully disagree with the referee on this point. The figures presented represent the data for statistical analysis because between the experimental groups the days of onset of rejection were compared and all these data are in the graph. However, as suggested by the reviewer, we have included a table with medians of the rejection-onset times, which Mann-Whitney U test uses for statistical comparison.

4) The paper is quite mouse oriented. Please include a paragraph indicating how the acquired knowledge from the mouse corneal transplantation data acquired in this study could be implemented in clinical practice.

The suggested paragraph was now included in the Discussion section.
**Reviewer: Andrew JT George**

Experimental methodology.

The authors used adoptive transfer experiments to determine the role of regulatory cells. Failing to see any increase in graft survival following transfer of T cells led them to conclude that there was no significant role for regulation. However, the splenocytes were obtained from animals whose grafts had survived 40 days. In untreated animals graft survival was between ~12 and ~50 days (figure 1, grey line). Therefore the spleen cell donors may been slow rejectors, rather than non rejectors. The authors may want to argue that they know that lymph node removal results in prolonged graft survival in all animals, but it would be good to see data or discussion of this point.

**This point is now discussed in the Discussion section.**

In addition there is no ‘positive’ control for this experiment. How do the authors know that their transfer protocol is effective at transferring regulation. It would be necessary to show that it worked in another system.

**The transfer protocol regarding other systems is now discussed in the Discussion section.**

An alternative way to address this question would be to remove the regulatory cells and see the effect on graft survival. Some of the early data indicating the presence of regulatory cells has come from autoimmune models (for example by D Mason) in which depletion of cells results in disease.

**We consider the suggested experiments beyond the scope of this study but we agree with the referee that these experiments would be very interesting for the future work.**

An alternative way to address the problem would be to see if the animals were tolerant. This could be done by performing a corneal graft in the contralateral eye in animals showing long term graft survival (assuming that lymph node removal only affects graft rejection on the appropriate side). Alternatively skin grafts might be performed, though I accept that would be a ‘tough’ challenge!

**We agree with the reviewer on the refinements of his experiments for demonstrating tolerance in mice with clear corneal grafts after LN removal. Unfortunately the regulations in UK in performing corneal grafts on the fellow eye are very strict and permission is very difficult to obtain without strong justification. We further agree with**
the referee that performing a skin graft instead of a second corneal graft introduces probably insurmountable complications to the immunology.

If the authors wish to argue that skin grafts are inappropriate due to tissue specific antigens then they might consider doing a heterotopic corneal transplant into the skin.

**Further, we are uncomfortable with the use of heterotopic corneal grafts, since the absence of immune privilege weakens this experiment as a control.**

In general the authors do not perform a mock surgery control in their experiments. Have they previously shown that this is not necessary?

**A comment on this point is now included in the Discussion section.**

The experiments performed to look at presensitization by prior grafting are not totally convincing. The abstract says that removal of the lymph node delayed graft rejection by around a week. However, the rejection times are (as estimated roughly from the graph)

Treated group 10 17 20 20 >60 >60 Control group 10 10 12 33 >60

I do not find this convincing – and the statistics do not indicate significance. However, the authors repeatedly indicate (abstract, results and discussion) that lymph node removal prolonges graft survival.

**We have now included a table, which indicates prolongation of graft survival in mice after LN removal and we have indicated in the abstract that this does not reach statistical significance.**

It might be useful to look at minor antigen (eg H-Y) mismatches at the skin graft and so see if the challenge is weaker whether lymph node removal could protect from corneal graft rejection.

**We agree entirely with these comments, but think that these experiments are beyond the scope of this manuscript.**

Comments on the manuscript

The authors need to be clear about whether they have seen tolerance or not. In general (for example abstract, start of the discussion) they use tolerance when all that has been demonstrated is prolonged (or even indefinite) graft survival. I would argue that this is an inappropriate usage of the word, as it encompasses immunological ignorance and
immunosuppression. In general tolerance should be reserved for those settings when it is shown that subsequent challenge with antigen via a route that will normally induce an immune response fails to induce an immune response. Formally one should also show that responses to third party antigens are preserved.

The relevant parts of Abstract, Introduction and Discussion have now been altered accordingly.

The spelling of whether (weather) is incorrect throughout.

We apologize profusely for the English errors which crept into the text. We hope we have managed to deal with them.

On page 4 2.5% rather than 2.5%. Page 6 should be ‘prior TO the test graft’. On Page 11 John Forrester conceived the study rather than conceived on the study (unless his laboratory has a more exciting social life than mine!!).

The errors have been corrected.

Major compulsory revisions.

Need to change use of tolerance throughout. Need to consider the comments on the regulatory cell experiments. Need to consider comments on the presensitisation with prior corneal graft. These comments were dealt with in the above section.