Reviewer's report

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

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Reviewer: Andrew JT George

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In this paper the authors describe a series of experiments which address the role of the draining lymph node in corneal allograft rejection. In addition they present data concerning the role of regulatory cells in preventing rejection.

This is an important topic because, contrary to conventional wisdom, corneal allografts do undergo rejection. Indeed, the 5 year graft survival data for these graft is now broadly similar to other solid organ transplants (as survival has improved for these other organs). Given that corneas are the most commonly transplanted organ (~40 000 a year in the USA), then a fuller understanding of rejection, and the development of strategies to overcome rejection, is of importance. In addition, there are a subset of patients who are at high risk of rejection. This includes patients with ongoing inflammation, paediatric recipients and those that have rejected previous grafts. This group of patients have a very poor prognosis, and so are good candidates for the development and testing of new treatments.

The work in this paper builds on previous work from this group looking at the importance of lymph nodes in corneal allograft survival. They have previously shown that priming of the alloresponse following corneal grafting is dependent on the submandibular lymph node. However, the mechanisms responsible for the prevention of graft rejection following lymph node removal are not known. One possibility raised by the authors is that removal of the lymph node results in a failure to prime the immune system against the graft, resulting in a state of ignorance. The second is that removal of the lymph node tilts the balance between the induction of regulatory and effector T cells, resulting in overall tolerance.

In order to address this question the authors carry out adoptive transfer of spleen cells from mice which show prolonged graft survival into naïve recipients, which then received a corneal graft. If the prevention of rejection is due to regulatory cells then one might expect to see prolonged graft survival in these animals. They did not see this, suggesting that regulatory cells may not be important.

They also looked at whether lymph node removal had any benefit in ‘high risk’ recipients. I would suggest that this is an important experiment as it paves the way for a similar manipulation in high risk patients receiving grafts (though one may choose to ablate the lymph nodes by local irradiation rather than surgery). Given the poor prognosis then I believe that, if backed up by experimental data, such interventions might be ethically justifiable. The authors generated a high risk recipient bed by inducing corneal vascularisation with a stitch, or by using animals that had previously rejected a graft. In these animals lymph node removal was of benefit and resulted in prolonged graft survival.

Further experiments were carried out in animals that had been sensitised by a previous skin graft. As might be expected lymph node removal had no effect in the face of such a strong immunisation.

As indicated above, these experiments address an important series of questions.
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 have 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.

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.

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.

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! 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.

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

The experiments performed to look at presensitisation 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.

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.

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 spelling of whether (weather) is incorrect throughout. 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!!).

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.

Minor compulsory revisions
Spelling and grammar as indicated.

Discretionary revisions
Consider other comments above.

What next?: Accept after minor essential revisions

Level of interest: An article of importance in its field

Quality of written English: Acceptable

Statistical review: No

Declaration of competing interests:
none