Summary

Using a murine MHC-mismatched renal transplant model, the authors demonstrated that exogenous activation of Tie2 with vasculotide (VT) was graft-protective as reflected in significantly diminished expression of peritubular and glomerular endothelial adhesion molecules, infiltration of inflammatory cells, fibrogenesis, and improved survival rates after kidney transplantation. Overall, it is a meticulous study with the inclusion of adequate parameters in a technically challenging animal model to elucidate the point that Tie2 activation promotes an anti-inflammatory, pro-survival, and anti-permeability phenotype of the vasculature. On the other hand, some issues need to be clarified.

We highly appreciate the reviewers' positive comments on our study, especially acknowledging the technical challenge of our animal model.We hope that we can adequately answer his/her questions to improve our initial manuscript.

Major comments

In the introduction section, ischemia-reperfusion injury (IRI) and acute rejection, which are two different causes of graft injury after organ transplantation, were mixed up. The authors may discuss them separately. On the other hand, since the topic is about rejection, why bother to discuss IRI in the first place? If a transplantation group using C57Bl/6 mice as both donors and recipients, the effect of IRI could theoretically be taken out of the picture. The issue can then be focused on acute rejection itself. Besides, parameters for monitoring IRI (e.g., myeloperoxidase, oxidative stress) were not included and the animals were not sacrificed at an acute time point for this purpose.

Response: We totally agree with your suggestion to remove the slightly confusing ischemia reperfusion injury (IRI) part from our initial introduction as we did not focus on appropriate IRI readouts. We thank the reviewer for bringing this aspect to our attention.

Changed paragraph (Page 5):
"*Graft failure and ultimately graft loss are still major problems in solid organ transplantation. The endothelium hereby plays a pivotal role in mediating inflammation and subsequent organ dysfunction.* ~~*Solid organ transplantation is a classical scenario of ischemia reperfusion injury (IRI) in which the restoration of blood flow and reoxygenation is associated with an exacerbation of tissue injury and a profound inflammatory response (literally the 'reperfusion injury').*~~ *"*

The dosage of VT used in this study (i.e., 500 ng) should be justified and referenced.

Response: We would like to apologize that we obviously forgot to justify the amount of VT we used for our kidney transplant model. We used a dose of 500ng VT as this has not only proven potent Tie2 phosphorylation but also functional efficacy in terms of hard outcome measures in several published studies before. 500ng of VT ameliorated outcome in murine models of endotoxemia, abdominal sepsis and influenza[1, 2, 3]. In our revised manuscript, we included the primary reference into our Material & Methods section.

Changed paragraph (Page 7):
"Dosage of VT was carefully adjusted before [18]."

1. Information about the number of animals used and animal grouping should be made available to the readers in the Methods section. Did the experiment include C57Bl/6 to C57Bl/6 transplantation to serve as controls?

Response: We thank the reviewer for careful reading of our manuscript and for raising this thoughtful point. Numbers of animals per group are now included into our revised Material & Methods section. Given the technical complexity and required human resources (surgical skills) to successfully perform the murine kidney transplant model we were not able to include an additional control group of C57Bl/6 to C57Bl/6 transplantation. Although from the experimental set-up point of

view this would have been extremely elegant.

Changed paragraph (Page 7):
"*Briefly, kidneys from C57Bl/6 male (donor) were transplanted into Balb/c female (recipient) (n=23). Donor mice received 500 ng VT (n=11) or vehicle (PBS) (n=11) intraperitoneally (i.p.) 1h prior to surgery.*"

2. In the Methods section, the authors mentioned that "Cold ischemia time is 60, and warm ischemia time 30 minutes. After explantation, kidneys are stored in vehicle solution at 4°C for 45 minutes…". Since cold ischemia time is defined as the time between the chilling of a tissue, organ, or body part after its blood supply has been reduced or cut off and the time it is warmed by having its blood supply restored, it seems that the procedure did not quite match the definition. In addition, warm ischemia time, which is the time a tissue, organ, or body part remains at body temperature after its blood supply has been reduced or cut off but before it is cooled or reconnected to a blood supply, was just 30 minutes according to the authors. Does it mean that all vascular reconstructions were finished within 30 minutes? That would be really amazing! Congratulations if it was the case. The authors may just confirm the correctness of the data.

Response: We highly appreciate this comment from our reviewer acknowledging the fine work from the murine core facility of our renal division. Here, we obviously made a mistake. The cold ischemia time (and thereby the time the kidney was stored at 4°C) was indeed 60min. The surgeon (Dr. Song Rong) who performs murine renal transplantation for us is a board certified vascular surgeon in China who nowadays exclusively works (as a member of a core facility) in our mouse laboratory. He transplants murine kidneys on a daily basis and is highly skilled and experienced in this procedure. His routine allows fast, precise and reproducible experiments. For your reference please compare a methodological paper by Rong *et al.*[4].

Change paragraph (Page 7):
"Mice were anesthetized with isoflurane and the donor kidney, ureter, and bladder were harvested en block, including the renal artery with a small aortic cuff and the renal vein. Cold ischemia time is 60, and warm ischemia time 30 minutes. After explantation, kidneys are stored in vehicle solution at 4°C for 60 minutes."

Minor comments

1. Since serum creatinine and urea levels are not sensitive indicators of changes in renal function, it is generally suggested that metabolic cages should have been used to collect urine for computing changes in glomerular filtration rates for small animals. On the other hand, taking into account the technical difficulty of the procedure, the authors had already done a nice job.

Response: We totally agree with the reviewers' comment that a proper analysis of the glomerular filtration rate and urine output (e.g. in metabolic cages) would have been of great interest / impact for our studies. We do appreciate the limitations of serum-creatinine and BUN measurements. Unfortunately, we cannot go back to perform these test and have just recently started to establish more sophisticated GFR measurements (e.g. FITC-labeled inulin clearance). We apologize for not being able to present GFR data from this project.

2. Regarding the dearth of murine renal tissue from each mouse that may not be enough for Western blotting, this author is wondering whether the authors used tissue pooling for analysis in this aspect? The information should be available to the readers.

Response: We did not pool tissue for any analysis. To clarify our proceedings, we included this point into our Material & Methods section.

Changed paragraph (Page 7):
"Within a given experiments / analysis, we only used samples from single mice. We did not pool samples to increase protein

amounts."

References

1. David S, Ghosh CC, Kumpers P, *et al.* Effects of a synthetic PEG-ylated Tie-2 agonist peptide on endotoxemic lung injury and mortality Am J Physiol Lung Cell Mol Physiol 2011: 300; L851-862
2. Kumpers P, Gueler F, David S, *et al.* The synthetic tie2 agonist peptide vasculotide protects against vascular leakage and reduces mortality in murine abdominal sepsis Crit Care 2011: 15; R261
3. Sugiyama MG, Armstrong SM, Wang C, *et al.* The Tie2-agonist Vasculotide rescues mice from influenza virus infection Sci Rep 2015: 5; 11030
4. Rong S, Lewis AG, Kunter U, *et al.* A knotless technique for kidney transplantation in the mouse J Transplant 2012: 2012; 127215

This is an interesting study in which authors show that vasculotide -a synthetic Tie2 agonist- may improve renal transplant outcome.

We thank the reviewer for careful reading of our manuscript and we are more than happy to comment on his/her suggestions to improve our manuscript.

I suggest that:

1. A sentence regarding the effects of the different angiopoietin ligands (agonistic, antagonistic) could be added after citations 6,7) in the second paragraph of the introduction

Response: We totally agree with the reviewers' comment that we should include some complementary information about Angiopoietins into the introduction.We added two additional sentences better describing the role of the ligands.

Changed paragraph (Page 5):

"*Angpt-1 which is mainly secreted by pericytes binds Tie2 as a natural agonist thereby promoting vascular quiescence [21]. Canonical downstream effects of Tie2 signaling are activation of PI3K/Akt [8,14], inhibition of the inflammatory transcription factor NFκB [7] and consecutive control of adhesion molecule expression [9] as well as cytoskeletal regulation via the scaffolding protein IQGAP1 [4]. All together Tie2 activation promotes an anti-inflammatory, pro-survival, and anti-permeability phenotype of the vasculature. In contrast, Angpt-2 which is released from ECs upon pro-inflammatory stimuli inhibits Tie2 phosphorylation and consequently disrupts protective Tie2 signaling [15]."*

2. Authors describe more in detail the aims of the study. For instance after the last sentence of the introduction, perhaps they could explain how they evaluate the protective effects of vasculotide (for instance "...., assessing inflammatory infiltration, fibrous tissue deposition,... renal function and survival".

Response: We thank the reviewer for his/her suggestion to better describe the aims of the study. The revised manuscript now contains additive information about our aims.

Changed paragraph (Page 6):

"To test this, we exogenously activated the Tie2 receptor with VT ~~and investigated its effects in a murine kidney transplant model.~~ The aim of our study was to investigate the potential beneficial effects of VT treatment in a murine kidney transplant model on graft function. We analyzed inflammation, fibrous tissue deposition, renal function and overall survival to better understand if Tie2 activation might improve outcome after transplantation."

3. It seems that some results do not reach statistical significance due to a type II error. A sentence relative to this possibility should be added in the discussion.

Response: We thank the reviewer for this comment and included a small paragraph highlighting the difficulty of statistical significance in small sample size. The rather simple statistical analysis in this project was done with help of an investigator experienced in statistical analysis.

Changed paragraph (Page 14):

"Due to the small number of animals that survived until day 28, we were not able to include more animals into our studies. Nevertheless, our VT-treated mice show a clear trend towards improvement after kidney transplantation indicating a potential type II error in our statistical analysis."

4. Some results could be better defined (i.e.,"immense","profound")

Response: We highly appreciate the reviewers' comment to better define the strength of data in our results. We included some specifications into the results sections.

Changed paragraph (Page 10):

"One can easily appreciate the ~~profound~~ glomerular as well as the ~~immense~~ interstitial inflammatory infiltrates in the vehicle-treated mice on day 28 after transplantation (Fig. 2a+c, left side)."

Changed paragraph (Page 10):

"Keeping in mind the profound histological changes indicating that VT prevents infiltration of immune cells, we wanted to further analyze vascular inflammation and the infiltrative cell population."

Changed paragraph (Page 12):

"*Despite the ~~profound~~ differences on protein level demonstrating that VT-treatment indeed reduces inflammation in a murine renal transplant model, differences on the transcriptional are not present on day 28 after transplantation (Fig.5).*"