nature biomedical engineering

PEER-REVIEW INFORMATION

Prevention of vascular-allograft rejection by protecting the endothelial glycocalyx with immunosuppressive polymers

Corresponding author: Jayachandran N. Kizhakkedathu

Editorial note

This document includes relevant written communications between the manuscript's corresponding author and the editor and reviewers of the manuscript during peer review. It includes decision letters relaying any editorial points and peer-review reports, and the authors' replies to these (under 'Rebuttal' headings). The editorial decisions are signed by the manuscript's handling editor, yet the editorial team and ultimately the journal's Chief Editor share responsibility for all decisions.

Any relevant documents attached to the decision letters are referred to as **Appendix #**, and can be found appended to this document. Any information deemed confidential has been redacted or removed. Earlier versions of the manuscript are not published, yet the originally submitted version may be available as a preprint. Because of editorial edits and changes during peer review, the published title of the paper and the title mentioned in below correspondence may differ.

Correspondence

Tue 12 May 2020
Decision on Article nBME-20-0729

Dear Prof Kizhakkedathu,

Thank you again for submitting to *Nature Biomedical Engineering* your Article, "Localized Organ Engineering and Immunomodulation Protect Vascular Transplants from Immune-mediated Rejection ". The manuscript has been seen by four experts, whose reports you will find at the end of this message. You will see that although the reviewers have some good words for the work, they articulate concerns about the degree of advance that this study represents over relevant published work, as well as about the experimental models used, and in this regard provide useful suggestions for improvement.

We hope that with significant further work you can address the criticisms, increase the level of significance of the study, and convince the reviewers of its merits. In particular, we would expect that a revised version of the manuscript provides:

* data from additional animal models to better substantiate the claims as requested from reviewers #1, #3 and #4, namely in what concerns the comments from reviewer #1 on the limitations of the aortic interposition model, and those of reviewer #3 on the limited advance in light of other strategies tested in syngeneic mouse models of transplantation. For supporting the translational potential of the work, larger mammal models (e.g. pigs) should be considered.

* data to better substantiate the extent and multiple aspects of immune protection elicited, including to support the claims of localized immune suppression as highlighted by reviewer #1.

* a longer assessment of immune rejection in the kidney transplantation model.

* a functional assessment of the treated endothelial cells, including a characterization of their glycocalyx in comparison with untreated cells, and data on endothelial-cell-specific cell death.

* the claims that the strategy can be applied in the context of organ preservation should be supported by the data or alternatively toned down.

When you are ready to resubmit your manuscript, please upload the revised files, a point-by-point rebuttal to

the comments from all reviewers, the (revised, if needed) reporting summary, and a cover letter that explains the main improvements included in the revision and responds to any points highlighted in this decision.

Please follow the following recommendations:

* Clearly highlight any amendments to the text and figures to help the reviewers and editors find and understand the changes (yet keep in mind that excessive marking can hinder readability).

* If you and your co-authors disagree with a criticism, provide the arguments to the reviewer (optionally, indicate the relevant points in the cover letter).

* If a criticism or suggestion is not addressed, please indicate so in the rebuttal to the reviewer comments and explain the reason(s).

* Consider including responses to any criticisms raised by more than one reviewer at the beginning of the rebuttal, in a section addressed to all reviewers.

* The rebuttal should include the reviewer comments in point-by-point format (please note that we provide all reviewers will the reports as they appear at the end of this message).

* Provide the rebuttal to the reviewer comments and the cover letter as separate files.

We hope that you will be able to resubmit the manuscript within 25 weeks from the receipt of this message. If this is the case, you will be protected against potential scooping. Otherwise, we will be happy to consider a revised manuscript as long as the significance of the work is not compromised by work published elsewhere or accepted for publication at *Nature Biomedical Engineering*. Because of the COVID-19 pandemic, should you be unable to carry out experimental work in the near future we advise that you reply to this message with a revision plan in the form of a preliminary point-by-point rebuttal to the comments from all reviewers that also includes a response to any points highlighted in this decision. We should then be able to provide you with additional feedback.

We hope that you will find the referee reports helpful when revising the work. Please do not hesitate to contact me should you have any questions.

Best wishes,

João

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Dr João Duarte
Senior Editor, *Nature Biomedical Engineering*

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Reviewer #1 (Report for the authors (Required)):

In their article, Siren et al tested the hypothesis that enzymatic polymer ligation allows for endothelial cell surface engineering that is compatible with clinically relevant transplant conditions. The concept is that steric and bioactive properties of ligated polymers would restore the natural protection provided to graft endothelial cells by their glycocalyx, which is damaged by ischemia reperfusion. Ultimately, this technique could provide localized immune therapy to minimize transplant damage after surgery and prevent transplant rejection.
This concept represents an interesting refinement of the “immunocloaking” therapy as proposed by Brasile et al a decade ago (Transplantation. 2010 Dec 27; 90(12):1294-8).
The paper is well written and easy to follow but the data presented do no always support the conclusions made by the authors.
Following are my comments:

Major comments:

1) (mandatory) The fact that enzymatic polymer ligation restores endothelial glycocalyx is central to the theory tested by the authors, yet this aspect is never directly and formally demonstrated.
Analyses of the glycocalyx (by electron microscopy? see for instance Okada, H., Crit Care 21, 261; 2017) from treated/untreated endothelial cells, both in culture wells and in murine kidney grafts, are necessary.

2) It would be important to test how endothelial cell surface engineering affects endothelial functions. In this regard the RNA-seq analysis provided figure S8 is interesting but insufficient. Functional assays would be far more informative.
In addition, all the in vitro experiments have been conducted with human endothelial cell line (Ea.hy926 cells). However, it is conceivable that i) the nature of endothelial cells affects endothelial cell surface engineering, and that ii) endothelial cell surface engineering may impair the function of some specialized endothelial cell subsets (such as glomerular endothelial cells for instance). Could the authors compare the efficiency and functional impact of cell surface engineering on primary cells vs cell line / macro vs microvascular endothelial cells?

3) (mandatory) The models used to test the protection offered by endothelial cell surface engineering against rejection are not appropriate.

Rejection is mainly an adaptive immune process, driven either by allospecific cytotoxic T cells (cellular rejection) or donor-specific antibodies (humoral rejection). These are the two processes that should be controlled by endothelial cell surface engineering if this strategy is to have any relevance against rejection. Of note, the incidence of cellular rejection decreases beyond 12 months post transplantation. It is therefore antibody-mediated rejection (first cause of graft loss) that represents the main challenge in transplantation.
i) In vitro:
* Only the protection against cellular rejection is tested. What about antibody-dependent cytotoxicity driven by complement and ADCC?
* In contrast with innate immune effectors (such as NK and monocytes, for which priming with cytokine is sufficient), alloreactive CD8+ T cells need to be primed through their TCR before responding to an allogeneic simulation. The response obtained for CD8+ T cells with IL2 is likely due to memory CD8+ T cells with “innate like function” (Seyda et al, Trend in Immunol, p546-556, 2016). Using this experimental setting it is not surprising that the authors conclude that “the protective effect of LPG-Q-Sia3Lac mainly involved inhibition of NK cell-mediated lysis” (Page 9). Using primed allospecific CD8+ T cells will likely trigger much more endothelial death than the 15% observed… and could drastically change the conclusions.
*the evaluation of the endothelial cell death by LDH dosage in culture supernatant is not appropriate because a lot of immune effectors (in particular activated CD8+ T cells) also die during these cocultures (contributing to LDH release). A read out analyzing only endothelial cell death is mandatory.
ii) In vivo:
*The aortic interposition model is simple, but it has several limitations to study rejection (discussed in Pober in Am J Transplant. 2002 Mar;2(3):201-2). Briefly, aortic graft has no real “function” (except conducting blood, which is not affected by rejection). Therefore, evaluation of rejection is based only on indirect read outs…
* Day 2 post Tx is too early to analyze acute rejection (the adaptive immune system of naïve recipients is not primed yet). At this time, only ischemia/reperfusion injuries can be seen. In addition, to evaluate acute cellular rejection, graft infiltration by recipients’ adaptive immune effectors (T cells) shouldn’t be evaluated in the endothelium but in the adventitia (15 to 21 days post Tx).
*The alloimmune humoral response (i.e. generation of donor-specific antibodies) should also be evaluated (see comment above on humoral rejection).
*In the aortic interposition model, the intensity of chronic rejection is often (as in this study) judged upon the size of the neointima. However, neointima is a non-specific feature that develops after almost any kind of arterial injury, including perturbations of laminar flux due to end to end anastomoses. Therefore, analysis of the size of neointima to evaluate chronic rejection (i.e. the consequence of chronic alloimmune response against the graft) can lead to misinterpretation, especially when: i) the group of animals are small (here $n<5$), ii) nothing is made to ensure that the distance between the anastomosis and the aortic cross-section analyzed is the same in all samples (This confounding factor is quite difficult to control and the authors provided no detail about how they dealt with it).
Based on all these comments I would strongly advise to conduct the in vivo experiments aiming at evaluating the impact of endothelial cell surface engineering on acute and chronic rejection in the murine kidney transplantation model. Both histological and functional (renal function, recipient survival) data should be provided.

4) (mandatory) The authors claim that “polymer-mediated organ engineering approach […] leads to […] localized immune suppression”. However, they never demonstrate that recipients respond normally to i) an (unmodified) graft from the same donor placed elsewhere, and ii) another immune stimulus (vaccination? or 3rd party allograft). These experiments are needed because I do not understand how the very transient presence (1/2 life is 8H according to figure 1e) of the polymer on graft endothelial cells can induce such a long-term protective effect against alloimmune response. Could it be that endothelial cell surface engineering induces immune tolerance (which can be allospecific or not)…Experiments should be performed to clarify this point. (mandatory)

Minor comment:
1) (figure 2g) Activation status of NK is usually evaluated by measuring: CD107a on the surface of the cells and/or cytokines or chemokines (IFN, MIP1b) in their cytoplasm. I have no idea what more (or less) SHP-1 protein in NK means regarding the activation status of the cells. SHP-1 is a phosphatase, which regulates some signaling pathways, the activation status of which is usually determined by performing WB of phosphorylated substrate proteins.
The question of the activation status of NK is however interesting because: in one hand endothelial cell surface engineering could prevent the ligation of “stress induced ligands” to NK activating receptors and/or even deliver inhibitory signals through siglecs (but not all NK subsets express siglecs)… However on the other hand, endothelial surface engineering could also impede the delivery of inhibitory signals, thereby triggering “Missing self”-induced activation of NK, a process that has been recently shown to promote rejection (Koenig et al, Nat Commun. 2019 Nov 25;10(1):5350).

Reviewer #2 (Report for the authors (Required)):

In this manuscript the authors describe an approach for engineering the surface of blood vessel endothelial cells with glyco-polymers that prevents immune cell targeting and damage. The major strengths of the report are the elegant method for decorating the surface of endothelial cells with glyco-polymers and demonstrating the impact of the engineered vessels organs on suppression of immune attack upon transplantation into animals.

The polymer design using a functionalized polyglycerol with alkyne clickable handles linked to a peptide that serves as a substrate for pig liver tissue transglutaminase, which allows for enzymatic attachment of the polymer to lysines exposed on cell surface membrane proteins. The alkyne handle(s) allow for facile attachment of glycans with azide terminated linkers using copper catalyzed ‘click’ chemistry. Although the copper is very toxic, it can be readily removed from the polymer before exposure to cells.

Engineering of cell surfaces of an epithelial cell line monolayer with the glycopolymers in organ storage solution at 4° C is efficient, and the polymers protect against damage/cytotoxicity to PBMCs, in particular, NK cells. The hypothesis is that sialic acid containing glycans will suppress immune responses that cause tissue rejection by recruiting inhibitory Siglecs that recognize sialic acid containing glycans as their ligands. While the hypothesis is attractive and consistent with the findings, there is really little data showing that Siglecs are involved in the protection.

While the mechanism may not be pinned down, pretreatment of the organs with the glycopolymer engineering protocol ex vivo is shown to impressively reduce/prevent tissue damage and rejection of syngeneic Balb/c blood vessels and kidneys upon transplantation into C57BL/6 mice.

Specific comments:

1. While the suggestion that the glyco-polymers recruit siglecs to suppress immune responses is attractive, evidence that they are playing a role is lacking. Simple experiments showing that Siglecs are expressed on PBMCs using commercially available antibodies and showing that these Siglecs bind to the modified cells using commercially available Siglec-Fc chimeras would be supportive.

2. The text imply that T cells express Siglecs, but dogma has it that T cells in PBMCs in mice do not express Siglecs. There are several reports that suggest human T cells express Siglecs, particularly in the context of tumor infiltrating T cells. But these experiments are in mice.

3. The data in Figure 2f are particularly informative, but it is not clear that unpaired T tests are an appropriate test for comparisons between groups. Better might be one-way Anova. It appears that removal of NK cells has significant impact on protection of endothelial cells, while removal of T cells or monocytes does not. While this is implied in the text, it is not shown statistically.

4. UW should be added as an abbreviation. This reviewer had to search Google to understand what UW was (e.g. cold preservation solution). This article is intended for a general audience.

Reviewer #3 (Report for the authors (Required)):

This is an innovative manuscript using cell surface engineering of the endothelial layer to minimize damage and rejection locally during organ preservation and subsequent transplantation. The protocol uses enzymatic polymer ligation to the endothelial surface and is likely to be scalable to human organs as it is a relatively easy method to apply. The authors claim near complete prevention of acute and chronic rejection. The manuscript is well executed, the results are exciting, and the idea is innovative. However, there are several issues that need to be addressed.

The literature coverage of the state-of-the art for both new approaches to immune suppression and organ preservation are completely absent and not acceptable. The authors need to present their idea within the context of the state-of-the-art.

The paper makes a lot of statements about preservation but this is clearly over-reaching as there is really no study of preservation except few conditions like 4 hours on ice. This is not considered proper organ preservation. The paper needs to be significantly re-written to avoid any claims that are not supported by the results.

The animal model used is not gold standard but rather the lowest bar that can be used for a preliminary testing. There have been multiple exciting papers published in the last 3 decades or more on complete abolishing of the immune rejection in syngeneic mouse models. The claims again significantly over-reach from what can be supported by the data and the animal model used to test.

In short, it is an interesting idea, the materials science aspect is very strong but the coverage of the prior art and the animal model used for testing are very weak, and equally importantly the claims are not supported by the results. This needs serious re-writing.

Reviewer #4 (Report for the authors (Required)):

The manuscript by Siren et al. describes a novel approach for localized immune therapy
to minimize transplant damage after surgery and prevent transplant rejection. Specifically, the authors utilize immune-deactivating polymers localized to the lumen of the blood vessels to prevents graft injury and transplant. Polymers were synthesized and evaluated for immune suppressive and anti-inflammatory activity in vitro, followed by in vivo evaluation in two different mice models. Overall, the approach is interesting and shows potential for preventing transplant rejection. Modification of grafts in static cold storage condition is also impressive. The manuscript is well written and the experiments are thorough. However, there are a few concerns, which if clarified will further improve this work.

1. Would be great if the authors can explain the rationale for choosing day 42 as the time point to evaluate chronic rejection in murine aortic allograft transplant model? Is it possible to look beyond day 42? The reviewer understands that repeating such a long experiment to look at further time points might be difficult, but it would be great if the authors can discuss this at least.
2. Is there a positive control that can be included in Fig. 3b and c? It would be interesting to see how their results compare to the positive control.
3. The authors mention that “LPG-Q-Sia3Lac also reduced histological features of medial inflammation such as medial necrosis and leukocyte infiltration (Fig. 3b-d).” However, the figures don’t show anything related to necrosis or leukocyte infiltration.

4. In the in vivo studies described in Fig. 3, its interesting to see that LPG-Q Sia3Lac shows better results than LPG-Q. Since LPG-Q did not show any significant effect compared to UT, one crucial question is what would happen if only sialic acid is grafted on to the tissue without the polymer. It would be great if authors can demonstrate that, even during the early time point.
5. For kidney transplant model, did the authors follow animals after day 7? Although the effect is great at day 7, the manuscript will become really strong if the authors can include data for longer period, even if its for 20-30 days. Day 7 is too short for evaluation of immunosuppression.
6. The authors say “Confocal microscopy confirmed covalent attachment”. This is a bit incorrect as confocal microscopy cannot confirm “covalent attachment”. It can only confirm “attachment”.
7. “Bioavailability of ICAM” is an inappropriate term. “Bioavailability” is usually used in the context of pharmacokinetic studies.
8. Authors used a scale of “0-4” to score histology in figure 3. What does each point on the score indicate?
9. The discussion can be strengthened a bit more. For example, authors should discuss the choice of time points for measuring end points in the animal model. Similarly, the authors need to comment on how do they envision the clinical re-application of this technology, i.e. once the polymer is cleared (as demonstrated by authors in cell studies), do they envision this polymer to be reapplied. If yes, how will that be feasible?

Thu 27 May 2021
Decision on Article nBME-20-0729

Dear Prof Kizhakkedathu,

Thank you for your revised manuscript, "Localized Organ Engineering and Immunomodulation Protect Vascular Transplants from Immune-mediated Rejection". Having consulted with the original reviewers (whose comments you will find at the end of this message), I am pleased to write that we shall be happy to publish the manuscript in *Nature Biomedical Engineering*, provided that the points specified in the attached instructions file are addressed.

When you are ready to submit the final version of your manuscript, please upload the files specified in the instructions file.

For primary research originally submitted after December 1, 2019, we encourage authors to take up transparent peer review. If you are eligible and opt in to transparent peer review, we will publish, as a single supplementary file, all the reviewer comments for all the versions of the manuscript, your rebuttal letters, and the editorial decision letters. **If you opt in to transparent peer review, in the attached file please tick the box 'I wish to participate in transparent peer review'; if you prefer not to, please tick 'I do NOT wish to participate in transparent peer review'.** In the interest of confidentiality, we allow redactions to the rebuttal letters and to the reviewer comments. If you are concerned about the release of confidential data, please indicate what specific information you would like to have removed; we cannot incorporate redactions for any other reasons. If any reviewers have signed their comments to authors, or if any reviewers explicitly agree to release their name, we will include the names in the peer-review supplementary file. More information on transparent peer review is available.

Please do not hesitate to contact me should you have any questions.

Best wishes,

Pep

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Pep Pàmies
Chief Editor, *Nature Biomedical Engineering*

P.S. Nature Research journals encourage authors to share their step-by-step experimental protocols on a protocol-sharing platform of their choice. Nature Research's Protocol Exchange is a free-to-use and open resource for protocols; protocols deposited in Protocol Exchange are citable and can be linked from the published article. More details can be found at www.nature.com/protocolexchange/about.

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Reviewer #1 (Report for the authors (Required)):

I have read carefully the revised version of the MS proposed by Siren et al.

Authors should be commended for their impressive efforts to address the comments that were made.

They have satisfactorily addressed my concerns. I have no further suggestion and strongly support the publication of this very interesting piece of work.

Reviewer #2 (Report for the authors (Required)):

The authors have adequately addressed the concerns of this referee concerning over reaching their conclusions and statistical analysis of the data.

Reviewer #3 (Report for the authors (Required)):

The revised paper addresses most issues raised in the original review; however, the literature coverage is still not appropriate. The most exciting new work for immune suppression relates to bone marrow transplantation for chimerism, engineered T-cells, local immuno-suppression among others. It is important to properly present this work within the context of new and exciting avenues and not just the typical approaches.

Reviewer #4 (Report for the authors (Required)):

The authors have addressed all the comments!! Thank you.

Rebuttal 1

Detailed response to the reviewer's comments for the manuscript "Localized organ engineering and immunomodulation protect vascular transplants from immune-mediated rejection" (Manuscript ID: nBME-20-0729)

We thank editor and reviewers for providing their feedback and, here, we fully address all the comments raised by the reviewers and performed many additional experiments.

Summary of Revision performed:

We performed major revisions based on the reviewer's feedback. Here are the summarized key findings.

1. Glycocalyx quantification data of endothelial monolayers. We quantified the amount of glycocalyx on the endothelial monolayers under various conditions using flow cytometry to reveal minimal perturbation of the glycocalyx caused by the CSE technique.
2. Functional data of endothelial monolayer. We performed transendothelial protein passage experiments, which determine that the CSE technique does not affect endothelial monolayer function to transport proteins across a cell monolayer.
3. We performed in vitro cytotoxicity experiments using highly cytotoxic A2-CAR $CD8^+$ T cells to reveal another mode of immunomodulation beyond the previously reported PBMC data. We have generated chimeric antigen receptor (CAR) T cells expressing a CAR that recognizes human leukocyte antigen-A2 (HLA-A2) on the surface of target cells. LPG-Q-Sia3Lac was able to reduce endothelial cytotoxicity in the new co-culture model.
4. Acute rejection model of allogeneic aortic graft model was performed by analyzing vessel histology 15 days post-transplantation. Scoring revealed that LPG-Q-Sia3Lac treatment lowered acute rejection compared to untreated control grafts.
5. Sera from mice 42 days post-transplantation was analyzed for donor specific antibodies. It was revealed that LPG-Q-Sia3Lac treated mice had sera containing less donor specific antibodies than that of untreated mice.
6. Skin allografts were performed on recipients of aortic allografts at 28 days after aortic transplantation to examine the effect of LPG-Q-Sia3Lac on systemic immunosuppression and tolerance. Skin graft rejection, of either artery donor-matched or third party skin, was non-significant between recipients of LPG-Q-Sia3Lac treated aortic transplants and untreated controls. This data reveals that the modification of the blood vessel does not impart immunomodulatory properties onto a secondary transplant, further supporting our hypothesis that the current CSE technique is a localized treatment.
7. A new data set for an allogeneic kidney transplant model was performed. The length of the transplant was 30 days before sacrificing the mice for histological analysis of the organ. Blood urea nitrogen data and histological scoring concluded that organ health and function was superior in LPG-Q-Sia3Lac treated mice groups compared to untreated mice group.

Reviewers' comments:

Reviewer #1 (Remarks to the author): In their article, Siren et al tested the hypothesis that enzymatic polymer ligation allows for endothelial cell surface engineering that is compatible with clinically relevant transplant conditions. The concept is that steric and bioactive properties of ligated

polymers would restore the natural protection provided to graft endothelial cells by their glycocalyx, which is damaged by ischemia reperfusion. Ultimately, this technique could provide localized immune therapy to minimize transplant damage after surgery and prevent transplant rejection. This concept represents an interesting refinement of the "immunocloaking" therapy as proposed by Brasile et al a decade ago (Transplantation. 2010 Dec 27; 90(12):1294-8). The paper is well written and easy to follow but the data presented do no always support the conclusions made by the authors.

Response: We thank the reviewer for their interest in the concept and technology described in our manuscript. The thoughtful and critical comments directed us to perform additional experiments which significantly improved the data quality and scope of the manuscript. Thank you! After considering the comments by this reviewer on the scope of claim for our manuscript and the strength of our animal models, we decided to perform several additional experiments both *in vitro* and *in vivo*. We have further assessed our CSE technology *in vitro* by analyzing the glycocalyx structure using flow cytometry and by examining health of the endothelial monolayer. Moreover, a new immune cell killing experiment using HLA-A2-reactive effector CAR-T cells and HLA-A2-expressing endothelial cells as targets was performed to more precise examine the ability of the technology to inhibit cytotoxic T cell responses. *In vivo*, we extended the histological analysis of blood vessel rejection by scoring acute rejection of the artery and determined the effect of the technology on antibody responses. Importantly, we performed allogeneic aortic interposition transplants lasting 15 days to examine acute rejection, evaluated the effect of the technology on systemic immunosuppression and tolerance using secondary skin grafts on artery transplant recipients. Allogeneic kidney transplants lasting 30 days were also examined to study the potential application of this technology to organ transplant rejection. The rigorous additional experiments included in our updated manuscript should enhance and better support our hypothesis.

Comment #1: The fact that enzymatic polymer ligation restores endothelial glycocalyx is central to the theory tested by the authors, yet this aspect is never directly and formally demonstrated.

Response: We thank the reviewer for raising this point. There was some confusion in the choice of the words we used in the previous version of the manuscript. We reworded our manuscript to reflect that our CSE technology recapitulates most functions of the glycocalyx but may not directly rebuild its physical structure entirely. We acknowledge that visualization of the glycocalyx by electron microscopy (EM) would be informative. However, this analysis is extremely challenging because the glycopolymer we use for the CSE technology has a base molecular weight of 14 kDa (5-6 nm in diameter). Current techniques, including high resolution scanning electron microscopy (SEM) and transmission electron microscopy (TEM) techniques, are not be able to achieve a resolution high enough to directly visualize our LPG-Q-Sia3Lac on the glycocalyx structure of endothelial cells. Although we cannot visualize the CSE modification directly using EM, have used flow cytometry to quantify glycocalyx abundance using wheat germ agglutinin (WGA) stain. This stain quantifies the sugar structures of the glycocalyx. Results are presented in main text Figure 2, panel 'e' (Fig. 2e). Our findings show that the CSE technology is not affecting the amount of glycocalyx present on unstimulated endothelial cells. Importantly, CSE based incorporation of LPG-Q-Sia3Lac is able to reduce TNF-α induced glycocalyx shedding. Together with the functional data presented in Figure 3 of the manuscript and confocal microscopy and flow cytometry data (Figure 2 a, b), we believe the data strongly support the conclusion that the current CSE modification is recapitulating the functional properties of the glycocalyx. This has been discussed in the revised manuscript.

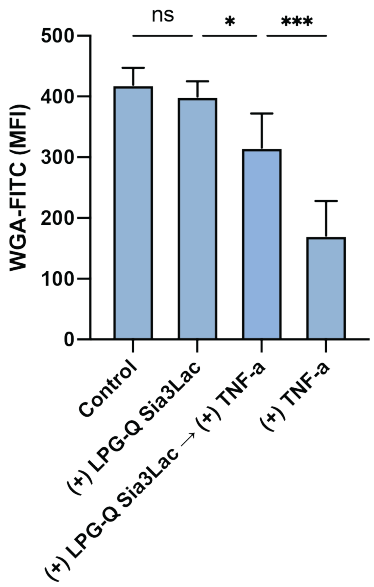


Figure 2e. Effects of cell surface engineering on endothelial glycocalyx structure. Enzyme mediated ligation does not induce shedding of the glycocalyx structure in Ea.hy926 cells. Ligation of LPG-Q-Sia3Lac decreases glycocalyx shedding induced by TNF-α. The glycocalyx structure was labelled using FITC modified wheat germ agglutinin and analyzed using flow cytometry. Ea.hy926 cells were activated using TNF-α (2000 U/mL) for 3 hours at 37°C and modified by incubating with UW solution supplemented with 0.5 mM Q-tagged polymer (acyl donor), 0.2 U/mL gtTGase, 3 mM GSH, 5 mM CaCl2 for 30 min at 4 °C. Error bars represent 95% confidence intervals. Unpaired comparisons using a non-parametric t-test are significant with $p > 0.05$ (ns), $p \leq 0.05$ (*), $p < 0.01$(**), $p < 0.001$ (***).

Comment #2: It would be important to test how endothelial cell surface engineering affects endothelial functions. In this regard the RNA-seq analysis provided figure S8 is interesting but insufficient. Functional assays would be far more informative.
In addition, all the in vitro experiments have been conducted with human endothelial cell line (Ea.hy926 cells). However, it is conceivable that i) the nature of endothelial cells affects endothelial cell surface engineering, and that ii) endothelial cell surface engineering may impair the function of some specialized endothelial cell subsets (such as glomerular endothelial cells for instance). Could the authors compare the efficiency and functional impact of cell surface engineering on primary cells vs cell line / macro vs microvascular endothelial cells?

Response: Additional functional assays that examine effects of the CSE modification on endothelial cell biology and examining the effect of the CSE technology on endothelial cell subsets are important considerations. We have addressed both considerations and describe them below.

In addition to the RNA-seq analysis, we have performed several functional assays to illustrate the point. We have added new data on the barrier function of endothelial monolayers by measuring the transendothelial passage of macromolecules (FITC-labeled albumin) across HMEC-1 cell monolayers (**Figure 2f**). The data establishes that LPG-Q-Sia3Lac does not change the permeability of resting endothelial layers. In response to inflammatory activation with TNF-a, the CSE technology reduced the permeability of endothelial cell monolayers which further highlights its anti-inflammatory effects. We have also examined the ability of the CSE modification to protect against T cell-mediated injury (see comment #3). When taken together with other functional assays in the original submission such as the shielding of ICAM-1 accessibility, prevention of PBMC adhesion and prevention of PBMC mediated cytotoxicity, these data support the conclusion that the our CSE technology recapitulates the functional activity of endothelial cell surfaces.

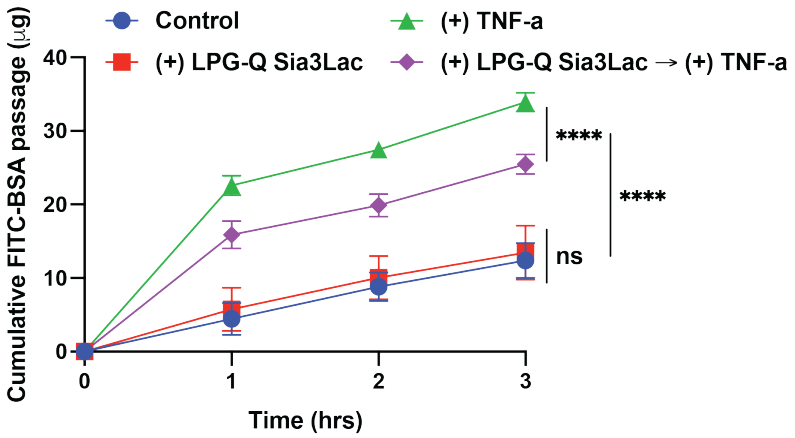
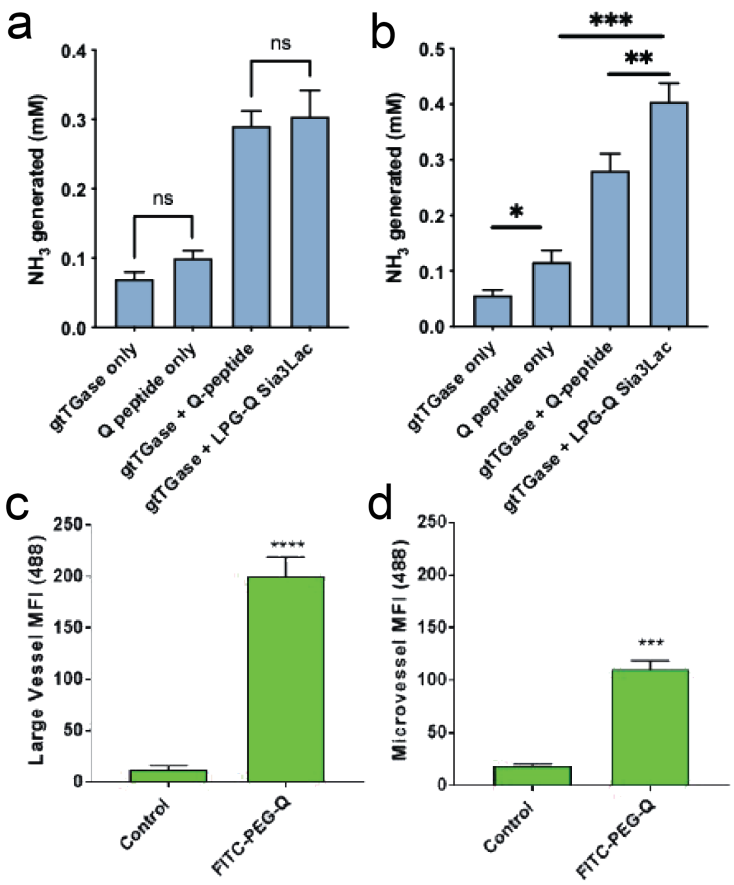


Figure 2f. Effects of cell surface engineering on endothelial barrier function: Enzyme mediated ligation of HMEC-1 cell monolayers does not affect transendothelial passage. Transendothelial passage experiments were performed by tracking FITC-labelled bovine serum albumin (BSA) passage across HMEC-1 cell monolayers. HMEC-1 cells were activated using TNF-α (2000U/mL) for 3 hours at 37 °C and modified by incubating with UW solution supplemented with 0.5 mM Q-tagged polymer (acyl donor), 0.2 U/mL gtTGase, 3 mM GSH, 5 mM CaCl2 for 30 min at 4°C. Error bars represent 95% confidence intervals. Unpaired comparisons using a non-parametric t-test are significant with $p > 0.05$ (ns), $p \leq 0.05$ (*), $p < 0.01$(**), $p < 0.001$ (***).

With regard to differences in primary cells and endothelial cell subsets, we have performed several additional experiments to illustrate that the CSE is applicable to endothelial cells from different vascular beds and to the vasculature of whole kidney. In the revised manuscript, we include experiments on human umbilical vein endothelial cell (HUVECs) and human microvascular ECs (HMEC-1 cells; Supplementary Fig. S6-7) (both primary and immortalized cell lines) to show that CSE modifies these types of endothelial cells in a similar manner as Ea.hy926 cells. This shows that the CSE approach is generalizable to primary endothelial cells in different vascular beds.



Figures S6-7: Cell surface engineering of endothelial glycocalyx using guinea pig liver transglutaminase. All polymer ligations were done in UW solution fortified with 0.5 mM FITC-PEG-Q or 0.56 mM LPG-Q-Sia3Lac, 3 mM GSH, 5 mM $CaCl_2$, 0.2 U/mL gtTGase over 30 minutes at 4 °C. Enzyme mediated ligation occurs on **a)** HUVEC and **b)** HMEC-1 cell surfaces. [redacted] Data is shown in figure 'c' and 'd'.

Taken together, these two sets of data described above clearly illustrate the functional recapitulation of the glycocalyx by CSE and its application to different types of endothelial cells. This has been incorporated in the revised manuscript.

Comment #3: In contrast with innate immune effectors (such as NK and monocytes, for which priming with cytokine is sufficient), alloreactive CD8+ T cells need to be primed through their TCR before responding to an allogeneic simulation. The response obtained for CD8+ T cells with IL-2 is likely due to memory CD8+ T cells with "innate like function" (Seyda et al, Trend in Immunol, p546-556, 2016). Using this experimental setting it is not surprising that the authors conclude that "the protective effect of LPG-Q-Sia3Lac mainly involved inhibition of NK cell-mediated lysis" (Page 9). Using primed allospecific CD8+ T cells will likely trigger much more endothelial death than the 15% observed… and could drastically change the conclusions.

Response: This is a very important consideration and we thank the reviewer for highlighting it. We have performed additional experiments to specifically examine the effect of CSE modification on T

cell-mediated killing of endothelial cells. Activation and expansion of alloreactive effector T cells that target endothelial cells is challenging because it requires endothelial cell-matched antigen presenting cells (i.e., from the same donor) or long-term co-culture of T cells with endothelial cells, the latter scenario being restricted to expansion of memory T cells (Dengler et al., J Immunol, 2001). As an alternative, we have examined the effect of CSE modification on killing of endothelial cells by chimeric antigen receptor (CAR) T cells (CAR T cells) expressing a CAR that is reactive to HLA-A2, an HLA type that we confirm is expressed by Ea.hy926 cells (Supplementary Fig. S26). In this system, the effector CAR T cells recognize their antigen on the surface of all endothelial cells and induce substantial cytotoxicity. Although CSE modification of endothelial cells with LPG-Q reduced endothelial cytotoxicity mediated by HLA-A2 reactive CAR T cells, the incorporation of sialic acid in the polymer was able to significantly and, in 1:10 effector to target cell ratio, almost completely inhibit CAR-T cell induced endothelial cell cytotoxicity (Fig. 3i). These findings establish that CSE modification is capable of inhibiting T cell-mediated cytotoxicity in endothelial cells using a robust and specific assay that is relevant to the transplant scenario. We have revised the manuscript accordingly and added these data.

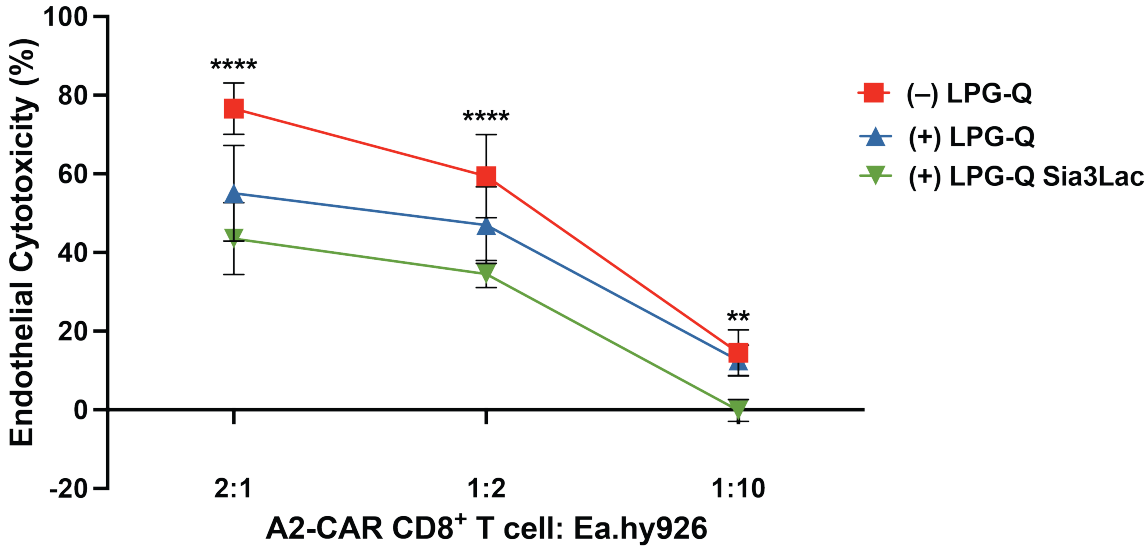


Figure 3i. Polymer-mediated cell surface engineering of endothelial monolayers inhibits activation of A2-CAR T cells in co-cultures: LPG-Q-Sia3Lac modification suppresses endothelial cell death mediated by HLA-A2 reactive CAR-T cells (A2-CAR CD8+ T cells). The experiment was repeated using A2-CAR $CD8^+$ T cells from 2 different donors. Error bars represents 95% confidence intervals. Unpaired comparisons using a non-parametric t-test between the (-) LPG-Q and (+) LPG-Q Sia3Lac group are significant with $p > 0.05$ (ns), $p \leq 0.05$ (*), $p < 0.01$(**), $p < 0.001$ (***), $p < 0.0001$ (****).

Comment #4: the evaluation of the endothelial cell death by LDH dosage in culture supernatant is not appropriate because a lot of immune effectors (in particular activated CD8+ T cells) also die during these cocultures (contributing to LDH release). A read out analyzing only endothelial cell death is mandatory.

Response: In our development of the LDH assay**,** we ensured that LDH released by PBMCs is accounted for in quantification of endothelial cell death. Specifically, LDH release from PBMCs without endothelial cells is measured and then subtracted from LDH release in PBMCs co-cultured with endothelial cells. This method has been used previously by other researchers (Zeng et al. Transplantation Proceedings, 2006; Taheri et al. IUBMB Life, 2019). We will consider readapting the methods using calcein or propidium iodide labelling for future experiments.

$$\text{cytotoxicity} = \frac{\text{(total LDH rel.) – (LDH rel. after polymer att) – (PBMC LDH rel.)-(LDH rel. 100\% alive)}}{\text{(LDH rel. 100\% dead EA.hy 926)}}$$

Comment #5: The aortic interposition model is simple, but it has several limitations to study rejection (discussed in Pober in Am J Transplant. 2002 Mar;2(3):201-2). Briefly, aortic graft has no real "function" (except conducting blood, which is not affected by rejection). Therefore, evaluation of rejection is based only on indirect read outs…

Response: We appreciate the opportunity to clarify the clinical relevance of the aortic interposition model. Pober et al. highlight limitations of the model for recapitulating the full pathogenesis of human transplant arteriosclerosis, although all mouse models are limited in this capacity. One main consideration of most murine transplant models, including the aortic interposition model, is the lack of immunosuppression and memory T cell responses that are components of transplant rejection in humans. In the aortic interposition model where transplants are performed across a complete MHC mismatch, there is extensive immune-mediated damage of the artery that recapitulates high-grade acute rejection. Intimal thickening follows from this acute rejection and it is important to note that acute rejection is a predictor of transplant arteriosclerosis in the clinical setting (Nakagawa et al., Circulation, 1995; Higuchi et al., Transplantation, 1999). There may be some components that contribute to intimal thickening in the aortic interposition model that minimally contribute to this arterial change in human transplants because of the extent of arterial injury. However, considering the aggressiveness of immune injury, the aortic interposition model is a useful and stringent model to study therapies that inhibit vascular rejection. Moreover, the extensive injury that results from lack of immunosuppression is valuable when evaluating potential therapies for eliminating or reducing systemic immunosuppression. Also, rejection in this model involves all aspects of immune responses that contribute to clinical rejection, including T cell-mediated injury as well as de novo development of donor specific antibodies (von Rossum et al., Transplantation, 2016). Finally, vascular rejection is recognized as a central component of heart transplant rejection but is applicable to understanding all organ transplants so our studies are broadly applicable (Duong van Huyen et al., Am J Transplant, 2020).

Given the limitations of all murine models for studying human conditions, it is important to examine potential therapies in several models to enhance potential clinical application. In our study, we have also examined application of the CSE technology for prevention of kidney transplant injury using an allogeneic kidney transplant model and observe that CSE reduces features of acute rejection-mediated injury. We believe that our collective data using two models of transplant rejection are sufficient to establish the potential of our technology for minimizing transplant rejection.

Comment #6: Day 2 post Tx is too early to analyze acute rejection (the adaptive immune system of naïve recipients is not primed yet). At this time, only ischemia/reperfusion injuries can be seen. In addition, to evaluate acute cellular rejection, graft infiltration by recipients' adaptive immune effectors (T cells) shouldn't be evaluated in the endothelium but in the adventitia (15 to 21 days post Tx).

Response: We agree that day 15 post-transplantation is an optimal time-point to examine acute rejection and have performed these experiments. In the aortic interposition model, acute rejection is characterized by infiltration of the adventitia and media by leukocytes, which leads to medial injury. There may also be early initiation of intimal thickening. As such, we evaluated the histology of arteries at day 15 post-transplantation and graded rejection based on these features. We see

that CSE modification significantly reduces acute rejection at this time-point (Fig. 4 f & g). All together, we show that CSE of blood vessels reduces early inflammation (day 2), acute rejection (day 15) and chronic rejection (day 42).

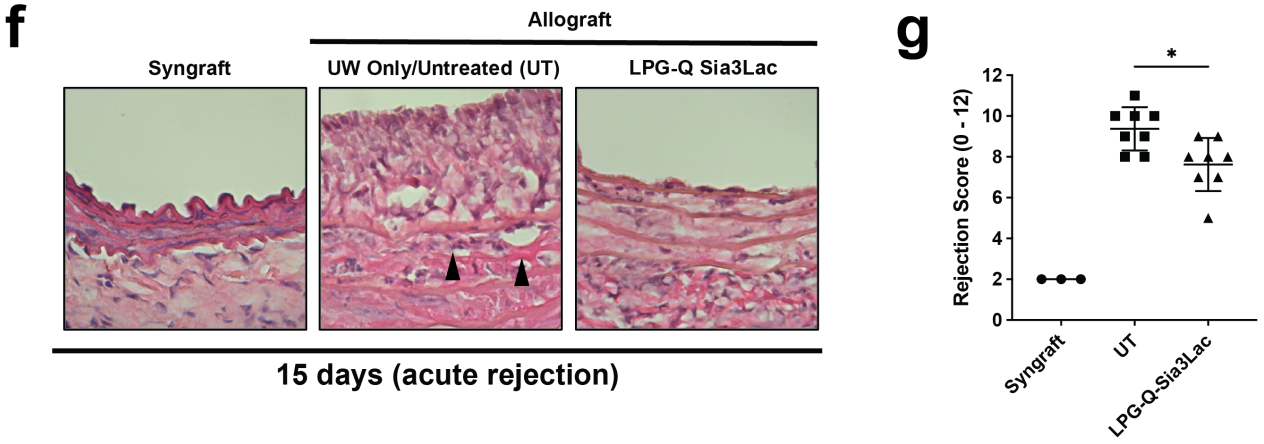


Figure 4f & g: Organ engineering via polymer-based CSE of blood vessel lumen. All polymer ligations were done in UW solution fortified with 0.5 mM LPG-Q or 0.56 mM LPG-Q-Sia3Lac, 3 mM GSH, 5 mM $CaCl_2$, 0.2 U/mL gtTGase for 1 hour at 4 °C. Untreated (UT) and syngraft groups were treated with UW solution fortified with 3 mM GSH, 5 mM $CaCl_2$ and 0.2 U/mL gtTGase. **f**) Treatment of blood vessels with LPG-Q-Sia3Lac (n=8) inhibits acute rejection 15 days post-transplant compared to UT allografts (n=8). Grafts were harvested 15 days post-transplantation and cross sections were stained with H&E to reveal effects of acute rejection. **g)** treatment of blood vessels with LPG-Q-Sia3Lac reduced rejection score 15 days post-transplantation. Rejection of grafts was scored on a 0 – 12 scale. Error bars represents 95% confidence intervals. Unpaired comparisons using a non-parametric t-test are significant with $p > 0.05$ (ns), $p \leq 0.05$ (*), $p < 0.01$(**), $p < 0.001$ (***).

Comment #7: The alloimmune humoral response (i.e. generation of donor-specific antibodies) should also be evaluated (see comment above on humoral rejection).

Response: We thank the reviewer for this comment. We have quantified donor specific antibodies in our transplant model and find that the CSE modification of allograft arteries significantly reduces development of donor specific antibody expression compared to an untreated control grafts (Fig. 4j).

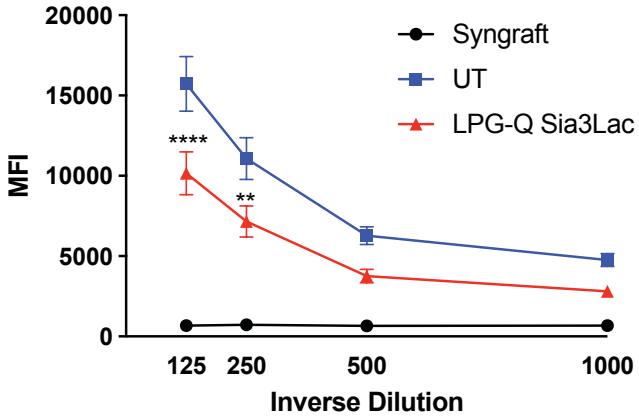


Figure 4J: Donor specific antibodies levels in organ engineering via polymer-based CSE of blood vessel lumen. Donor specific antibodies levels were quantified from sera collected at 42 days post-transplantation. Treatment of blood vessels with LPG-Q-Sia3Lac reduced donor specific antibodies in recipient mice. Error bars represents 95% confidence intervals. Unpaired comparisons using a non-parametric t-test are significant with $p > 0.05$ (ns), $p \leq 0.05$ (*), $p < 0.01$(**), $p < 0.001$ (***).

Comment #8: In the aortic interposition model, the intensity of chronic rejection is often (as in this study) judged upon the size of the neointima. However, neointima is a non-specific feature that develops after almost any kind of arterial injury, including perturbations of laminar flux due to end to end anastomoses. Therefore, analysis of the size of neointima to evaluate chronic rejection (i.e. the consequence of chronic alloimmune response against the graft) can lead to misinterpretation, especially when: i) the group of animals are small (here n<5), ii) nothing is made to ensure that the distance between the anastomosis and the aortic cross-section analyzed is the same in all samples (This confounding factor is quite difficult to control and the authors provided no detail about how they dealt with it).

Response: We agree that a careful and systematic approach for measuring intimal thickness is important to ensure that we are evaluating arterial changes that are driven by allogeneic immune responses. The standard protocol that we have used and published involves examining cross-sections of artery grafts obtained by sectioning 100 um past the sutures to ensure that the intimal thickening measured is not a result of the surgical procedure or alterations in flow that can occur at the site of anastomosis. In order to ensure that our findings are robust, we have now evaluated additional sections in all the allograft arteries examined at day 42 post-transplantation. Intimal thickening was quantified in two additional sections of all allograft arteries that were ~50 and 100 um further into the harvested artery sections (ie. further past the suture sites). All together, intimal thickening was quantified in three sections/artery graft that were 100, 150 and 200 um past the suture site. The mean of these measurements was calculated to determine the intimal thickening in each graft. When this additional analysis is performed, the findings are the same as what we report in the original manuscript. CSE modification of artery grafts substantially and significantly reduces intimal thickening (Fig. 4i). The Methods section has been revised to clearly describe this method for quantifying intimal thickening.

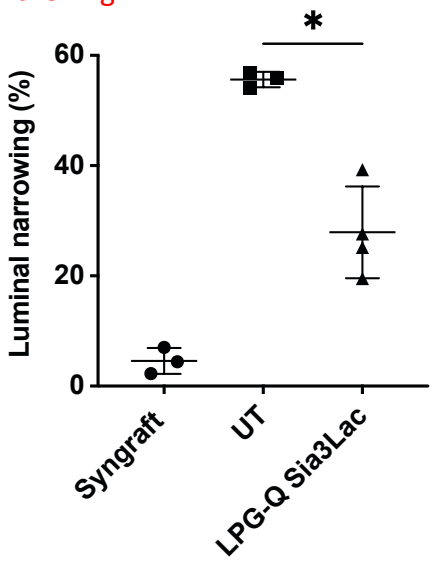


Figure 4 i. Luminal narrowing in grafts 42 days post-transplantation. Grafts were harvested at day 42 post-transplantation and cross-sections stained with H&E. Quantification of luminal narrowing in grafts. Error bars represents 95% confidence intervals. Unpaired comparisons using a non-parametric t-test are significant with $p > 0.05$ (ns), $p \leq 0.05$ (*), $p < 0.01$(**), $p < 0.001$ (***).

Comment #9: The authors claim that "polymer-mediated organ engineering approach […] leads to […] localized immune suppression". However, they never demonstrate that recipients respond normally to i) an (unmodified) graft from the same donor placed elsewhere, and ii) another immune stimulus (vaccination? or 3rd party allograft). These experiments are needed because I do not

understand how the very transient presence (1/2 life is 8H according to figure 1e) of the polymer on graft endothelial cells can induce such a long-term protective effect against alloimmune response. Could it be that endothelial cell surface engineering induces immune tolerance (which can be allospecific or not)…Experiments should be performed to clarify this point.

Response: This is a very important point and we thank the reviewer for suggesting the experiments. To examine whether the immunosuppressive effect of localized treatment of grafts with LPG-Q-Sia3Lac was restricted to grafts or led to systemic immunosuppression or tolerance, skin grafts were performed onto artery graft recipients at day 28 post-transplantation. There was no rejection of syngeneic skin grafts but skin grafts from Balb/c donors (the same donor strain as artery grafts) were rapidly rejected by day 10 after skin transplantation (Fig 4k). Also, third party skin grafts from C3H donors rejected with similar kinetics as Balb/c grafts (Fig. 4l). These findings establish that the protective effects of the CSE approach are limited to the treated graft and do not result in systemic immunosuppression or tolerance. We agree that the mechanism by which this occurs is interesting and this is the focus of current studies.

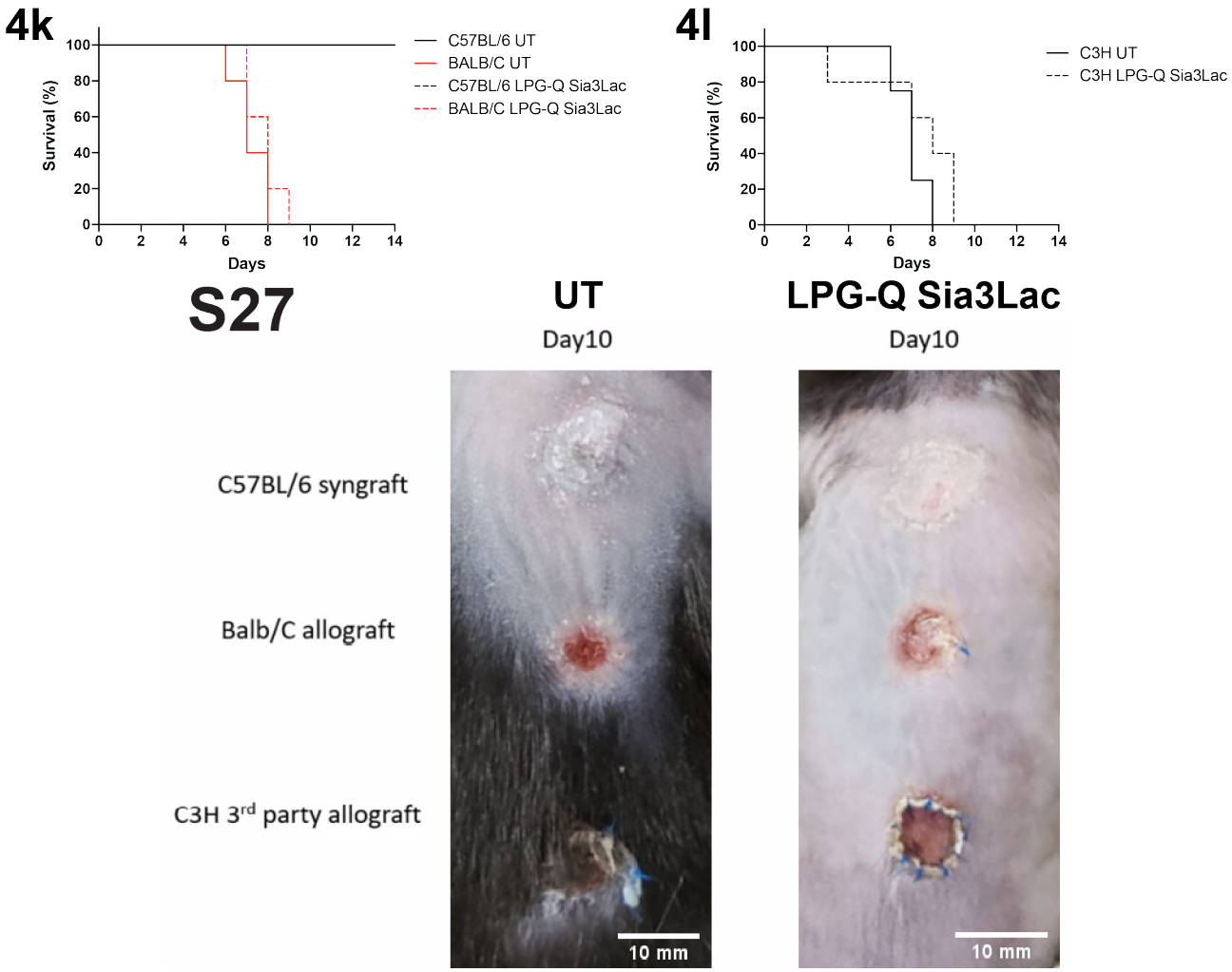


Figure 4k,l and supplementary figure S27. Survival of skin grafts in artery transplanted mice. Skin from syngeneic and allogeneic (Balb/c and C3H third party) donors were grafted onto mice that received untreated and LPG-Q-Sia3Lac treated allograft blood vessels 28 days after artery transplantation. Survival of skin grafts in artery transplanted mice. **k)** Balb/c and **l)** C3H skin grafts reject rapidly and similarly in mice that received artery grafts treated with LPG-Q-Sia3Lac as compared to UT arteries. **S27)** Representative images of skin grafts.

Comment #10: (figure 2g) Activation status of NK is usually evaluated by measuring: CD107a on the surface of the cells and/or cytokines or chemokines (IFN, MIP1b) in their cytoplasm. I have no idea

what more (or less) SHP-1 protein in NK means regarding the activation status of the cells. SHP-1 is a phosphatase, which regulates some signaling pathways, the activation status of which is usually determined by performing WB of phosphorylated substrate proteins.
The question of the activation status of NK is however interesting because: in one hand endothelial cell surface engineering could prevent the ligation of "stress induced ligands" to NK activating receptors and/or even deliver inhibitory signals through siglecs (but not all NK subsets express siglecs)… However, on the other hand, endothelial surface engineering could also impede the delivery of inhibitory signals, thereby triggering "Missing self"-induced activation of NK, a process that has been recently shown to promote rejection (Koenig et al, Nat Commun. 2019 Nov 25;10(1):5350).

Response: The examination of SHP-1 expression was originally intended to examine potential activation of NK cells. Although expression of total SHP-1 is increased by CSE modification and this suggests that there reduced NK cell activation, we agree that this data is not as accurate as examining phospo-SHP-1. As such, we have removed this data from the manuscript. We believe that the experiments showing reduced protection of CSE modification when NK cells are depleted from PBMCs and our new experiments showing that CSE protects endothelial cells from CAR T cell-mediated killing robustly demonstrates that the technology inhibits NK cell and T cell responses relevant to transplant rejection. A discussion of possible mechanisms by which this could occur is included in the Discussion.

Reviewer #2

(Remarks to the Author): In this manuscript the authors describe an approach for engineering the surface of blood vessel endothelial cells with glyco-polymers that prevents immune cell targeting and damage. The major strengths of the report are the elegant method for decorating the surface of endothelial cells with glyco-polymers and demonstrating the impact of the engineered vessels organs on suppression of immune attack upon transplantation into animals.

The polymer design using a functionalized polyglycerol with alkyne clickable handles linked to a peptide that serves as a substrate for pig liver tissue transglutaminase, which allows for enzymatic attachment of the polymer to lysines exposed on cell surface membrane proteins. The alkyne handle(s) allow for facile attachment of glycans with azide terminated linkers using copper catalyzed 'click' chemistry. Although the copper is very toxic, it can be readily removed from the polymer before exposure to cells.

Engineering of cell surfaces of an epithelial cell line monolayer with the glycopolymers in organ storage solution at 4° C is efficient, and the polymers protect against damage/cytotoxicity to PBMCs, in particular, NK cells. The hypothesis is that sialic acid containing glycans will suppress immune responses that cause tissue rejection by recruiting inhibitory Siglecs that recognize sialic acid containing glycans as their ligands. While the hypothesis is attractive and consistent with the findings, there is really little data showing that Siglecs are involved in the protection.

While the mechanism may not be pinned down, pretreatment of the organs with the glycopolymer engineering protocol ex vivo is shown to impressively reduce/prevent tissue damage and rejection of syngeneic Balb/c blood vessels and kidneys upon transplantation into C57BL/6 mice.

Response: We thank the reviewer for positive outlook for our manuscript and support. We have performed several additional experiments that, we believe, substantially improve the manuscript. We provide details below.

Comment #1: While the suggestion that the glyco-polymers recruit siglecs to suppress immune responses is attractive, evidence that they are playing a role is lacking. Simple experiments showing that Siglecs are expressed on PBMCs using commercially available antibodies and showing that these Siglecs bind to the modified cells using commercially available Siglec-Fc chimeras would be supportive.

Response: We thank the reviewer for bringing this point. Our early investigations and preliminary evidence suggest the involvement of siglecs. However, although siglecs are potentially a contributing factor there may be other mechanisms. [redacted] We believe that these experiments are out of scope for this manuscript considering the amount of data presented in the revised manuscript (6 main figures and 28 supplemental figures) and that the data is the first presentation of the CSE approach in transplantation rejection. Characterizing the expression of siglecs on PBMCs would be interesting but would not definitively establish these receptors in the mechanism of action of our CSE modification. In light of this, we have decided to reword the mechanistic aspect in the manuscript to not overreach any claims. We propose possible mechanisms in the revised manuscript. Hopefully the reviewer can agree with this.

Comment #2: The text imply that T cells express Siglecs, but dogma has it that T cells in PBMCs in mice do not express Siglecs. There are several reports that suggest human T cells express Siglecs, particularly in the context of tumor infiltrating T cells. But these experiments are in mice.

Response: Our study provides proof-of-principle for the local immunosuppressive potential of a novel CSE modification and the reviewer is correct that the mechanism by which it acts remains unclear. Siglecs are likely candidate receptors that may be controlling the immune inhibitory effects that we observe. Members of this receptor family are known to be expressed by NK cells, monocytes/macrophages, dendritic cells and B cells in mice (Duan and Paulson, Ann Rev Immunol, 2020). We do not know the expression of siglecs in T cells in our mouse studies. If mouse T cells do not express any siglec, the immunosuppressive effect of CSE on T cells that we observe may involve indirect effects of the CSE approach through actions in dendritic cells or could be mediated by direct interactions with molecules that are not siglecs. We have clarified these possibilities in the discussion of the revised manuscript.

Comment #3: The data in Figure 2f are particularly informative, but it is not clear that unpaired T tests are an appropriate test for comparisons between groups. Better might be one-way Anova. It appears that removal of NK cells has significant impact on protection of endothelial cells, while removal of T cells or monocytes does not. While this is implied in the text, it is not shown statistically.

Response: We thank the reviewer for this suggestion. The statistical analysis has been reperformed using a one-way ANOVA test. The data is now moved to Figure 3f in the revised manuscript. We have updated the contents of the paragraph to highlight the difference between different immune cell suppressed cohorts.

Comment #4: UW should be added as an abbreviation. This reviewer had to search Google to understand what UW was (e.g. cold preservation solution). This article is intended for a general audience.

Response: We thank the reviewer for this suggestion and have updated our abbreviation list to include UW.

Reviewer #3

(Remarks to the Author): This is an innovative manuscript using cell surface engineering of the endothelial layer to minimize damage and rejection locally during organ preservation and subsequent transplantation. The protocol uses enzymatic polymer ligation to the endothelial surface and is likely to be scalable to human organs as it is a relatively easy method to apply. The authors claim near complete prevention of acute and chronic rejection. The manuscript is well executed, the results are exciting, and the idea is innovative. However, there are several issues that need to be addressed.

Response: We thank the reviewer for the positive outlook for our manuscript. In the following paragraphs, we respond to the specific comments. We have also performed several additional experiments to further solidify the concept.

Comment #1: The literature coverage of the state-of-the art for both new approaches to immune suppression and organ preservation are completely absent and not acceptable. The authors need to present their idea within the context of the state-of-the-art

Response: We thank the reviewer for their comment. We recognize that we needed to supplement our current introduction with previously reported technology. We now have included commonly used immunosuppressive therapeutics such as tacrolimus and mycophenolate mofetil in the introduction. We acknowledge their administration but also their limitations in global immunosuppression. We present solutions by previous researchers using encapsulation technology like immune-triggered hydrogels for their safe release and its administration ex vivo pre-transplantation as a gel. Moreover, we also state a nano-barrier membrane made of proteins to achieve immunocloaking. However, this technology requires ex vivo warm perfusion in order to be effective. We then ultimately detail the advantage of our technology being compatible with the more facile and commonly employed cold static storage for the organ preservation step. We believe that the presentation of these previous technologies support the motivation for our current technology and provide a good comparison with different technologies.

Comment #2: The paper makes a lot of statements about preservation but this is clearly over-reaching as there is really no study of preservation except few conditions like 4 hours on ice. This is not considered proper organ preservation. The paper needs to be significantly re-written to avoid any claims that are not supported by the results.

Response: We thank the reviewer for raising this point. Our illustration of the technology examines only one condition. There is certainly important research that is being performed on development of novel technologies for organ preservation (Friend, Peter J. Transplantation, 2020; Eshmuminov et al. Nature Biotech, 2020; Vries et al., Nature Biotech, 2019). Thus, we have rewritten the manuscript to ensure that we focus on the local immunomodulatory/immunosuppressive aspect of the technology after transplantation into the recipient rather than effects during preservation. In

principle our CSE approach could be used in conjunction with many of the current organ preservation approaches. We have expanded the discussion to clearly articulate the potential application of the CSE technology.

Comment #3: The animal model used is not gold standard but rather the lowest bar that can be used for a preliminary testing. There have been multiple exciting papers published in the last 3 decades or more on complete abolishing of the immune rejection in syngeneic mouse models. The claims again significantly over-reach from what can be supported by the data and the animal model used to test.

Response: We thank the reviewer for raising this point. Please see response to reviewer 1 comment # 5 that describes the value of the aortic interposition model for studying transplant rejection. We have also further developed the allogeneic aortic interposition model to include 3 different time-points to reflect early inflammation, and acute and chronic rejection (Fig. 4a-j). Lastly, we have also confirmed the localized immunosuppressive aspect of the CSE approach by performing secondary skin grafts on recipients of artery transplants (Fig. 4k,l).
Notably, in the revised manuscript, we have performed an allogeneic kidney transplantation in mice in addition to the aortic interposition and syngeneic kidney transplant models. The previous syngeneic kidney transplant model was performed to examine protection against cold ischemic perfusion injury. The new data using an allogeneic kidney transplant model is directly applicable to kidney transplant rejection and is reported in Figure 6 of the revised manuscript. It demonstrates significant protection offered by the CSE approach for reducing damage of allogeneic kidney transplants.
We also agree that mice data does not represent the full scale of immune responses that drive transplant rejection in humans. We have carefully worded our discussion to make sure that we are not over claiming.

Reviewer #4

(Remarks to the Author): The manuscript by Siren et al. describes a novel approach for localized immune therapy to minimize transplant damage after surgery and prevent transplant rejection. Specifically, the authors utilize immune-deactivating polymers localized to the lumen of the blood vessels to prevents graft injury and transplant. Polymers were synthesized and evaluated for immune suppressive and anti-inflammatory activity in vitro, followed by in vivo evaluation in two different mice models. Overall, the approach is interesting and shows potential for preventing transplant rejection. Modification of grafts in static cold storage condition is also impressive. The manuscript is well written and the experiments are thorough. However, there are a few concerns, which if clarified will further improve this work.

Response: We thank the reviewer for highly positive feedback and outlook for our paper. Our response to the specific comments is below.

Comment #1: Would be great if the authors can explain the rationale for choosing day 42 as the time point to evaluate chronic rejection in murine aortic allograft transplant model? Is it possible to look beyond day 42? The reviewer understands that repeating such a long experiment to look at further time points might be difficult, but it would be great if the authors can discuss this at least.

Response: It is important to understand the kinetics of arterial changes that occur in the aortic interposition transplant model and we appreciate the reviewer asking for this clarification. This

model involves early inflammation followed by acute and chronic rejection. Chronic rejection is reflected in the development of intimal thickening that occurs as a result of immune-mediated arterial injury and cytokine production. This intimal thickening is robust by day 30 post-transplantation and can be very extensive by day 60 post-transplantation (von Rossum et al., Transplantation, 2016). We chose day 42 to examine intimal thickening in order to observe a time-point in which this arterial change is robust but therapeutic inhibition of its development can be measured accurately. We agree with the reviewer that a more extensive analysis of the effects of the CSE modification on rejection is valuable and have examined an earlier time-point (day 15) to precisely examine acute rejection. Consistent with our observation that CSE modification inhibits early inflammation and chronic rejection, it also significantly reduces acute rejection. In the revised manuscript, we have described reasons for examining artery grafts at the time-points chosen (days 2, 15, 42) (Figure 4).

Comment #2: Is there a positive control that can be included in Fig. 3b and c? It would be interesting to see how their results compare to the positive control.

Response: We are happy to clarify the experiments and comparisons that the reviewer inquires about. The experiments presented in Fig. 3b and c (which are now Fig. 4b and c in the revised manuscript) examines the protective effect of the CSE modification. The grafts bathed in UW grafts (referred to as untreated) are unmodified grafts that serve as the positive control (i.e. they undergo extensive inflammation and eventual rejection). The CSE modified grafts are compared to these unmodified grafts to determine efficacy.

Comment #3: The authors mention that "LPG-Q-Sia3Lac also reduced histological features of medial inflammation such as medial necrosis and leukocyte infiltration (Fig. 3b-d)." However, the figures don't show anything related to necrosis or leukocyte infiltration.

Response: The authors appreciate the comment made by the reviewer. The figure 3 has been changed to Figure 4 with more data in the revised manuscript. We understand that this may not have been clear in our previous version of the manuscript. We have included arrows to indicate leukocyte infiltration and arrowheads to highlight areas of medial injury, the latter involving structural changes indicative of early injury but not necessarily necrosis. These features have also been clarified in the text of the revised manuscript.

Comment #4: In the in vivo studies described in Fig. 3, its interesting to see that LPG-Q Sia3Lac shows better results than LPG-Q. Since LPG-Q did not show any significant effect compared to UT, one crucial question is what would happen if only sialic acid is grafted on to the tissue without the polymer. It would be great if authors can demonstrate that, even during the early time point.

Response: This is an interesting point. Our goal in this paper was to utilize both the steric property and multivalency of glycopolymers in immunosupression as a mimic of native glycocalyx. The native glycocalyx offers both steric as well as immunosuppressing properties. Thus, we have sought to use the two polymer models employed here.

It would be interesting to examine how directly incorporating sialic acid onto the graft affects rejection. However, this is very challenging under the conditions of graft perfusion and storage. [redacted] However, these experiments are in the very early stages and, we believe, beyond the scope of the current manuscript because the revised manuscript already includes 6 main

figures and 28 supplemental figures dedicated to robustly demonstrating the protective effect of the CSE technology in transplantation. Hopefully the reviewer can agree on this.

Comment #5: For kidney transplant model, did the authors follow animals after day 7? Although the effect is great at day 7, the manuscript will become really strong if the authors can include data for longer period, even if its for 20-30 days. Day 7 is too short for evaluation of immunosuppression.

Response: We acknowledge the point brought forth by the reviewer. We agree that the our CSE approach would be more convincing with data incorporating longer time points. In light of the reviewer comments, we have performed several additional experiments to strengthen this aspect of the manuscript.

Our initial aortic interposition graft model in mice (Day 15 [new data] and Day 42) illustrate the potential of CSE-based organ protection in an allogenic model. We further demonstrate using a skin graft model (3rd party skin grafts in an artery transplanted mice) that the effect is localized to the transplant.

In light of this data, we examined the ability of the CSE approach to inhibit rejection of allogeneic kidney transplants. This data is presented in figure 6 of the revised manuscript. The allogeneic model we use is long enough to evaluate the immunological impact of our CSE approach as per the reported literature (Qiu et al. Kidney International, 2020). This experiment provides proof-of-principle data establishing the ability of the CSE approach to reduce acute kidney transplant rejection. In future experiments we will perform additional long-term experiments. Hopefully the reviewer can agree that the new experiments we have performed establish the therapeutic potential of the CSE approach in kidney transplantation.

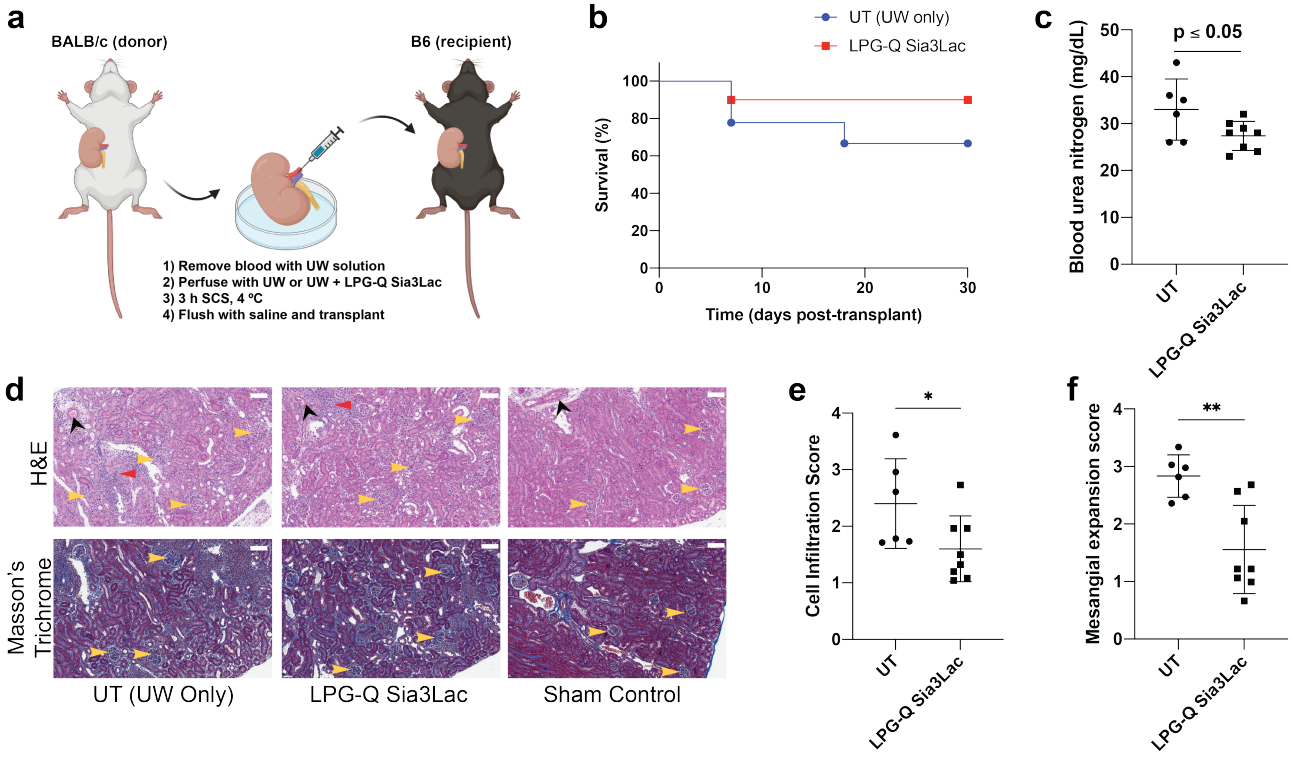


Figure 6: Organ engineering via polymer-based CSE imparts improved protection against immune mediated injury in an allogenic renal transplant mouse model. All polymer ligations were done in UW solution fortified with 0.56 mM LPG-Q-Sia3Lac, 3 mM GSH, 5 mM $CaCl_2$, 0.2 U/mL gtTGase for 3 hours at 4 °C. Untreated (UT) groups were treated with UW solution fortified with 3 mM GSH, 5 mM $CaCl_2$ and 0.2 U/mL gtTGase. **a**) Overview of allogeneic renal transplant model, kidneys from Balb/c donors into B6 recipients. **b**) Treatment of kidney grafts with LPG-Q-Sia3Lac

improves survival of mice (N=8) 30 days post-transplantation. **c**) Blood urea nitrogen levels were improved in LPG-A-Sia3Lac treated mice. **d**) kidneys were harvested 30 days post-transplantation and cross sections were stained with H&E and Masson's Trichrome. Glomerulus is indicated by yellow arrows, arcuate artery by black arrows and severe cell infiltration by red arrows. Scale bars (white) = 100 μm. **e**) Treatment of kidneys with LPG-Q-Sia3Lac reduces cellular infiltration of renal tissue analyzed using histological scoring. **f**) Mesangial expansion of the glomeruli in renal tissue was scored using Masson's Trichrome stain to reveal transplant rejection. Treatment of LPG-Q-Sia3Lac reduced mesangial expansion. Error bars represents 95% confidence intervals. Unpaired comparisons using a non-parametric t-test are significant with $p > 0.05$ (ns), $p \leq 0.05$ (*), $p < 0.01$(**), $p < 0.001$ (***), $p < 0.0001$ (****).

Comment #6: The authors say "Confocal microscopy confirmed covalent attachment". This is a bit incorrect as confocal microscopy cannot confirm "covalent attachment". It can only confirm "attachment".

Response: We thank the reviewer for bringing this point. We acknowledge that the terminology is incorrect and that the type of attachment cannot be confirmed using confocal microscopy. We removed the word "covalent."

Comment #7: "Bioavailability of ICAM" is an inappropriate term. "Bioavailability" is usually used in the context of pharmacokinetic studies.

Response: We thank the reviewer for bringing this point. We acknowledge this error and have corrected the terminology from "bioavailability of ICAM" into the "accessibility of ICAM."

Comment #8: Authors used a scale of "0-4" to score histology in figure 3. What does each point on the score indicate?

Response: We thank the reviewer for bringing this point. We have described in greater detail the scoring methodology in the revised manuscript. We have detailed the histological features that are considered and also included a reference of the same method used in previous literature. These references have been added or additional information has been added in the supplementary information.

Histological features of arterial injury and inflammation at day 2 post-transplantation were graded in a blinded manner on a 0 – 4 scale that considered the amount of medial injury and leukocyte infiltration. Medial thickness was quantified using ImageJ software (NIH) as described (Rey at al. Transplantation, 2018). Histological features of acute rejection at day 15 post-transplantation were graded in a blinded manner on a 0 – 12 scale because acute rejection involves more extensive features of immune reaction and injury compared to day 2 post-transplantation. Specifically, there is extensive infiltration of the arterial media by host leukocytes that causes medial damage characterized by swelling, death of smooth muscle cells, damage to the elastic laminae, fibrin deposition in severe cases, and early development of intimal thickening. Leukocyte infiltration, medial injury and intimal thickening were each graded on a 0 – 4 scale and an aggregate score calculated. For evaluation of chronic rejection, intimal thickening at day 42 was quantified using ImageJ software (Enns et al. Journal visual experiment, 2015; Choy et al. Americn Journal of Transplantation, 2005; von Rossum et al. Journal of Immunology, 2013).

Comment #9: The discussion can be strengthened a bit more. For example, authors should discuss the choice of time points for measuring end points in the animal model. Similarly, the authors need to comment on how do they envision the clinical re-application of this technology, i.e. once the polymer is cleared (as demonstrated by authors in cell studies), do they envision this polymer to be reapplied. If yes, how will that be feasible?

Response: We thank the reviewer for this point. We have expanded the discussion to explain the time-points chosen in the animal models as well as the clinical application and limitations of the CSE approach.

See pages 9-10, 12 for the modification.