nature biomedical engineering

PEER-REVIEW INFORMATION

**Synthetically glycosylated antigens for the antigen-specific suppression of established immune responses**

Corresponding author: Jeffrey Hubbell

## 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

Fri 30 Sep 2022
**Decision on Article nBME-22-2035**

Dear Prof Hubbell,

Thank you again for submitting to *Nature Biomedical Engineering* your manuscript, "Suppression of Established T Cell Immunity and Disease by Immunotherapy with Synthetically Glycosylated Antigen". The manuscript has been seen by three experts, whose reports you will find at the end of this message.

You will see that the reviewers appreciate the work. However, they express concerns about the degree of support for the claims, raise a substantial number of questions, and provide useful suggestions for improvement. We hope that with significant further work you can address the criticisms and convince the reviewers of the merits of the study.

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 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 16 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*.

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,

Pep

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Pep Pàmies
Chief Editor, *Nature Biomedical Engineering*

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

In this manuscript, the authors evaluate immune responses to their previously reported tolerance inducing therapeutic, pGal-Antigen, in several models. These include murine models of OVA-specific tolerance induction after immunization to OVA, as well as two murine EAE models of multiple sclerosis, and a non-human primate model involving antigen-specific tolerance induction after immunization. Treatment with pGal-Antigen constructs suppresses T-cell responses in an antigen-specific manner, and resolution of EAE symptoms by pGal-MOG treatment is particularly impressive. Antigen-specific T-cell responses in immunized non-human primates are also reduced after subsequent treatment with pGal-Nef. This non-human primate model developed to evaluate immune tolerance inducing treatments may be of interest to those developing novel immunotherapeutics. Collectively, these studies yield an improved understanding of the mechanisms of tolerance induction by pGal-Antigen in the context of
pre-existing immunity, and support translational potential of this therapeutic approach, which currently is being evaluated in two clinical trials for autoimmune diseases (celiac disease and multiple sclerosis). The evaluation of the immunotherapeutic in multiple animal models, robust therapeutic responses in the murine EAE models, and demonstrated effects in a translational non-human primate model would be of interest to the readers of Nature Biomedical Engineering.

Major Technical Criticisms or Questions:

1) In Figure 5c, treatments with pGal-MOG18-62 and free MOG35-55 peptide are compared. Since these treatments differ in terms of both pGal and peptide length/sequence, the results of this experiment do not conclusively demonstrate the requirement of pGal in this model. Free MOG18-62 is a more appropriate control, and repeating the experiment with free MOG18-62 could support the following claim made in lines 198-200: “In contrast to pGal-MOG18-62 treated groups, groups treated with free MOG35-55 on days 0, 3, and 6 were unable to control disease, indicating the importance of the pGal-mediated tolerance induction in this model (Figure 5c).” If this experiment is not repeated with the MOG18-62 control, the presence of a confounding variable (i.e., use of a different free peptide, MOG35-55) should be noted in the results, and the conclusion rephrased to acknowledge the fact that the different peptide sequence could potentially affect the results.

2) Lines 262-264: “There were no global changes in CD3+, CD4+, CD8+, NK cells, monocytes or B cells (Supplemental Table 14) further confirming that these results were due to antigen specific tolerance rather than bulk immunosuppression.” – Non-specific immunosuppression does not necessarily lead to substantial changes in immune cell frequencies, as a percentage of total PBMCs (as reported). Since NHP were immunized against multiple antigens (Nef, Gag, Env, and Pol) and only tolerized with pGal-Nef, demonstrating comparable antigen-specific responses to one or more of the other antigens in the control and pGal-Nef treatment groups would provide stronger evidence for immune competence (lack of non-specific immunosuppression). Alternatively, complete blood counts (T cells, NK cells, etc. per mL blood) would also provide more evidence of immune competence, or lack thereof.

3) Reporting of statistical differences is inconsistent throughout the figures and supplemental figures. In particular, the methods state: “for showing statistical significance ***P≤0.001; **P≤0.01; *P≤0.05, unless otherwise stated” (Lines 454-455); however, these symbols are used only in Figures 6A-B, 7C, S1, S2A-D, S5B, and S8A. All other figures report specific p-values. Furthermore, some graphs include specific p-values > 0.05, while others do not show p-values for non-significant differences, and this may even vary within the same figure. Finally, some figure legends (Figures S5, S6, and S9) list symbols for p-values when actual p-values are presented in the figures, and others (Figure S8A) use symbols in the figure, but do not provide a key in the legend. Reporting of statistical differences should be made consistent throughout the manuscript to facilitate better evaluation of results.

Minor Technical Criticisms or Questions:

1) The authors should provide a brief justification for why 10-fold different doses of anti-PD1 (100 µg) and isotype control antibody (1 mg) were used for the antibody blockade experiment (Supplemental Materials, Lines 18-19; main document, Line 548). Could this have any effect on the therapeutic outcomes?

2) Lines 123-127: “In the LmOVA model, pGal-OVA treatment reduced antigen-specific CD8+ T cell expansion and peptide-responsive cytokine-secreting cells (Supplemental Figure 6a,b,g,h).” – No stats are shown in Figure S6A,B,G,H to indicate a significant reduction in OTI frequency, absolute number, or cytokine production. Since the effects of pGal-OVA on these cells are not significant in this particular model, the corresponding statement should be revised to avoid overstating the results.

3) Lines 87-88: “Upon peptide re-stimulation, the remaining OTIs produced less IFNγ and TNFα while the OTIIs produced less IFNγ (Figure 1e) after all antigen therapies, however only after pGal-antigen therapy did the OTIIs also produce less TNFα in the spleen indicating an exhausted state (Figure 1d)25,26.” – Since Figure 1e shows secretion of only IFNγ, but not TNFα, should the first statement in this sentence instead refer to Figures 1d-e?

4) Please specify the parent population for the flow plots in Figures 1B and 2B.

5) Flow cytometry plots in Figures S3A-C, S8G, and S10A-B are difficult to read due to low resolution images and small font size. Figure S7 is unreadable at the current resolution, and the first line of the y-axis label in Figure 7C is not legible. Improved figure resolution and/or increased font size for the axis labels and gating frequencies would be helpful.

6) Lines 105-106: “Analysis of cytokine production after six-hour peptide restimulation revealed that pGal-OVA treatment resulted in lower frequencies of IFNγ and TNFα-secreting OTI cells (Figure 2d).” – Figure 2d shows that pGal-OVA treatment resulted in lower frequencies of IFNγ+ OTI, but not TNFα+ OTI, cells in the LN, as well as lower frequencies of TNFα+ OTII cells in the spleen. This statement should be revised to better reflect the data presented in Figure 2d.

7) Figure S6B – Please note the parent population for these flow plots in the figure caption. Also, the axes labels (H2Kb:SIINFEKL and TCRβ) appear to be reversed in this figure. Assuming the parent population is total CD8+ T cells, the axes labels would indicate there is a large population of Pentamer+ TCRβ– cells (?), rather than Pentamer– TCRβ+ non-specific T cells.

8) Figure S8 is missing the volcano plot for pGal vs. OVA, CD4 genes.

9) For the EAE experiments in Figure 5, anti-VLA-4 was administered every other day for what period? The entire length of the study (0-22 days)? Please specify this in the figure caption and/or methods.

10) Lines 205-207: “Phenotypic analysis of the donor T cells demonstrated a trend towards reduced recovery of donor T cells in pGal-MOG18-62 treated groups, suggesting that deletion of active T cells may contribute to reduced disease scores (Figure 5e).” – Reduced recovery of donor T cells could be due to deletion (as suggested) and/or reduced proliferation of these cells after adoptive transfer. Unless there is additional evidence to support deletion, rather than anergy or exhaustion, these alternative mechanisms should also be acknowledged.

11) Lines 156-158: “In OTI cells, TP53-regulated pathways were inhibited, while pathways involving KLF3, TCF7, and MYC were activated. In OTII cells, KLF3 and TCF3 were also activated, and beta-estradiol and TCR-regulated pathways were inferred to be inhibited.” – A brief explanation of the relevance of these pathways to this particular therapeutic model would be helpful.

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

The team previously developed an antigen delivery strategy that targeted hepatic antigen-presenting cells in tolerogenic environments. The strategy is based on N-acetyl galactosamine-functionalized polymer (pGal) that carries conjugated protein antigen. In this manuscript, the team reported that a model antigen, OVA, delivered by pGal was able to suppress antigen-specific T cell responses, in both the effector and the memory stages. The team also provided data that connected the suppression effect of pGal-OVA to the PD-1 immune checkpoint and the B7-H3-mediated inhibitory pathways. Further, pGal-delivered EAE antigens resulted in clear suppression of EAE in mice. Lastly, the pGal-delivered antigen, Nef, accelerated the diminishment of Nef-specific T cell responses.

The reported study is on an interesting and important topic, antigen-specific immune tolerance. Information presented is useful to researches on this topic to an extent. The results related to EAE are impressive and promising in terms of therapeutic development. However, the information, overall lacks a significant innovation and the contents reported in this manuscript may not represent a significant advance in concept, technology, translational capacity, or fundamental understanding on antigen-specific immune tolerance. The weaknesses in coherence, description, study design and interpretation as detailed below cast further doubt on overall conclusions and impact of of this manuscript.

Major weaknesses:
1, pGal was reported in the team's 2019 publication, "Synthetically glycosylated antigens induce antigen-specific tolerance and prevent the onset of diabetes". There is no innovation in engineering, method, or technology. The efficacy of pGal-delivered antigen was reported in T1D model in the 2019 paper. The results of EAE reported in this manuscript is new but not groundbreaking.

2, The preparation of manuscript needs further refinement. Descriptions of research results are incomplete and incohesive, and important details are missed in all sections. Some paragraphs are inserted rather arbitrarily, such as paragraphs under "Tolerance via pGal-OVA therapy is evident in an endogenous repertoire". The writing of this section is challenging to understand as well. Many labels on figures are too small, blurring, and/or messy. Examples include Figure 1B, 2B, 3C, 7C, etc. The low-quality figures, combined with unclear texts and figure legends, impeded the understanding and judge of conclusions of this study.

3, The memory and effector stages, as highlights of this study, were not clearly defined. What are the molecular or cellular characterizations of these stages? It is uncertain that results reported are indeed related to memory and effector stage of cells? Memory and effector T cells were not phenotypically characterized and evaluated.

4, It is counter-intuitive to examine OTII (CD4 T)-mediated responses when OVA peptide, SIINFEKL, was used as an antigen. SIINFEKL is an MHC class I epitope that only interact with TCR of CD8 T cells. This design issue affects all studies/sections using both SIINFEKL and OTII cells. An explanation on how SIINFEKL induced tolerance is needed. In the group's 2019 publication, the whole OVA was used as the antigen.

5, When SIINFEKL is used as pGal delivered antigen, the free antigen control, logically should be the

peptide as well. However, in this study, whole OVA protein is used as control. In addition, the pGal should have been used a vehicle control, but not included. Without the vehicle control, all results of pGal-OVA can be attributed to pGal as well.

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

The authors present work that uses an N-acetyl galactosamine-functionalized polymer (pGal) fused to a protein antigen to induce antigen-specific tolerance therapeutically. The authors previously showed the prophylactic efficacy of this approach. The main model used here is EAE (chronic and relapsing-remitting experimental autoimmune encaphalitis) and an OVA-specific adoptive T cell transfer system. The latter uses the widely used TCR transgenic OTI and OTII mice as a source of T cells of defined specificity. The authors also include preliminary data in a non-human primate (NHP) study. The latter dataset is of limited scope and mechanistically is less important, although it does speak to translatability. On the whole, the manuscript describes a complex and rather heterogeneous dataset. My question of suitability for Nat Biomed Eng is really related to the issue of generalizability (see 9 below).

The novelty of this work lies in the delineation of a more detailed molecular mechanism (transcriptomic analyses) to explain the responses of antigen-specific CD4 and CD8 T cells when mice were treated with pGal-OVA. The NHP study aims at a similar goal. The authors have published a study that uses the pGal delivery system in a T1D model (Wilson et al, 2019).

Specific comments:

1. In the manuscript, the authors carry out RNAseq analyses on adoptively transferred CD4 and CD8 T cells (OTII and OTI cells respectively). These cells were obtained following immunization with OVA and challenged with pGal-OVA, saline or OVA. The authors also conducted RNAseq analyses in the prophylactic setting. RNAseq was performed on pooled RNA from spleen, liver, and peripheral lymph nodes. Is there a reason why the RNAseq analyses were not performed in an organ-specific manner?

2. Aside from PD1, have the authors investigate other potentially tolerogenic pathways implicated in the mechanism of action of pGal-OVA?

3. Since the PD1 pathway is involved, have the authors investigated whether the APCs that acquire pGal-OVA carry PD1 ligands?

4. In the chronic EAE model, have the authors tried to treat mice that reached clinical score 3 (complete hindleg paralysis), i.e. not when mice were asymptomatic?

5. The authors showed 3 doses of treatment to achieve the indicated therapeutic efficacy. What happens if animals receive one or two doses?

6. Minor: The y axis legend of Figure 7C is superimposed with random symbols.

7. Since the authors highlight the importance of PD1 pathway as one of the possible tolerogenic mechanisms, have the authors checked for upregulation of PD1 in PBMCs in the NHP model?.

8. Have the authors explored whether treatment with pGal-OVA results in any bystander immune suppression? Does treatment with pGal-OVA affect epitope spreading?

9. Do the authors have information on the use of pGal-modified antigens in mouse models other than for OVA? For example, would the use of pGAL-fluNP affect CD8 T cell responses against flu in the B6 mouse? This question really speaks to the extent that this treatment can be generalized. The authors are well-aware of the limitations of the various mouse models (T1D in the NOD mouse; EAE etc). It would be great if it could be shown that a CD8 T cell response against a dominant pathogen-derived epitope (Db-ASNENMETM) could be modulated, for example in the course of a flu infection.

Tue 09 May 2023
**Decision on Article nBME-22-2035A**

Dear Prof Hubbell,

Thank you for your patience in waiting for the feedback on your revised manuscript, "Suppression of Established T Cell Immunity and Disease by Immunotherapy with Synthetically Glycosylated Antigen". Having consulted with the original Reviewers #1 and #2 (whose comments you will find at the end of this message; Reviewer #3 unfortunately did not act on our chasing messages), I am pleased to write that we shall be happy to publish the manuscript in *Nature Biomedical Engineering*.

We will be performing detailed checks on your manuscript, and in due course will send you a checklist detailing our editorial and formatting requirements. You will need to follow these instructions before you upload the final manuscript files. In the meantime, please consider the minor points from Reviewer #1.

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

Best wishes,

Pep

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Pep Pàmies
Chief Editor, *Nature Biomedical Engineering*

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

Summary of Improvements Included in the Revision:
The authors have adequately addressed each of my comments from the original submission. In addition to adding clarifying text and discussion to the manuscript, they performed an experiment to demonstrate that treatment of NHP with pGal-Nef does not alter responsiveness of polyclonal T cells, supporting the hypothesis of antigen-specific tolerance induction rather than non-specific immunosuppression.

Minor Technical Criticisms or Questions:
1) The type of ELISPOT used for the new Supplemental Figure 18 should be noted in the figure and/or caption. Was this an IFNg ELISPOT, similar to the Nef-specific IFNg ELISPOT in Figure 7?

2) In the revised abstract, the authors claim “there are currently no antigen-specific technologies capable of inducing tolerance during an established immune response without incorporating non-specific immunosuppressive signaling molecules.” Several studies, however, have shown induction of antigen-specific tolerance without non-specific immunosuppressive drugs during established immune responses (e.g., in relapsing-remitting EAE and/or chronic EAE models). Such studies include: (1) Casella et al. Sci Transl Med (2020), PMID: 33148622 and (2) Krienke et al. Science (2021), PMID: 33414215. Demonstrating tolerance induction during active inflammatory responses is, as the authors note, important for translation and the current approach does appear to have translational potential. The authors should focus on these benefits without suggesting this is the first approach to induce tolerance during established immune responses without non-specific immunosuppressive drugs.

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

It is acknowledgeable that authors improved quality of figures and made important clarifications regarding confusing and deficient descriptions in the first draft. It is commendable that authors conducted further experiments and provide additional (control) data to strengthen conclusions.

**Rebuttal 1**

We greatly appreciate the responses from all reviewers. Based on this feedback we have updated the manuscript to address their concerns and we respond to each of their points below. In the course of revising this manuscript we have also incorporated some additional changes. For clarity, we updated the presentation of T cell cytokine expression data Fig 1d, Fig 2d, and Fig 4c. Additionally we concluded that a one-way ANOVA analysis was more appropriate for these comparisons than the two-way ANOVA we performed originally and have updated the statistics accordingly. Lastly, in the course of incorporating revision experiments we noticed an inconsistency in the gating of CD4+ FR4+ CD73+ anergic T cells and have updated the analysis and manuscript using a revised consistent gating scheme.

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

*Major Technical Criticisms or Questions:*

*1) In Figure 5c, treatments with pGal-MOG18-62 and free MOG35-55 peptide are compared. Since these treatments differ in terms of both pGal and peptide length/sequence, the results of this experiment do not conclusively demonstrate the requirement of pGal in this model. Free MOG18-62 is a more appropriate control, and repeating the experiment with free MOG18-62 could support the following claim made in lines 198-200: "In contrast to pGal-MOG18-62 treated groups, groups treated with free MOG35-55 on days 0, 3, and 6 were unable to control disease, indicating the importance of the pGal-mediated tolerance induction in this model (Figure 5c)." If this experiment is not repeated with the MOG18-62 control, the presence of a confounding variable (i.e., use of a different free peptide, MOG35-55) should be noted in the results, and the conclusion rephrased to acknowledge the fact that the different peptide sequence could potentially affect the results.*

We thank the reviewer for pointing this out. Figure 5C was mislabeled. The control peptide should be labeled as MOG30-60. While the pGal-MOG18-62 peptide construct is different than the free MOG30-60, both peptides contain the immunodominant MOG35-55 epitope used for vaccination and induction of the pro-inflammatory T cell responses and prevent the development of EAE in a similar manner. We have updated the manuscript accordingly:
"While the $MOG_{18-62}$ peptide is longer, both $MOG_{18-62}$ and $MOG_{30-60}$ contain the relevant immunodominant $MOG_{35-55}$ epitope used for vaccination. The ineffectiveness of $MOG_{30-60}$ treatment suggests the importance of pGal in disease suppression (**Figure 5c**)." (page 9)

*2) Lines 262-264: "There were no global changes in CD3+, CD4+, CD8+, NK cells, monocytes or B cells (Supplemental Table 14) further confirming that these results were due to antigen specific tolerance rather than bulk immunosuppression." – Non-specific immunosuppression does not necessarily lead to substantial changes in immune cell frequencies, as a percentage of total PBMCs (as reported). Since NHP were immunized against multiple antigens (Nef, Gag, Env, and Pol) and only tolerized with pGal-Nef, demonstrating comparable antigen-specific responses to one or more of the other antigens in the control and pGal-Nef treatment groups would provide stronger evidence for immune competence (lack of non-specific immunosuppression). Alternatively, complete blood counts (T cells, NK cells, etc. per mL blood) would also provide more evidence of immune competence, or lack thereof.*

We acknowledge the points brought up in this comment. We have included additional data in Supplemental Figure 15 representing the polyclonal T cell response in the NHP groups after PBMC stimulation with PMA at each bleed timepoint, quantified via ELISPOT. These data indicate there is no difference in the capacity for T cells to respond to stimulation after treatment with p(Gal)-Nef. We have also updated the manuscript accordingly:
"There were no global changes in CD3+, CD4+, CD8+, NK cells, monocytes or B cells (**Supplemental Table 14)** and no difference was found in the capacity of bulk T cells to respond to PMA simulation **(Supplemental Figure 18),** further confirming that these results were due to antigen-specific tolerance rather than bulk immunosuppression" (Page 12)

*3) Reporting of statistical differences is inconsistent throughout the figures and supplemental figures. In particular, the methods state: "for showing statistical significance ****P≤0.0001; ***P≤0.001; **P≤0.01; *P≤0.05, unless otherwise stated" (Lines 454-455); however, these symbols are used only in Figures 6A-B, 7C, S1, S2A-D, S5B, and S8A. All other figures report specific p-values. Furthermore, some graphs include specific p-values > 0.05, while others do not show p-values for non-significant differences, and this may even vary within the same figure. Finally, some figure legends (Figures S5, S6, and S9) list symbols for p-values when actual p-values are presented in the figures, and others (Figure S8A) use symbols in the figure, but do not provide a key in the legend. Reporting of statistical differences should be made consistent throughout the manuscript to facilitate better evaluation of results.*

The manuscript has been updated for consistency with the methods. For showing statistical significance ****P≤0.001; ***P≤0.001; **P≤0.01; *P≤0.05, unless otherwise stated. The threshold for significance was fixed at $p < 0.05$ and non-significant p values are not listed unless referenced in the manuscript.

*Minor Technical Criticisms or Questions:*

1) *The authors should provide a brief justification for why 10-fold different doses of anti-PD1 (100 μg) and isotype control antibody (1 mg) were used for the antibody blockade experiment (Supplemental Materials, Lines 18-19; main document, Line 548). Could this have any effect on the therapeutic outcomes?*

A 10-fold different dose of isotype control antibody was used compared to anti-PD1 to control for another unpublished group in the experiment utilizing a higher dose of antibody. Administering high doses of exogenous antibodies during the antigen-therapy treatment window could affect therapeutic outcomes by interfering with the circulation, uptake and effector functions of OVA-specific antibodies which could bind to pGal-OVA during this treatment window due to increased competition for Fc-receptors binding. If this is the case we should see it exacerbated in a higher dose of isotype control antibody. However using a high dose of isotype control antibody in the pGal-OVA treatment group does not result in abrogated tolerogenic effects, as seen when the OTI and OTII recovery is compared to that seen in Figure 1 pGal-OVA treatments. It is not expected that smaller doses of antibody such as those used in the anti-PD1 treatment would impact therapeutic outcomes to a greater extent than the high dose.
The manuscript has been updated to read:

"Since it is not expected that smaller doses of antibody would impact therapeutic outcomes to a greater extent than a high dose, Rat IgG2a Isotype control (Clone C1.182) antibody 1 mg per injection were purchased from BioXCell to account for another unpublished group in the experiment" (Supplemental materials page 1)

2) *Lines 123-127: "In the LmOVA model, pGal-OVA treatment reduced antigen-specific CD8+ T cell expansion and peptide-responsive cytokine-secreting cells (Supplemental Figure 6a,b,g,h)." – No stats are shown in Figure S6A,B,G,H to indicate a significant reduction in OTI frequency, absolute number, or cytokine production. Since the effects of pGal-OVA on these cells are not significant in this particular model, the corresponding statement should be revised to avoid overstating the results.*

Statistics for Supplemental Figure 6g (now listed as Supplemental Figure 11) were missing. The figure has been revised to show a statistically significant reduction in IFNγ produced upon restimulation in the pGal-OVA treatment group compared to saline. We have also revised the written statement to say "In the LmOVA model, pGal-OVA treatment led to a consistent, though non-significant trend towards reduced antigen-specific CD8+ T cell expansion and a significant decrease in peptide-responsive cytokine-secreting cells (Supplemental Figure 11a,b,g,h)." (Page 6)

3) *Lines 87-88: "Upon peptide re-stimulation, the remaining OTIs produced less IFNγ and TNFα while the OTIIs produced less IFNγ (Figure 1e) after all antigen therapies, however only after pGal-antigen therapy did the OTIIs also produce less TNFα in the spleen indicating an exhausted state (Figure 1d)25,26." – Since Figure 1e shows secretion of only IFNγ, but not TNFα, should the first statement in this sentence instead refer to Figures 1d-e?*

The written statement has been revised to refer to Figure 1d-e.

4) *Please specify the parent population for the flow plots in Figures 1B and 2B.*

The figure captions have been revised to specify the parent population is live CD19-F4/80-CD3+ CD4+ T cells in Figure 1 and live CD19- F4/80- CD3+ CD8+ T cells in Figure 2 .

5) *Flow cytometry plots in Figures S3A-C, S8G, and S10A-B are difficult to read due to low resolution images and small font size. Figure S7 is unreadable at the current resolution, and the first line of the y-axis label in Figure 7C is not legible. Improved figure resolution and/or increased font size for the axis labels and gating frequencies would be helpful.*

Figures have been enlarged and resolution has been increased.

6) *Lines 105-106: "Analysis of cytokine production after six-hour peptide restimulation revealed that pGal-OVA treatment resulted in lower frequencies of IFNγ and TNFα-secreting OTI cells (Figure 2d)." – Figure 2d shows that pGal-OVA treatment resulted in lower frequencies of IFNγ+ OTI, but not TNFα+ OTI, cells in the LN, as well as lower*

*frequencies of TNFα+ OTII cells in the spleen. This statement should be revised to better reflect the data presented in Figure 2d.*

Statement has been revised to say "Analysis of cytokine production after six-hour peptide restimulation revealed that pGal-OVA treatment resulted in lower frequencies of IFNγ-secreting OTI cells and TNFα-secreting OTII cells (Figure 2d)." (Page 5)

7) *Figure S6B – Please note the parent population for these flow plots in the figure caption. Also, the axes labels (H2Kb:SIINFEKL and TCRβ) appear to be reversed in this figure. Assuming the parent population is total CD8+ T cells, the axes labels would indicate there is a large population of Pentamer+ TCRβ– cells (?), rather than Pentamer– TCRβ+ non-specific T cells.*

The axes have been fixed and parent population is listed as CD8+ T cells.

8) *Figure S8 is missing the volcano plot for pGal vs. OVA, CD4 genes.*

The prophylactic gene overlay volcano plot for pGal vs OVA, CD4 genes is found in Figure 3D. This has been clarified in the figure caption.

9) *For the EAE experiments in Figure 5, anti-VLA-4 was administered every other day for what period? The entire length of the study (0-22 days)? Please specify this in the figure caption and/or methods.*

Anti-VLA-4 was administered every other day for the entire length of the study. This has been clarified in the methods.

10) *Lines 205-207: "Phenotypic analysis of the donor T cells demonstrated a trend towards reduced recovery of donor T cells in pGal-MOG18-62 treated groups, suggesting that deletion of active T cells may contribute to reduced disease scores (Figure 5e)." – Reduced recovery of donor T cells could be due to deletion (as suggested) and/or reduced proliferation of these cells after adoptive transfer. Unless there is additional evidence to support deletion, rather than anergy or exhaustion, these alternative mechanisms should also be acknowledged.*

The manuscript has been edited to say " Phenotypic analysis of the donor T cells demonstrated a trend towards reduced recovery of donor T cells in pGal-MOG18-62 treated groups, suggesting that deletion or impaired proliferation of active T cells due to anergy or exhaustion may contribute to reduced disease scores (Figure 5e)." (Page 9)

11) *Lines 156-158: "In OTI cells, TP53-regulated pathways were inhibited, while pathways involving KLF3, TCF7, and MYC were activated. In OTII cells, KLF3 and TCF3 were also activated, and beta-estradiol and TCR-regulated pathways were inferred to be inhibited." – A brief explanation of the relevance of these pathways to this particular therapeutic model would be helpful.*

The manuscript has been edited accordingly to provide more context on these pathways. "In OTI cells, TP53-regulated pathways were inhibited which is known to be associated with antigen-specific proliferative responses, while pathways involving KLF3 associated with lymphocyte, T cell master regulator TCF7, and MYC were activated. In OTII cells, KLF3 and TCF3, a transcription factor reported to regulate FoxP3 expression, were also activated, and beta-estradiol, known to enhance Th1 responses, and TCR-regulated pathways were inferred to be inhibited." (Page 7)

*Reviewer #2*

*1, pGal was reported in the team's 2019 publication, "Synthetically glycosylated antigens induce antigen-specific tolerance and prevent the onset of diabetes". There is no innovation in engineering, method, or technology. The efficacy of pGal-delivered antigen was reported in T1D model in the 2019 paper. The results of EAE reported in this manuscript is new but not groundbreaking.*

The pGal-antigen delivery system was designed to increase the delivery of antigen to APCs in the presence of the anti-inflammatory milieu of the liver and absence of inflammation to drive tolerogenic signatures. Previous published studies investigated pGal-antigen delivery as a prophylactic treatment in a system without inflammation. In this setting, naïve T cell responses can be fine-tuned from their first antigen encounter towards a tolerogenic fate and strong B cell responses can be avoided. There is a much higher barrier to generating tolerance during an active immune response or a memory response. Challenges include the circulating antigen-specific antibodies that may engage with antigen therapies and memory T cells poised for increased activation upon encounter with their cognate antigen. Furthermore in settings of autoimmune disease there is typically systemic inflammatory signals due to the damage inflicted by the immune system which risks pro-inflammatory antigen presentation during antigen immunotherapy which could exacerbate disease. Currently there are no antigen-specific technologies capable of inducing tolerance during these immune responses without including non-specific immunosuppressive signaling molecules. Therefore, the performance of pGal-antigen to induce tolerogenic signatures in settings of strong immune memory (such as CFA immunization) and systemic inflammation (such as relapsing-remitting EAE and d7 treated chronic EAE) is groundbreaking and critical for its translation as autoimmune diseases are treated only once clinical pathology is identified and strong immune responses have been established. The abstract has been edited to emphasize this.

*2, The preparation of manuscript needs further refinement. Descriptions of research results are incomplete and incohesive, and important details are missed in all sections. Some paragraphs are inserted rather arbitrarily, such as paragraphs under "Tolerance via pGal-OVA therapy is evident in an endogenous repertoire". The writing of this section is challenging to understand as well. Many labels on figures are too small, blurring, and/or messy. Examples include Figure 1B, 2B, 3C, 7C, etc. The low-quality figures, combined with unclear texts and figure legends, impeded the understanding and judge of conclusions of this study.*
We have edited the manuscript for clarity on page 5 and increased the quality of the figures.

*3, The memory and effector stages, as highlights of this study, were not clearly defined. What are the molecular or cellular characterizations of these stages? It is uncertain that results reported are indeed related to memory and effector stage of cells? Memory and effector T cells were not phenotypically characterized and evaluated.*

In this study we are defining the stages of an immune response as 'effector' and 'memory' phases based on the fundamental immunology definitions in Janeway's Immunobiology. It is well established that after T lymphocyte activation, there is a period of clonal expansion and differentiation into effector cells, which takes 4-5 days. This is the effector phase, or primary response. These effector cells can be cytotoxic T cell or helper T cells and they are found at the sites of immune insult and lymphoid tissues. Most of these T cells generated in the immune response will eventually die once the antigen has been eliminated. The T cells that persist after antigen has been eliminated are known as memory T cells. Once the effector cells have contracted, this is the memory phase of the response. These cells are characterized most fundamentally based on their ability to respond faster and with a greater magnitude than naïve T cells during a secondary challenge. In this study we are using the effector and memory stages of an immune response as dosing windows for treatment with pGal-antigen to examine the response under both conditions.

In order to ensure we were treating during a memory response we measured the proliferation of OVA-specific OTI and OTII cells after primary and secondary challenge of CFA/OVA followed by IFA/OVA 28 days later and measured the cellular response defined as the number of OTI and OTII cells in the spleen and dLN 5 days after each challenge. Upon secondary challenge we saw robust OTI and OTII responses which trended higher than the primary challenge, consistent with what is expected from memory T cells.

We also saw robust proliferation of the OTIs after secondary challenge with R848 compared to primary challenge, which indicates that there was immune activation after the primary challenge and thus an effector response. R848 also enabled us to treat in the 5 day window after initial challenge without the extended antigen-depot effect observed with CFA vaccination. These data are represented in Supplemental Figure 3.

To further validate these findings, we performed flow cytometry on the OTI and OTIIs at each timepoint and looked for phenotypic markers of effector and memory T cells. Supplemental Figure 4 demonstrates that CFA/OVA treated mice had an increased percentage of CD44+ antigen-experienced T cells as a percentage of both OTI and OTII cells as well as a decrease in the percentage of CD44-CD62L+ CCR7+ naïve T cells compared to the relevant naïve controls. R848 treatment was found to increase the number of CD44+ Ki67+ proliferating antigen-specific T cells compared to other groups, indicating a stronger effector response is underway at this timepoint. CFA/OVA vaccination showed an increase in CD44+ CD62L+ central memory T cells in both the OTI and OTII compartments as well as an increase in CD8+ CD44+ CD62L- CXCR3+ KLRG1- TIM3- Tox- effector memory and CD8+ CD44+ TCF1+ PD1+ stem-like memory cells in the spleen compared to the naïve control at day 28.

We have updating the manuscript accordingly: "We confirmed an increase in antigen-specific memory T cell subsets after CFA/OVA vaccination and an increase in proliferating antigen-experienced effector cells after R848 compared to naïve mice (**Supplemental Figure 4**)." (Page 3)

*4, It is counter-intuitive to examine OTII (CD4 T)-mediated responses when OVA peptide, SIINFEKL, was used as an antigen. SIINFEKL is an MHC class I epitope that only interact with TCR of CD8 T cells. This design issue affects all studies/sections using both SIINFEKL and OTII cells. An explanation on how SIINFEKL induced tolerance is needed. In the group's 2019 publication, the whole OVA was used as the antigen.*

We should clarify that the only experiment in which pGal-SIINFEKL was used is in Supplemental Figure 1, in which OTI responses are recorded to investigate dosing kinetics. All other OTI and OTII experiments were done using the full OVA protein as the antigen which contains both OTI and OTII epitopes. When SIINFEKL is used for restimulation purposes, cytokine secretion is measured in OTI cells only. We have updated the manuscript for clarity on page 3.

*5, When SIINFEKL is used as pGal delivered antigen, the free antigen control, logically should be the peptide as well. However, in this study, whole OVA protein is used as control. In addition, the pGal should have been used a vehicle control, but not included. Without the vehicle control, all results of pGal-OVA can be attributed to pGal as well.*

We acknowledge the value of additional controls and have included free pGal and an unconjugated mix of pGal and OVA as controls to account for any results attributed to pGal alone. These data are represented in Supplemental Figure 6. We have updated the manuscript accordingly: "pGal alone had no impact on OTI or OTII responses compared to saline, and an unconjugated pGal and OVA control failed to induce tolerance (**Supplemental Figure 6**)." (Page 4).

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

1. *In the manuscript, the authors carry out RNAseq analyses on adoptively transferred CD4 and CD8 T cells (OTII and OTI cells respectively). These cells were obtained following immunization with OVA and challenged with pGal-OVA, saline or OVA. The authors also conducted RNAseq analyses in the prophylactic setting. RNAseq was performed on pooled RNA from spleen, liver, and peripheral lymph nodes. Is there a reason why the RNAseq analyses were not performed in an organ-specific manner?*

The RNAseq analyses were not performed in an organ-specific manner because pooling of the OTI and OTII cells from different organs was required to yield a high enough number of cells for RNAseq analysis. Specifically the saline-treated group which did not see cognate antigen and did not proliferate had limited recovered cell numbers even after optimizing sorting protocols. While organ-specific immune responses would be interesting to pursue, it was not technically feasible in this experiment.

2. *Aside from PD1, have the authors investigate other potentially tolerogenic pathways implicated in the mechanism of action of pGal-OVA?*

We did not investigate any other pathways in the scope of this study.

3. *Since the PD1 pathway is involved, have the authors investigated whether the APCs that acquire pGal-OVA carry PD1 ligands?*

We have previously demonstrated that pGal-OVA is taken up by dendritic cells, Kupffer cells, LSECs, and hepatocytes (Wilson et al. *Nat. Biomed. Engin*. 2019). While we did not look directly at these cells it has been widely published that PD-L1 is normally expressed by hepatocytes (von Knethen et al 2019 10.7150/thno.28057), LSECs, as well as Kupffer cells (Hutchins et al 2013 10.1189/jlb.0113051, Iwai et al 2003 10.1084/jem.20022235, Dong er al Immunity 2004 10.1016/s1074-7613(04)00050-0). We have updated the manuscript to emphasize this: "Transcriptional analysis revealed an important role for co-inhibitory ligands in T cell programming by pGal-OVA treatment, among which PD-L1 is known to be expressed on LSECs and other liver APCs such as hepatocytes and Kupffer cells, the known target of pGal-antigen therapies [22, 44-47]" (Page 7)

4. *In the chronic EAE model, have the authors tried to treat mice that reached clinical score 3 (complete hindleg paralysis), i.e. not when mice were asymptomatic?*

In Figure 5d, we show treatment of mice in the chronic EAE model starting on day 7 post adoptive transfer. At this timepoint the mice have begun showing early signs of disease. The average clinical score at this time was 1.3 which corresponds to clinical symptoms of limp tail and reduced hindlimb mobility. We chose to treat at this timepoint because the mice have developed symptoms, but may not yet have severe spinal cord damage due to demyelination. We hypothesize that pGal-antigen treatment can reduce damaging T cell immune responses which begin to escalate at this timepoint and trigger demyelination. However we do not necessarily expect pGal-antigen treatment to induce remyelination and we aimed to deliver treatment during a window in which the T cell response was active, and mice were developing clinical symptoms but before too much irreparable damage to the myelin was done.

5. *The authors showed 3 doses of treatment to achieve the indicated therapeutic efficacy. What happens if animals receive one or two doses?*

In order to address this question we performed an experiment to investigate the effects of the number of doses of pGal-MOG on EAE progression. We found that mice treated with two total doses on days 0 and 3 or three total doses on days 0, 3, and 6 were completely protected from the onset of EAE pathology. In contrast, mice treated only once on day 0 did show progression of EAE. These data are represented in Supplemental Figure 16 and suggest the importance of multiple doses of pGal-antigen required for therapeutic effect. The manuscript has been updated accordingly:

"At least two doses of pGal-MOG$_{18\text{-}62}$ were found to be required for protection from EAE pathology (**Supplemental Figure 16).**" (Page 9)

6. *Minor: The y axis legend of Figure 7C is superimposed with random symbols.*

The format of the figure has been revised.

7. *Since the authors highlight the importance of PD1 pathway as one of the possible tolerogenic mechanisms, have the authors checked for upregulation of PD1 in PBMCs in the NHP model?*

Our previous work has demonstrated that pGal-antigen therapy delivers antigen to the liver. Since the PD-1 blockade demonstrated a role during treatment with the antigen and related pathways were upregulated in T cells based on RNAseq after antigen recognition, we expect the role of PD-1 to be greatest during T cell antigen experience and anticipate that this would take place in the liver based on previously published biodistribution data (Wilson et al 2019). Outside of the liver, T cell antigen experience may take place in secondary lymphoid tissues such as the spleen or the lymph nodes, but would not be mediated by PBMCs. Therefore we did not think it would be mechanistically relevant look at PD-1 on PBMCs.

8. *Have the authors explored whether treatment with pGal-OVA results in any bystander immune suppression? Does treatment with pGal-OVA affect epitope spreading?*

In order to address this question, we performed an experiment to investigate the capacity of pGal-antigen therapy to induce bystander suppression. We utilized the OTI/OTII adoptive transfer mouse model of antigen-specific immune tolerance in order to determine whether pGal glycopolymers induce CD4+ Tregs capable of inhibiting a CD8+ T cell response to a non-overlapping epitope. It has been previously reported that pGal-OVA induces expression of FoxP3, the transcriptional master regulator of Treg differentiation and effector function, in OTII T cell receptor (TCR) transgenic T cells (Wilson et al., 2019) that recognize a major histocompatibility complex class II (MHCII)-restricted (I-$A^b$) epitope from OVA (ISQAVHAAHAEI, hereafter referred to as ISQA, $OVA_{323-339}$). This study was designed to test whether induction of ISQA-specific CD4+ Tregs by pGal-ISQA suppresses the expansion and activation of CD8+ T cells from OTI mice that recognize a non-overlapping MHCI-restricted (H-$2K^b$) epitope from OVA (SIINFEKL, $OVA_{257-264}$).

On day 0, CD4+ T cells from OTII TCR transgenic mice expressing the CD45.2 pan-leukocyte marker were adoptively transferred intravenously (i.v.) into congenic wildtype recipient mice expressing the CD45.1 pan-leukocyte marker. On day 1, mice were intravenously administered with pGal conjugated to an antigen containing the OTII epitope ISQA (pGal-ISQA). On day 14, CD8+ T cells isolated from CD45.2-expressing OTI transgenic mice, alone or in combination with a second infusion of OTII T cells, were adoptively transferred i.v. to recipient mice. Mice were then challenged with OVA adjuvanted with lipopolysaccharide (LPS) on day 15. On day 19, cells from the spleen and lymph nodes were assessed for the activation of OTI CD8+ T cells. These experimental details have been added to the supplemental methods.

We found when groups had been previously treated with pGal-ISQA they had significantly fewer pro-inflammatory IFNγ+ OTI cells in the spleen after restimulation with the MHCI-restricted CD8+ T cell epitope SIINFEKL. These data demonstrate that pGal-antigen therapy with a CD4+ T cell specific epitope has the potential to reduce proinflammatory CD8+ T cell responses to different epitopes within the same antigen and enable bystander suppression.
These data have been added to Supplemental Figure 2 and the manuscript has been edited accordingly: "Having previously demonstrated efficacy of pGal-OVA prophylactically [11] and demonstrated the potential for pGal-antigen therapy to enable bystander suppression (**Supplemental Figure 2**), here we sought to test efficacy in models of active effector and immune memory response." (Page 3)

9. *Do the authors have information on the use of pGal-modified antigens in mouse models other than for OVA? For example, would the use of pGAL-fluNP affect CD8 T cell responses against flu in the B6 mouse? This question really speaks to the extent that this treatment can be generalized. The authors are well-aware of the limitations of the various mouse models (T1D in the NOD mouse; EAE etc). It would be great if it could be shown that a CD8 T cell response against a dominant pathogen-derived epitope (Db-ASNENMETM) could be modulated, for example in the course of a flu infection..*

While we recognize the value of testing a variety of antigens using our pGal platform, the use of pGal-fluNP is outside of the scope of this study. We have already demonstrated herein that pGal-antigen therapy induces T cell tolerance signatures in variety of models with a variety of diverse antigens. We have shown pGal-antigen therapy is effective using a vaccination model with OVA, a listeria infection with lmOVA, two different EAE models with different antigens as well as previously published T1D model. Furthermore we demonstrate tolerance in a NHP vaccination model with an entirely different antigen and adjuvant. We feel these experiments which show tolerogenic effects of pGal-antigen treatment when the antigen is a protein or peptide, during sterile inflammation from vaccination, inflammation from pathogen infection, and inflammation both before and after the onset of autoimmune-mediated pathology to demonstrate its broad generalizability in inducing tolerogenic T cell signatures across inflammatory contexts and animal models.

**Rebuttal 2**

For reference, this research follows our previous findings from 2019 in *Nature Biomedical Engineering* in which we report that treatment with synthetically glycosylated antigen (pGal-antigen) induces antigen-specific T cell tolerance signatures when administered prior to an immune challenge. In this manuscript our advancement is four-fold: 1) we demonstrate that pGal-antigen immunotherapy can induce T cell tolerance signatures not only prophylactically (as in 2019), but also after an initial immune challenge when administered during either the effector and memory stages of immune response, 2) we investigate the molecular mechanisms underlying this therapeutic tolerance, 3) next we find that pGal-antigen treatment after disease onset resulted in remission in multiple mouse models of multiple sclerosis, and 4) we further demonstrate antigen-specific T cell tolerance when administered to non-human primates after established vaccination. As to the translational potential of this work, the preclinical results we present herein were pivotal in launching two Phase 1 trials of this technology for tolerogenic vaccines, one in celiac disease (clinicaltrials.gov #NCT04248855) and another in multiple sclerosis (clinicaltrials.gov #NCT04602390).

We greatly appreciate the responses from the reviewers and editors. Based on this feedback we have updated the manuscript to address their concerns. In summary we have clarified the type of ELISPOT used in Supplemental Figure 13 (formerly Supplemental Figure 18) in the figure caption. Additionally we have updated the abstract and introduction section to highlight our technology as the first approach to induce tolerance during an established immune response.