nature biomedical engineering

PEER-REVIEW INFORMATION

Generation of functionally distinct T-cell populations by altering the viscoelasticity of their extracellular matrix

Corresponding author: David Mooney

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

Mon 24 Jan 2022
Decision on Article nBME-21-2791

Dear Prof Mooney,

Thank you again for submitting to *Nature Biomedical Engineering* your manuscript, "Matrix Viscoelasticity Regulates T Cell Phenotype". 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 many serious concerns about the degree of support for the claims, 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. In particular, we would expect that a revised version of the manuscript provides:

* Strong support for the main claim: that viscoelastic responses are a driving factor of T-cell phenotype. As pointed out by all reviewers, there are a range of potential confounders that would need to be explicitly ruled out.

* Extended characterization of the viscoelastic behaviour of the gels and the viscoelastic responses of the T cells.

* In appropriate tumour models, evidence of the in vivo outcomes of infused CAR T cells generated in matrices with different viscoelasticities, as suggested by Reviewers #1 and #3. Such evidence would increase the biomedical impact of the work.

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 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*.

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 present an ECM-based approach to generate a functionally distinct T cell population. They modulate the physical properties of collagen type 1 based ECM to allow for independent tuning of matrix stiffness and viscoelasticity. They apply the concept for the generation of T cells for CAR-T therapy. essence, the work is of translational importance, while simultaneously providing basic information on the use of tuning of ECM viscoelasticity for the generation of functionally distinct T cells. However, the work has significant weaknesses that reduce the enthusiasm of the reviewer.

These comments are summarized below.

1. The scientific premise and data in figure 1 is not supportive of the choice of collagen 1 as the only matrix necessary for demonstrating matrix viscoelastic effect. The data presented needs better clarity on what cell adhesion pathways are enriched.

2. The gene expression analysis itself is inconclusive. Authors should demonstrate using protein multiomics that specific ECM molecules, and if collagen only, is present near T cells in healthy and diseased tumor tissues.

3. In extended Fig 4, authors show that T cells in slow-relaxing matrices show higher expression of activation

and inhibitory markers, while those in fast-relaxing matrices express higher levels of memory-associated markers. ECM viscoelasticity was claimed as the main driver of the differences observed in T cell phenotype. However, these studies do not demonstrate whether these distinct phenotypes emerged as a function of time, or were present early on and persisted, and most importantly reversible by manipulating the viscoelastic properties of the ECM.

4. While the authors present impressive data on extracellular matrix viscoelasticity modulation of T cell transcriptomic profile, the manuscript does not really explain and mechanistically determine why AP1 enriches and regulates the memory-associated markers or activation and inhibitory markers. These studies are critical for the understanding of why ECM and mechanics would work and will drive the field.

5. It is unclear how the ECM viscoelasticity regulates the T cell repertoire and how this will change with donors and site specific T cells. This is critical information missing in the current manuscript.

6. The CAR-T data is quite preliminary. Fig 6d missed a critical control – CD19 CAR T cell not cultured in ECM but stimulated with similar acute exposure to different strengths. How long are the inhibitory and activation markers retained?

7. The co-culture experiments are quite rudimentary and authors should implant a CD19 tumor PDX, infuse CAR T cells obtained from various matrices and demonstrate the long term impact of these cells. It is also necessary to benchmark with state-of-the-art cultures.

8. A large number of hydrogel ECM culture platforms have been reported for T cell expansion. The current manuscript does not make efforts to compare, benchmark against current technologies other than stating that the collagen system is the first to report a crosslinking mechanism that successfully decouples ECM stiffness from viscoelasticity and that they explore tissue-level viscoelasticity. The manuscript leaves one thinking whether demonstrated effects are not possible with other reported systems and whether collagen is the only suitable matrix, specifically because fibrin etc have been reported for improving in vivo CAR-T efficacy.

9. What is the longevity of T cells isolated from the viscoelastaic ECMs, what is their proliferation potential?

10. Can the CAR-T cells or other tissue specific T cells be serially passaged in these ECMs and what is the impact of viscoelastic properties on maintaining activation/memory phenotypes?

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

The authors generate two 3D gel matrix systems. One is a classic collagen matrix system and the other a formulation with the goal of producing a more viscoelastic environment. The overall goal is to determine how a viscoelastic environment can influence T cell behavior, which may have implications for how therapeutic T cells are prepared. The authors identify key shifts in T cell behavior and identify signaling pathways implicated in T cell phenotype and function and study how these may relate to cancer and fibrotic disease. Overall, the concept of methods to alter T cell behavior for therapeutic benefit prior to transfer experiments or treatment is very exciting. The study has many strengths, mostly in the detailed characterization of T cell responses.

However, major issues are present with the 3D matrix approach. It is not clear that a different viscoelastic response is the driving factor influencing the observed shifts in cell behavior. The characterization of the viscoelastic response is not rigorous enough to accurately determine potential changes in viscoelastic behavior or their implications. Rigorous analysis is the viscoelastic response is not included and the language and conclusions regarding viscoelastic behavior are in error in multiple portions of the manuscript. Likewise, a deep characterization of the 3D matrices in not included. While the examination of fibers and pore size is appreciated, a much deeper analysis is certainly needed. Along these lines, key controls for 3D system are missing. It is not clear that that the linker and click reactions steps are not impacting cell behavior, that the presence of difference materials is not influencing T cell behavior directly and that adhesion in the matrices is
equivalent. It is not clear that the matrices underwent the same processes in terms of time under different conditions (temperature, vehicle solutions etc.). Lastly, while the analysis of the T cell that is included in the

manuscript is very exciting and impactful, key direct measure of T cell function are somewhat limited. Yet, this Reviewer finds that the manuscript could provide fundamental new information and could be of interest to the Nature Biomedical Engineering readership following very significant revisions. The following comments are provided as constructive criticism to help improve the manuscript.

Major Comments:

1) The characterization of potentially distinct viscoelastic responses is not rigorous. As this is the foundation of all conclusions in the manuscript it is not possible to fully interpret most of the findings. While the methods discuss both stress-controlled testing and stress relaxation, neither is adequately described and key viscoelastic mechanical data is not presented in the manuscript. Fundamental language for viscoelastic behaviors (e.g. creep and stress relaxation) are often intermixed, creating additional confusion. So there are strong issues with the bulk matrix viscoelastic description. Also, collagen matrices can possess nonlinear viscoelastic responses where the rate of relaxation is dependent on the input strain (likewise the rate of creep can depend on input stress) with the rate being very fast for very low strains or stresses. As cell produce very low traction stresses the local rates would be extremely fast and it is not clear that even if there are difference
in the bulk gels this would be detectable by cells. Likewise, the moduli ranges presented are not particularly relevant related to the tissues discussed in the manuscript. For instance the ~100-700 Pa range is lower than gummy bears ~70kPa), raisins (~220kPa) etc. and clearly not indicative of the “stiffness range of multiple soft tissues (Extended Fig. 1b), as well as pre-malignant and malignant tumors” except for the cases where those tissues are not mechanically tested correctly (e.g. AFM etc. pushing on the side of a fiber or network of fibers where collagen has a bending modulus that is orders of magnitude lower than in under tensile loading, which is what cells produces from traction stresses applied to the fibers or fiber network). Overall the description of viscoelastic behavior on multiple scales would need to be profoundly more rigorous to support the conclusions presented in the manuscript.

2) Similar to point 1: The description of the matrices themselves and the potential direct impact on cells is lacking. While this reviewer greatly appreciates the analysis of fibers and pore size as much deeper analysis is needed (fiber width, angle, length, waviness etc.). Also, potential direct impact from the component and processing to make the Norbornene-modified collagen is not evaluated. These are critical controls. Along these lines it needs to be shown that other key aspects of cell behavior are not being impacted by the formulation on multiple levels (e.g. general ability to adhere etc.)

3) This is present at many locations in the manuscript, but as an example the statement “Pathway enrichment analysis showed that the SModule was enriched for pathways related to TCR signaling, CD8+ T cell activation and T cell cytotoxicity, indicating a more activated T cell state, while the FModule was enriched for genes related to leukocyte adhesion to endothelial cells (Fig.3f).” seems to suggest that more elastic reponses is a key driver of key behaviors evaluated in the manuscript, but the text consistently emphasizes viscoelasticity as the driver. If in fact there is a different viscoelastic response, it still seems clear that some key behaviors are driven by a more elastic environment and others by more viscous and the focus on more viscous is over-emphasized and interpreted. This is also true in areas there the authors conclude that “Our findings also suggest that therapies targeting the ECM viscoelasticity may directly impact T cell biology and fate”. This may be
true but it is also seems equally clear that targeting more elastic environments also directly impact T cell biology and fate. There are many instances throughout the manuscript where data doesn’t line up or is showing opposite behavior, which is fine but as currently written there appears to be too much emphasis on trying to fit all the findings into the viscoelasticity box. Lastly since tumors are very complex and heterogeneous with a lot of elastic and viscous components this is particularly relevant.

4) Deeper functional analysis of T cell would strengthen the manuscript, but this is difficult to evaluate at this time since the impact of the foundational platforms are not clear at this time.

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

The manuscript by Adu-Berchie et al., from the David Mooney lab addresses an interesting aspect of T cell engineering, which has gained importance in the recent years due to its high clinical efficacy and hence significance in cancer therapeutics. The manuscript explores the role of matrix stiffness and viscoelasticity as

a potential modulator of T cell phenotype and function in the context of different tumor types.
The topic addressed is interesting and the data reveal that mechanosensitive cues from synthethic matrices affect the AP1 pathway in a differential manner and that ECM viscoelasticity rather than stiffness can be utilized to fine tune generation of clinically relevant Therapeutic CAR T cells.

There are however some major caveats with the data analyses and interpretation as well as many missing details that make it difficult to assess the data and conclusions. These concerns are outlined below and need to be addressed-

Major points:
1. Figure1- Not clear what the data presents in Figs 1b and c…. 'tumor-tissue derived T cell gene signatures' needs to be defined…how is the module represented as a single expression number? Also, the data just shows that T cells from blood, normal tissue and tumor are distinct…which is to be expected.
…..the conclusion that the distinct ECM micro-environments affect T cell phenotype is not justified at least based on the current representation
Fig1 a…missing x, y axis titles

2. Rationale and succinct description of the CD3/CD28 dynabeads as a proxy for APCs for T cell activation should be described in results so that its clear how the T cells were activated before culture in slow/fast gels. It would also be interesting to additionally test CD3/28/137 combination that is used for expansion of effector/memory cells since fast relaxing conditions seem to naturally enrich the memory markers.

3. Since the T cells are activated prior to culture in fast/slow gels….it should be checked if the gels just lead to selective survival and proliferation of certain T cell subsets.
Live/dead cell staining can provide an idea.
Alternatively, the cells can be cultured in the gels first and then activated to confirm that the effect of the ECM viscoelasticity is on the T cell phenotype per se and not selective growth of subsets. These exps are described later in Fig5…as acute vs chronic activation but only the protein expression of the AP 1 pathway components is analyzed, which seems to be operational in both slow and fast gels
It is important to analyze sc-RNA data from these conditions and test if the data are similar in 'acute' vs 'chronic' conditions.

4. Figure 2- is the data in g and h marker expression from flow cytometry or gene expression? No introduction or details for the markers (including CD45Ra and CD62L) is provided which is needed to understand rationale.

5. In Figure 3a- this is single cell data so the cell clusters in the fast and slow gels should be defined and analyzed. What are the 3 colors in the UMAP?…

In Fig e, the fast module seems to represented in both fast/slow gels ….how are the differential modules determined? The informatics and statistics need to be detailed here

6. To validate that the ECM viscosity changes are transduced through the AP1 pathway, modulation of this pathways (activation and inhibition) should be tested in the fast/slow gels.

7. The CAR T data (Fig 6) is very interesting-howveer it has been carried out in 'acute' conditions only. Due to the reservations expressed earlier about the selective growth potential…..the CAR T cells should be cultured in the fast/slow matrices first and then activated to discern the difference between ECM viscoelasticity

The functional differences in tumor cell toxicity is exciting but has been carried out in a single tumor type-the raji cells are a cell line of lymphoblastic tumor…it would also be really interesting to test NSCLC, liver and colorectal cells lines that have differences in ECM viscoelasticity since the initial figure and data hypothesize that the tumor ECM differences will also affect the function of T cells.

8. If possible, testing the function of CAR T cells generated in different matrices against tumors in vivo would really underscore the clinical relevance of the study and the potential to use ECM matrices for the generation and modulation of CAR T and other therapeutic T cells.

Fri 20 Jan 2023
Decision on Article nBME-21-2791

Dear Prof Mooney,

Thank you for your patience in waiting for the latest feedback on your revised manuscript, "Matrix Viscoelasticity Regulates T Cell Phenotype", which has been seen by the original reviewers. In their reports, which you will find at the end of this message, you will see that the reviewers acknowledge the improvements to the work and that Reviewer #2 raises a few additional technical criticisms that I am hoping you will be able to address.

As before, 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.

We look forward to receive a further revised version of 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)):

The authors have done outstanding work revising the manuscript. This has improved the quality of the manuscript and the scientific findings would be of high interest to the field. I do not have any further comments.

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

Overall, this reviewer appreciates the significant effort the authors have made to perform additional experiments and clarify their findings in order to address the concerns of the three reviewers. The new data and clarifications have addressed a number of the raised concerns. However, it is this reviewer's opinion that a number of some concerns from all three reviewers were not adequately addressed and some of the new data is being over-interpreted.

Focusing on responses to this reviewer's comments, the characterization and presentation of viscoelastic behavior between the relaxing collagen gels vs. the crosslinked gels (termed fast vs. slow relaxing; this reviewer apologizes for the viscoelastic vs. elastic typo in the first review and hopes it did not create undo confusion) still lacks robust rigor. While the explanation defining the fundamentals of mechanics is an intriguing strategy, it is not convincing to a reviewer with extensive expertise in this area that the measures, data completeness, and language are rigorous. For instance, this reviewer is fully aware that the authors did not conduct creep experiments, but when the authors use terms such as "applied stress' for a displacement controlled relaxation experiments it can create confusion (which was part of the point about focusing the language used). Certainly stress is generated and there can be relaxation from the peak stress for a viscoelastic material, however, this is typically not a linear viscoelastic behavior for many biologic tissues and for collagen matrices.

Further confusion is added by the very limited presentation of data from both a shear relaxation test (as opposed to a more classic uniaxial stress relaxation test and where shear is not mentioned in the main text)

and a classic oscillatory rheology test. However, the data in supplementary figure 1c is a greatly appreciated. Yet, the analysis is still quite limited (the nano-indentation data does not add greatly to the analysis) and it is concerning that a more robust analysis of data and more rigorous discussion of viscoelastic behavior was not presented in the revision.

However, with all that said, this reviewer remains very excited about the work overall and concepts behind it. While this reviewer clearly has strong concerns about the testing and description of the base platform - that all conclusion derived from – it is also clear that the two gels are mechanically different. It may remain to be seen how the cells are sensing this difference, but for now the impact on T cell behavior is very impactful and interesting. As such, this reviewer believes the above points can be largely addressed to a reasonable degree with text edits (and more robust data analysis if available is welcomed) and that the manuscript could be acceptable for publication following these edits (and those noted below).

Additional comments:

1) For the new data in Extended Figure 3. It is greatly appreciated (along with the more in depth data analyzing the gel structures in supp. Figure 1), however, it seems to show more variability than the authors are noting. The cluster and PCA analysis show some strong differences, suggesting the autors are over-interpreting the findings. Also,a more cell level analysis would be insightful.

2) The new data in Figure 7 is also appreciated. However, Figure 7 is missing key controls. How do activated T cells that don't go into a 3D matrix behave? What if activated T cell that have never gone into a matrix out-perform the ones that are activated and then go into a matrix? This still would indicate very interesting differences from the matrices but may not indicate that going into 3D before infusion is a good clinical strategy. Likewise, for the tumor-only group it does not appear there was a control injection or introduction of non-specific T cells etc.

3) (minor) Along the lines of other mechanics issues, the figure listed showing the comparison to the 70 kPa gummy bear highlights the reviewers point as the other metrics are largely from innapropritate mechanical testing schemes such as indentation (oddly the 70 kPa for gummy bears comes from a classic stress strain test, unlike muscle which also has a hundreds of kPa to MPa modulus when tested in the correct configuration, e.g. uniaxial stress-strain test). Unfortunately, a disregard for an extensive body of tissue mechanics literature with enormous consensus data is quite problematic in the cancer mechanobiology field.

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

The revised manuscript by Adu-Berchie et al., from the David Mooney lab addresses an interesting aspect of T cell engineering, which has gained importance in the recent years due to its high clinical efficacy and hence significance in cancer therapeutics. The manuscript explores the role of matrix stiffness and viscoelasticity as a potential modulator of T cell phenotype and function in the context of different tumor types.

The authors have satisfactorily addressed all concerns that were raised-clarifying methods and text and adding new data. Added new experiments especially the in vivo studies, inhibitor studies as well as inclusion of additional tumor cell lines have significantly strengthened the study which in my opinion is now suitable for publication.

Tue 28 Feb 2023
Decision on Article nBME-21-2791

Dear Prof Mooney,

Thank you for your revised manuscript, "Matrix Viscoelasticity Regulates T Cell Phenotype". Having consulted with Reviewer #2 (whose brief 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*.

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.

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

The authors have sufficiently addressed this Reviewer's concerns and it is recommended that the manuscript is acceptable to publication in Nature BME.

Rebuttal 1

Adu-Berchie et al., Point-to-point response (reviewer comments in black text; responses in green text):

We thank the reviewers for their time and supportive comments. We have attached our response for each reviewers' comments below:

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

In this manuscript, the authors present an ECM-based approach to generate a functionally distinct T cell population. They modulate the physical properties of collagen type 1 based ECM to allow for independent tuning of matrix stiffness and viscoelasticity. They apply the concept for the generation of T cells for CAR-T therapy. essence, the work is of translational importance, while simultaneously providing basic information on the use of tuning of ECM viscoelasticity for the generation of functionally distinct T cells. However, the work has significant weaknesses that reduce the enthusiasm of the reviewer.

These comments are summarized below.

1. The scientific premise and data in figure 1 is not supportive of the choice of collagen 1 as the only matrix necessary for demonstrating matrix viscoelastic effect. The data presented needs better clarity on what cell adhesion pathways are enriched.

To better motivate the choice of collagen type 1, we analysed the TCGA dataset to look for expression of collagen type 1 (COL1A1) and lysyl oxidase (LOX) in tumours and normal tissues for patients with NSCLC, liver cancer and colorectal cancer. We found that both COL1A1 and LOX were differentially expressed in tumours compared with normal tissues. We have modified the manuscript as follows:

"To better correlate the observed transcriptomic differences between T cells isolated from the different tissues with their microenvironment, and to further inform our choice of ECM protein for subsequent studies, bulk sequencing from the TCGA dataset was analyzed to compare the relative expressions of canonical matrix proteins that are known to influence tissue mechanical properties. In particular, collagen type I (COL1A1) and lysyl oxidase (LOX), which is known to crosslink collagen fibers in vivo, were found to be differentially expressed in tumors relative to normal tissues for each tumor type (Fig. 1d)."

2. The gene expression analysis itself is inconclusive. Authors should demonstrate using protein multiomics that specific ECM molecules, and if collagen only, is present near T cells in healthy and diseased tumor tissues.

We would like to thank the reviewer again for this comment. We do not wish to claim that collagen type 1 is the only matrix necessary for demonstrating viscoelastic effect. In addition to the analysed TCGA dataset, we have updated the manuscript text citing previous studies that have demonstrated increased deposition of collagen 1 and LOX protein expression in tumours[1–3].

We have also extended our studies to investigate the effects of viscoelasticity within the context of a more complex ECM formulation by developing and characterizing a Matrigel-collagen type 1 interpenetrating network (IPN) with tunable viscoelasticity. Our results show that T cells

cultured in fast relaxing and slow relaxing Matrigel-collagen IPNs were phenotypically different. We have modified the text with the new findings as follows:

"Even though collagen type I forms a significant fraction of many tissue ECM, the effects of viscoelasticity within the context of a more complex ECM formulation was next studied. These studies were performed with an interpenetrating network (IPN) of Matrigel and collagen type I to mimic the complexity of tissue ECM. The viscoelasticity of the collagen-Matrigel IPN was again tuned by selectively and locally crosslinking collagen-Nb within the network with low molecular weight methyltetrazine crosslinkers. Bulk rheological characterization of the IPNs showed significant effects of click crosslinking on stress relaxation halftime and loss angle (Extended Fig. 4a). Importantly, similar to the observations made for the collagen-only matrices, alterations in viscoelastic properties led to significant changes in T cell phenotype (Extended Fig. 4b-e)."

3. In extended Fig 4, authors show that T cells in slow-relaxing matrices show higher expression of activation and inhibitory markers, while those in fast-relaxing matrices express higher levels of memory-associated markers. ECM viscoelasticity was claimed as the main driver of the differences observed in T cell phenotype. However, these studies do not demonstrate whether these distinct phenotypes emerged as a function of time, or were present early on and persisted, and most importantly reversible by manipulating the viscoelastic properties of the ECM.

We appreciate the reviewer's comment, and exploring this question has revealed new insights. To address this question, we performed single cell RNA and TCR sequencing of T cells cultured in fast relaxing and slow relaxing matrices, as well as plate-cultured T cells (without matrix culture). The T cells used for this study were from a different donor from the ones used in the initial RNA sequencing study. Our studies revealed that ECM viscoelasticity does not select for specific T cell clones that persist, but instead these phenotypes emerge as a function of viscoelasticity. We have updated the manuscript and figures with our findings (Figure 5 and Extended Figure 8). We summarize our findings here:

a. We investigated the profile of T cell receptor β (TRβ), a component of the TCR heterodimer. Similar frequency distributions were observed for the different TRβV genes as a function of ECM condition (Extended Fig. 8a). In addition, a Venn diagram of the TRβV genes showed that 96% of the genes were present in all the conditions (Extended Fig. 8b). Shannon entropy, which estimates the diversity within a specific population, showed similar TRβV diversity between T cells cultured in fast relaxing matrices, slow relaxing matrices, and plate-culture, as well as their parent T cell population (Pre-Gel) (Extended Fig. 8c). Importantly, when the Slow-Relaxing Module (SModule) generated from Fig 3 was applied to the T cells grouped by TRβV gene, the slow relaxing matrices had the highest expression across all TRβV genes (Extended Fig. 8d).
b. We next investigated the effects of ECM viscoelasticity on CDR3 profiles and the persistence of T cell clonotypes. First, it was observed that TCRβ CDR3 length distribution was similar across all the conditions, including in the parent T cell population (Fig. 5c). Assessment of TCR clones revealed that all the conditions had similar clonotypic diversity as estimated by Shannon entropy (Fig. 5d). The T cells generally had unique clonotypes, with only 3.7% of T cells being clonal (clones observed in >1 cell) (Fig. 5e). Dominant clones (clones observed in >3 cells) were not limited to specific conditions, but were shared across multiple conditions, with 5 clones being shared among all conditions (Fig. 5f). Importantly, T cells with shared TCRs derived from the various matrix conditions were transcriptomically different,

with the T cells cultured in slow relaxing matrices having the highest expression of the SModule (Fig. 5g).

c. We hypothesized that if the matrix is acting by selecting specific T cell clones, whose phenotypes persist over time, then there should be a correlation between the transcriptomic profiles of those T cell clones before and after matrix culture. However, correlation analysis between individual T cell clones before and after their culture in collagen matrices showed weak correlation for the fast relaxing matrix condition (Pearson correlation=0.38) and no correlation for the slow relaxing matrix condition (Pearson correlation=-0.089) (Fig. 5h-i). Together, these findings suggest that ECM viscoelasticity directly modulates T cell phenotype, and does not select specific T cell clones which persist over time.

We also performed additional experiments to address comments on both the reversibility of T cell phenotypes after manipulating ECM viscoelastic properties, and the serial passaging of T cells in ECM with different viscoelastic properties (#10). We have modified the manuscript text and figures as follows:

"To further explore the role of ECM viscoelasticity on imprinting long-term T cell phenotype, T cells were serially passaged as follows: from fast relaxing to fast relaxing matrices (Fast-Fast), from fast relaxing to slow relaxing matrices (Fast-Slow), from slow relaxing to fast relaxing matrices (Slow-Fast) and from slow relaxing to slow relaxing matrices (Slow-Slow) (Fig. 6a). Flow cytometry analyses revealed an impact of the prior matrix condition on the phenotype of the T cells in the latter matrix. In particular, Umap, K-means and PCA analyses showed that T cells from Fast-Fast and Slow-Slow conditions were the most different, with the Fast-Slow and Slow-Fast conditions being intermediate (Fig. 6b-e). Importantly, when the Euclidean distances between a) Fast-Fast and Fast-Slow and b) Slow-Slow and Slow-Fast conditions were compared, it was found that there was a greater distance between Fast-Fast and Fast-Slow (more dissimilar) than Slow-Slow and Slow-Fast conditions, suggesting that slow relaxing matrices had a greater impact on modulating T cells that have been previously cultured in fast relaxing matrices than vice versa (Fig. 6f)."

4. While the authors present impressive data on extracellular matrix viscoelasticity modulation of T cell transcriptomic profile, the manuscript does not really explain and mechanistically determine why AP1 enriches and regulates the memory-associated markers or activation and inhibitory markers. These studies are critical for the understanding of why ECM and mechanics would work and will drive the field.

The critical role of AP1 in modulating T cell activation, exhaustion and memory phenotype has been shown in multiple studies independent of extracellular matrix viscoelasticity. We have highlighted a few of these studies below:

a. Quigley, M. et al. Transcriptional analysis of HIV-specific CD8+ T cells shows that PD-1 inhibits T cell function by upregulating BATF. Nat Med 16, 1147–1151 (2010).
b. Transcription Factor IRF4 Promotes CD8+ T Cell Exhaustion and Limits the Development of Memory-like T Cells during Chronic Infection - ScienceDirect.
c. Papavassiliou, A. G. & Musti, A. M. The Multifaceted Output of c-Jun Biological Activity: Focus at the Junction of CD8 T Cell Activation and Exhaustion. Cells 9, 2470 (2020).
d. Kurachi, M. et al. The transcription factor BATF operates as an essential differentiation checkpoint in early effector CD8+ T cells. Nat Immunol 15, 373–383 (2014).

e. Lynn, R. C. et al. c-Jun overexpression in CAR T cells induces exhaustion resistance. Nature 576, 293–300 (2019).
f. Seo, H. et al. BATF and IRF4 cooperate to counter exhaustion in tumor-infiltrating CAR T cells. Nat Immunol 22, 983–995 (2021).
g. Koizumi, S. et al. JunB regulates homeostasis and suppressive functions of effector regulatory T cells. Nat Commun 9, 5344 (2018).
h. jun-B inhibits and c-fos stimulates the transforming and trans-activating activities of c-jun - ScienceDirect.
i. Chiu, R., Angel, P. & Karin, M. Jun-B differs in its biological properties from, and is a negative regulator of, c-Jun. Cell 59, 979–986 (1989).
j. Atsaves, V., Leventaki, V., Rassidakis, G. Z. & Claret, F. X. AP-1 Transcription Factors as Regulators of Immune Responses in Cancer. Cancers (Basel) 11, 1037 (2019).

While many other studies have demonstrated the role of AP-1 in the regulation of T cell phenotype, we show the effects of ECM viscoelasticity on AP-1 within the context of T cells. As we indicated in the discussion section of the manuscript, "*While our study took an agnostic approach to investigate the effects of ECM stiffness and viscoelasticity on T cells, the convergence of the data on AP-1 is indicative of the central role it plays in the cellular response to ECM mechanics.*"

We have extended our studies to directly test the role of viscoelasticity on AP-1, and on T cells by blocking c-Jun function with the SP600125 Jun N-terminal kinase inhibitor[4]. We saw that SP600125 abrogated the effects of matrix viscoelasticity and led to increased expression of memory markers. We have updated the manuscript text and figures with these findings (Fig 4d-g)

5. It is unclear how the ECM viscoelasticity regulates the T cell repertoire and how this will change with donors and site specific T cells. This is critical information missing in the current manuscript.

As mentioned in the third reviewer comment, single cell RNA and TCR sequencing showed that TCRβ CDR3 length distribution was similar across all the conditions, all the conditions had similar clonotypic diversity, dominant clones (clones observed in >3 cells) were not limited to specific conditions, but were shared across multiple conditions, and T cells with shared TCRs derived from the various matrix conditions were transcriptomically different, with the T cells cultured in slow relaxing matrices having the highest expression of the SModule (Fig 5)

6. The CAR-T data is quite preliminary. Fig 6d missed a critical control – CD19 CAR T cell not cultured in ECM but stimulated with similar acute exposure to different strengths.

We thank the reviewer for this comment. While we believe that it is difficult to directly compare plate cultured T cells with T cells cultured in 3D matrices we acknowledge the importance of having plate-cultured T cells (i.e., T cells not cultured in ECM) as a control. Using a different donor, we have consequently performed additional cytolysis experiments which includes plate-cultured T cells as a condition. We show that consistent with the previous cytolysis experiment, anti-CD19 T cells cultured in slow relaxing matrices exhibited the best in vitro killing. In response to reviewer 3, we also performed cytolysis studies using a different tumour model: Pmel-1 T cells, which recognize the gp100 epitope on B16-F10 melanoma cells. Here as well we included the plate-cultured T cell condition and again saw B16-F10 highest killing for Pmel-1 T cells cultured in slow relaxing matrices. We have updated the manuscript text and figures with

these findings (Extended Figure 13). In all, we have performed the following new studies/analyses with plate-cultured T cells as a condition:

a. Single cell RNA-sequencing: comparing the expression profiles of Fast-relaxing module (FModule) and slow-relaxing module (SModule) between plate-cultured T cells and T cells embedded in fast relaxing or slow relaxing collagen matrices (Fig. 3f)
b. Investigating the effects of cJun inhibitor on T cells cultured in plate-cultured and in collagen matrices with different viscoelasticity (Figure 4d-g)
c. TCR sequencing (Figure 5, Extended Figure 8)
d. Persistence of T cell phenotypes after they are harvested from matrices (Extended Fig 9)
e. Varying modes of prior T cell activation (Extended Figure 11)
f. In vitro T cell cytolysis studies using anti-CD19 CAR T cells and Pmel TCR T cells for different tumour models (lymphoma and melanoma), and species (human and mouse)

Despite the comprehensive comparison of plate-cultured T cells with T cells embedded in matrices summarized above, we would like to highlight that plate-culture provides a mechanically diverse environment to cells. Cells engaged with the surface encounter a highly inelastic surface while those in suspension are surrounded by a viscous cell culture medium. It is difficult to decouple the net mechanical effects of the viscous media from the inelastic surface of the plate.

How long are the inhibitory and activation markers retained?

When we harvest T cells from the various matrix conditions and culture them in suspension, the T cells maintain their phenotypic differences for at least 7 days (which is the longest we have cultured the cells). T cells that were embedded in matrices (or plate cultured) for 3 days, harvested and cultured in suspension for 4 days with or without dynabead restimulation were phenotypically different, based on their prior matrix culture conditions (Extended Figure 9). When T cells were harvested from collagen matrices on days 3 and 7, and subsequently cultured in suspension for 7 additional days, they maintained the T cell phenotype imprinted by the specific matrix mechanical properties in a manner dependent on the duration of initial gel culture (Extended Fig. 10f).

7. The co-culture experiments are quite rudimentary and authors should implant a CD19 tumour PDX, infuse CAR T cells obtained from various matrices and demonstrate the long term impact of these cells. It is also necessary to benchmark with state-of-the-art cultures.

We thank the reviewer for this comment. To address this comment, we have performed a lymphoma xenograft study with anti-CD19 CAR-T cells (Figure 7f-h) cultured in fast relaxing and slow relaxing collagen matrices. Similar to our in vitro cytolysis studies, CAR-T cells exposed to slow-relaxing matrices showed superior control of Raji tumours and resulted in significantly better mouse survival. Based on the extensive transcriptomic and phenotypic analyses performed on plate-cultured T cells in our studies, our functional in vitro T cell cytolysis studies using both anti-CD19 CAR T cells and Pmel-1 TCR T cells that included plate-cultured T cells, we would expect T cells cultured in slow-relaxing matrices to show superior control of mouse tumours than plate-cultured cells. We thus did not include plate-cultured T cells in the in vivo study to avoid unnecessary use of animals; the consistency between in vitro T cell cytolysis and the in vivo lymphoma xenograft study for cells cultured in the gels supports the predictive ability of the in vitro studies.

8. A large number of hydrogel ECM culture platforms have been reported for T cell expansion. The current manuscript does not make efforts to compare, benchmark against current technologies other than stating that the collagen system is the first to report a crosslinking mechanism that successfully decouples ECM stiffness from viscoelasticity and that they explore tissue-level viscoelasticity. The manuscript leaves one thinking whether demonstrated effects are not possible with other reported systems and whether collagen is the only suitable matrix, specifically because fibrin etc have been reported for improving in vivo CAR-T efficacy.

We thank the reviewer for their thoughts. We agree that there have been efforts to develop strategies for enhanced in vivo T cell delivery and expansion, as the fibrin study and other hydrogel-based studies have sought to achieve[5,6]. However, the goal of this manuscript is not to serve as an in vivo T cell delivery or expansion system, but rather demonstrate that changes in ECM viscoelasticity could affect T cell phenotype and function. This is a principle that we hope could potentially be used to improve current CAR-T cell manufacturing, even if the final product is not a collagen-based one. To demonstrate this principle, we had to develop a material system that decouples matrix stiffness from viscoelasticity, but we do not claim that this observation only applies to collagen-only systems. In fact, we have performed similar experiments with a more complex matrix system comprising an interpenetrating network (IPN) of Matrigel and collagen type I, where the stiffness and viscoelasticity is also decoupled, and we see differences in T cell phenotype as a function of viscoelasticity (Extended figure 4). These findings suggest that the effects of ECM viscoelasticity can be conserved across different systems.

9. What is the longevity of T cells isolated from the viscoelastaic ECMs, what is their proliferation potential?

We performed additional studies to address this comment. T cells were embedded in fast- and slow- relaxing ECMs for 3 days, harvested, and then kept in suspension culture for up to 4 days. T cells remained viable and highly proliferative, and their phenotypes from the different matrix conditions remained distinct. This trend was maintained when the T cells harvested from the various matrix conditions were re-stimulated with dynabeads in suspension. We have modified the manuscript text and figures as follows to address this comment:

“To investigate the extent to which the differences in T cell phenotypes persist after being exposed to matrices with different viscoelasticity, T cells were first embedded in either fast or slow relaxing collagen matrices for 3 days, after which they were cultured in suspension for 4 days. The T cells expanded and remained viable post matrix culture (Extended Fig. 9a). Umap, K-means and PCA analyses showed that T cell phenotypes remained distinct after 4 days of suspension culture, with T cells harvested from slow-relaxing matrices being enriched in clusters with higher expression of activation markers: clusters 4, 5, 6, 8 (Extended Fig. 9b-c). Importantly, this trend was maintained when the T cells harvested from the various matrix conditions were re-stimulated with dynabeads in suspension (Extended Fig. 9f-j).”

10. Can the CAR-T cells or other tissue specific T cells be serially passaged in these ECMs and what is the impact of viscoelastic properties on maintaining activation/memory phenotypes?

We thank the reviewer for the comment. We performed additional studies to address this comment and have modified the manuscript text and figures as follows:

“To further explore the role of ECM viscoelasticity on imprinting long-term T cell phenotype, T cells were serially passaged as follows: from fast relaxing to fast relaxing matrices (Fast-Fast), from fast relaxing to slow relaxing matrices (Fast-Slow), from slow relaxing to fast relaxing matrices (Slow-Fast) and from slow relaxing to slow relaxing matrices (Slow-Slow) (Fig. 6a). Flow cytometry analyses revealed an impact of the prior matrix condition on the phenotype of the T cells in the latter matrix. In particular, Umap, K-means and PCA analyses showed that T cells from Fast-Fast and Slow-Slow conditions were the most different, with the Fast-Slow and Slow-Fast conditions being intermediate (Fig. 6b-e). Importantly, when the Euclidean distances between a) Fast-Fast and Fast-Slow and b) Slow-Slow and Slow-Fast conditions were compared, it was found that there was a greater distance between Fast-Fast and Fast-Slow (more dissimilar) than Slow-Slow and Slow-Fast conditions, suggesting that slow relaxing matrices had a greater impact on modulating T cells that have been previously cultured in fast relaxing matrices than vice versa (Fig. 6f).”

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

We would like to thank the reviewer for your thoughtful comments. We have performed a series of additional experiments to address your comments and have also provided clarification where necessary.

The authors generate two 3D gel matrix systems. One is a classic collagen matrix system and the other a formulation with the goal of producing a more viscoelastic environment.

We would like to clarify that the goal of the second collagen formulation is to create a **more elastic** environment. The classic collagen matrix crosslinks by forming fibrils, which can be deformed by cells. When we locally introduce covalent crosslinking to the matrix, we make the matrix less deformable. In the manuscript, we have termed the classic collagen matrix system as fast relaxing (more viscous), and the locally covalently crosslinked matrix we developed as slow relaxing (more elastic). This is reflected in Figure 2, where the time to which the collagen matrix relaxes to 60% of its initial stress is just about 10s for the fast relaxing matrix and >1000s for the slow relaxing matrix.

The overall goal is to determine how a viscoelastic environment can influence T cell behavior, which may have implications for how therapeutic T cells are prepared. The authors identify key shifts in T cell behavior and identify signaling pathways implicated in T cell phenotype and function and study how these may relate to cancer and fibrotic disease. Overall, the concept of methods to alter T cell behavior for therapeutic benefit prior to transfer experiments or treatment is very exciting. The study has many strengths, mostly in the detailed characterization of T cell responses.

Thanks for these comments

However, major issues are present with the 3D matrix approach. It is not clear that a different viscoelastic response is the driving factor influencing the observed shifts in cell behavior. The characterization of the viscoelastic response is not rigorous enough to accurately determine potential changes in viscoelastic behavior or their implications. Rigorous analysis is the viscoelastic response is not included and the language and conclusions regarding viscoelastic behavior are in error in multiple portions of the manuscript.

We originally characterized matrix viscoelasticity with rheology by determining the loss angle, which is the relative energy that is lost versus stored when stress is applied to a matrix. A fast relaxing matrix (classic collagen matrix system) will have a higher loss angle than the slow relaxing matrix system we developed (please refer to Figure 2c). In addition, we performed stress relaxation measurements, which is a measure of the stress needed to hold a matrix at a specific strain (displacement). For a fast relaxing matrix, the stress needed to hold the matrix at a specific strain will decrease more rapidly than for a slow relaxing matrix. Thus, the time at which the stress decreases to 60% of its initial value will be faster for a fast relaxing matrix than for a slow relaxing matrix. This observation is seen in Figure 2b (right). In general, loss angle and stress relaxation are two commonly used tests used to define the viscoelasticity of materials[7–9].

To more thoroughly characterize the mechanical properties of our gels, we have performed additional nano-indentation studies on these matrices to estimate the local mechanical properties. Consistent with our observations from bulk rheology, the fast relaxing matrices have significantly higher loss (viscous) moduli than slow relaxing matrices.

Likewise, a deep characterization of the 3D matrices in not included. While the examination of fibers and pore size is appreciated, a much deeper analysis is certainly needed. Along these lines, key controls for 3D system are missing. It is not clear that that the linker and click reactions steps are not impacting cell behavior, that the presence of difference materials is not influencing T cell behavior directly and that adhesion in the matrices is equivalent.

We appreciate these comments. We have performed additional experiments and have provided a detailed response below.

It is not clear that the matrices underwent the same processes in terms of time under different conditions (temperature, vehicle solutions etc.).

The matrices underwent the same processes under the different conditions. Cells in fast relaxing matrices (ie. Classic collagen matrix) were exposed to the same temperatures and same amount of vehicle solution for the same time period. We have modified the Methods section of the manuscript to make these points more clear.

Lastly, while the analysis of the T cell that is included in the manuscript is very exciting and impactful, key direct measure of T cell function are somewhat limited.

To address this comment, we performed two additional in vitro T cell cytotoxic studies: one study with a different donor, and a second with a different tumor type (Extended Figure 13), as well as a lymphoma xenograft study in mice with anti-CD19 CAR-T cells (Figure 7f-h) to directly test T cell function in vivo.

Yet, this Reviewer finds that the manuscript could provide fundamental new information and could be of interest to the Nature Biomedical Engineering readership following very significant revisions. The following comments are provided as constructive criticism to help improve the manuscript.

We appreciate the reviewer’s comments. Please see below for detailed response.

Major Comments:

1) The characterization of potentially distinct viscoelastic responses is not rigorous. As this is the foundation of all conclusions in the manuscript it is not possible to fully interpret most of the findings. While the methods discuss both stress-controlled testing and stress relaxation, neither is adequately described and key viscoelastic mechanical data is not presented in the manuscript.

We thank the reviewer for these comments. Detailed descriptions for the various mechanical characterizations we performed have been updated in the methods section. These include oscillatory time-sweep to measure the modulus and loss angle of the matrix, and shear stress relaxation to characterize stress relaxation and viscoelasticity of the matrix test for bulk rheology, as well as nanoindentation for local mechanical characterization. We describe these mechanical characterizations in detail in the methods section of the manuscript.

Regarding key viscoelastic mechanical data, we provide data on loss angle measurements and stress relaxation showing both stress relaxation profiles (Extended Figure 1c) and time when the matrix relaxes to 60% (Figure 2b (right)) of initial stress for bulk rheology. These are considered as key data in defining the viscoelasticity of a bulk material and have been commonly used in literature. Additionally, we also provide storage and loss moduli for nanoidentation. Importantly, these independent measurements consistently show significant changes in viscoelasticity between fast relaxing and slow relaxing matrices

Fundamental language for viscoelastic behaviors (e.g. creep and stress relaxation) are often intermixed, creating additional confusion. So there are strong issues with the bulk matrix viscoelastic description.

We are sorry if there has been any confusion regarding the mechanical characterizations we performed. However, we have not performed any creep experiments for our studies, neither have we mentioned creep in our manuscript.

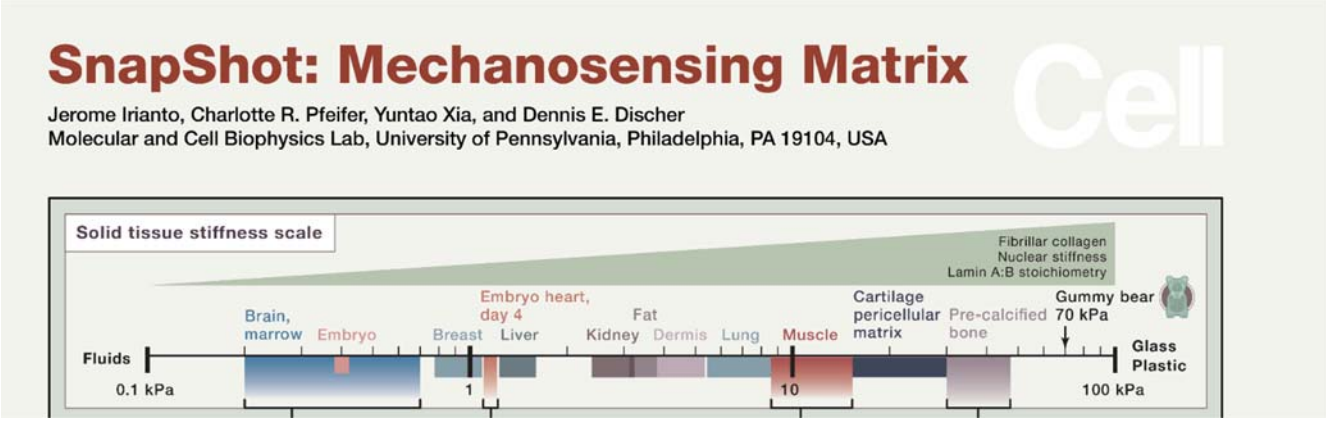
Also, collagen matrices can possess nonlinear viscoelastic responses where the rate of relaxation is dependent on the input strain (likewise the rate of creep can depend on input stress) with the rate being very fast for very low strains or stresses. As cell produce very low traction stresses the local rates would be extremely fast and it is not clear that even if there are difference in the bulk gels this would be detectable by cells.

Given collagen's non-linear viscoelastic response, we performed local nano-indentation measurements on fast- and slow-relaxing matrices with a fixed testing frequency of 110Hz (the nanoindenter's resonant frequency) and an oscillation amplitude of 500nm (0.5μm), translating to about 3300μm/min. Various studies have estimated T cell migration to be between 10μm/min and 25μm/min[10,11], which is significantly lower than our nano-indentation testing frequency. At our high testing frequency, we see that while these matrices exhibit similar storage modulus, slow-relaxing matrices had significantly lower loss modulus, supporting our claim that differences in viscoelasticity between fast- and slow-relaxing matrices could be detected on a micron-scale.

Likewise, the moduli ranges presented are not particularly relevant related to the tissues discussed in the manuscript. For instance the ~100-700 Pa range is lower than gummy bears ~70kPa), raisins (~220kPa) etc. and clearly not indicative of the "stiffness range of multiple soft tissues (Extended Fig. 1b), as well as pre-malignant and malignant tumors" except for the cases where those tissues are not mechanically tested correctly (e.g. AFM etc. pushing on the side of a fiber or network of fibers where collagen has a bending modulus that is orders of magnitude

lower than in under tensile loading, which is what cells produces from traction stresses applied to the fibers or fiber network). Overall the description of viscoelastic behavior on multiple scales would need to be profoundly more rigorous to support the conclusions presented in the manuscript.

As the figure[12] below suggests, the stiffnesses of individual collagen fibres are high, with some studies reporting GPa range[13] . However, as the figure also points out, most tissues (which have collagen as a substantial part of their ECM), are significantly softer than the individual fibrils and softer than gummy bears, suggesting that the long-range structures formed from collagen in tissues, which permits the sliding of fibres relative to each other[14], could be crucial to determining the tissue's overall mechanical properties.



While T cell phenotype and function could be influenced by the shorter, nanometer-scale fibre mechanical properties[14,15], our work is focused on how T cell phenotype is affected at the longer length scales.

2) Similar to point 1: The description of the matrices themselves and the potential direct impact on cells is lacking. While this reviewer greatly appreciates the analysis of fibers and pore size as much deeper analysis is needed (fiber width, angle, length, waviness etc.). Also, potential direct impact from the component and processing to make the Norbornene-modified collagen is not evaluated. These are critical controls. Along these lines it needs to be shown that other key aspects of cell behavior are not being impacted by the formulation on multiple levels (e.g. general ability to adhere etc.)

We thank the reviewer for their comment. We have performed additional analyses on the fiber width (Figure 2d), fiber length (Extended figure 1e), fiber waviness (Extended figure 1f), and angles between fibers (Extended figure 1g). Fast- and slow-relaxing matrices showed similar properties in all above-mentioned parameters, indicating they have similar collagen architecture.

As mentioned above, we ensure that T cells in both fast- and slow-relaxing gels are exposed to the same conditions including amount of collagen , temperature, vehicle solution, etc. during manufacturing. The only difference between fast and slow relaxing matrices is the presence of the methyltetrazine crosslinker, which is used to introduce local covalent crosslinks to the collagen.

We have also performed additional experiments to address the reviewer's comment regarding the impact of norbornene collagen modification and the presence of crosslinker on T cell adhesion and phenotype. Our data showed that collagen modification had little impact on T cell adhesion (Extended figure 3a), survival (Extended figure 3b) and phenotype (Extended figure 3c-e). Additionally, the presence of methyltetrazine crosslinkers had minimal effect on T cells cultured in unmodified collagen matrices (Col (+Linkers)), where the T cells were exposed to the crosslinker but the unmodified collagen would not become crosslinked. T cells however exhibited substantial phenotypic change when they were embedded in modified collagen crosslinked with methyltetrazine linkers (Col-Nb (+Linkers)). (Extended figure 3c-e). These data indicate that the differences we see are from changes of the viscoelasticity of the gel, rather than from collagen modification or the mere presence of methyltetrazine linkers. We have modified the manuscript text to address these points as follows:

"Potential effects of collagen modification and the presence of methyltetrazine crosslinkers on T cell adhesion and phenotype were next investigated. T cells that were seeded on top of unmodified and Nb-modified collagen with and without methyltetrazine crosslinkers adhered similarly to their respective collagen matrices (Extended Fig. 3a). In addition, T cells remained highly viable in all matrix conditions (Extended Fig. 3b) and had similar phenotypic profiles when they were embedded in unmodified collagen (Col) or in Nb-modified collagen (Col-Nb) (Extended Fig. 3c-e). The presence of methyltetrazine crosslinkers had minimal effect on T cells cultured in unmodified collagen matrices (Col (+Linkers)), but had significant effects on T cells cultured in modified collagen (Col-Nb (+Linkers)), as expected due to changes in matrix viscoelasticity from the resultant crosslinking in the latter (Extended Fig. 3c-e). Importantly, PCA analyses confirmed our observations, with Col-Nb (+Linkers) being most different along PC1 (Extended Fig. 3f)."

3) This is present at many locations in the manuscript, but as an example the statement "Pathway enrichment analysis showed that the SModule was enriched for pathways related to TCR signaling, CD8+ T cell activation and T cell cytotoxicity, indicating a more activated T cell state, while the FModule was enriched for genes related to leukocyte adhesion to endothelial cells (Fig.3f)." seems to suggest that more elastic reponses is a key driver of key behaviors evaluated in the manuscript, but the text consistently emphasizes viscoelasticity as the driver. If in fact there is a different viscoelastic response, it still seems clear that some key behaviors are driven by a more elastic environment and others by more viscous and the focus on more viscous is over-emphasized and interpreted. This is also true in areas there the authors conclude that "Our findings also suggest that therapies targeting the ECM viscoelasticity may directly impact T cell biology and fate". This may be true but it is also seems equally clear that targeting more elastic environments also directly impact T cell biology and fate. There are many instances throughout the manuscript where data doesn't line up or is showing opposite behavior, which is fine but as currently written there appears to be too much emphasis on trying to fit all the findings into the viscoelasticity box. Lastly since tumors are very complex and heterogeneous with a lot of elastic and viscous components this is particularly relevant.

We thank the reviewer for suggesting clarifications to the text. We would like to clarify by first defining what we mean by stiffness and viscoelasticity. We define stiffness as a property that describes the tissue's resistance to deformation in response to applied stresses. In contrast, viscoelasticity is a distinct feature from stiffness that confers a time dependent response to deformation: the stress needed to achieve a particular deformation decreases with time for a viscoelastic substrate but remains the same for a perfectly elastic material. The viscoelasticity of tissues, while previously underappreciated, is gaining increasing attention as growing evidence

suggests it can independently influence cellular behaviour. Thus, cells that are exposed to matrices with the same initial stiffness (initial elasticity), but with different time dependent responses to deformation of these matrices (viscoelasticity) could behave differently.

In the manuscript, when we say viscoelasticity is the driver for T cell phenotype, we are using it as an umbrella term to describe this distinct time dependent property, specifically looking at how the different regimes of viscoelasticity affect T cell profiles. While the collagen matrices we use in our studies are both viscoelastic, these are either more viscous (fast-relaxing) or more elastic (slow-relaxing) while having the same stiffness (soft or stiff matrices). Our work indicates that for the same initial stiffness, T cells respond differently to matrices with different viscoelasticity. So we are not claiming in our manuscript that the elastic component of the matrix has no effect on T cell phenotype. We are instead saying that for the same stiffness, the rate at which T cells can relax the matrix is an important modulator of their phenotype.

We have modified the following sentences in the introduction section of the manuscript to clarify our definitions of the various terms:
"Stiffness describes a material's resistance to instantaneous deformation, whereas viscoelasticity is a distinct feature from stiffness that refers to a material's time-dependent mechanical response to applied stresses and strains. The stress needed to maintain a particular deformation decreases more rapidly with time for minimally crosslinked, fast relaxing ECM (more viscous) than for more highly crosslinked, slow relaxing ECM (more elastic). Both tissue stiffness and viscoelasticity have been shown to independently influence the behavior of cells, including their proliferation, mobility and differentiation"

4) Deeper functional analysis of T cell would strengthen the manuscript, but this is difficult to evaluate at this time since the impact of the foundational platforms are not clear at this time.

We have performed extensive additional phenotypic and functional analyses of T cells in the matrices including a second single cell RNA-sequencing studies with a different donor, which includes TCR clonotypic analysis (Figure 5, Extended Figure 8), two additional in vitro T cell killing studies (using a different donor/model), and a mouse therapeutic study using a lymphoma xenograft model (Figure 7, Extended figure 12). We have also further characterized the persistence of T cell phenotypes after being exposed to matrices with different viscoelasticity, including performing a serial matrix culture experiment (Figure 6, Extended figure 9, Extended figure 10). We believe that these data give additional support to our claims.

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

The manuscript by Adu-Berchie et al., from the David Mooney lab addresses an interesting aspect of T cell engineering, which has gained importance in the recent years due to its high clinical efficacy and hence significance in cancer therapeutics. The manuscript explores the role of matrix stiffness and viscoelasticity as a potential modulator of T cell phenotype and function in the context of different tumor types.
The topic addressed is interesting and the data reveal that mechanosensitive cues from synthethic matrices affect the AP1 pathway in a differential manner and that ECM viscoelasticity rather than stiffness can be utilized to fine tune generation of clinically relevant Therapeutic CAR T cells.

We thank the reviewer for these comments

There are however some major caveats with the data analyses and interpretation as well as many missing details that make it difficult to assess the data and conclusions. These concerns are outlined below and need to be addressed-

Major points:
1. Figure1- Not clear what the data presents in Figs 1b and c.… 'tumor-tissue derived T cell gene signatures' needs to be defined…

We apologize for this confusion. To generate 'tumor-tissue derived T cell gene signatures', we performed differential gene expression analysis between T cells isolated from tumours and T cells isolated from normal tissue and blood. Genes that were differentially expressed in tumors (genes with adjusted p-value$\leqslant$0.1, average log2 fold change$\geqslant$0.1 and fraction gene expression in $\geqslant$0.4 of tumor T cells) were selected as the tumor-tissue derived T cell gene signature. To simplify the terminology, we have changed the term 'tumor-tissue derived T cell signatures' to 'tumor T cell signatures'. We have updated the manuscript text as follows:
"Tumor T cell gene signatures were generated for each tumor type by performing differential gene expression analysis between tumor derived T cells and T cells in normal tissue and blood (Supplementary Table 2)."

how is the module represented as a single expression number?
The aggregate expression of the tumor T cell signature was determined using Seurat's AddModuleScore function. The function calculates "the average expression levels of each module on a single cell level, subtracted by the aggregated expression of control feature sets" [16] The single expression number estimated is therefore the average expression of the genes within the module normalized to the average expression of randomly generated gene sets.

Also, the data just shows that T cells from blood, normal tissue and tumor are distinct…which is to be expected. …..the conclusion that the distinct ECM micro-environments affect T cell phenotype is not justified at least based on the current representation

While we observed that T cells with TCR clones shared between blood, normal tissue and tumors had different transcriptomic profiles across the three tissue types, we agree that this observation does not necessarily mean that the transcriptomic differences are specifically attributable to the tissue micro-environment. Instead, we believe that the results imply that the tissue microenvironment may be playing a role. As indicated in the introduction section of manuscript, prior studies have also suggested that T cell phenotype could be inherently linked to their location[17–19].

We now edit this text to make clear that this analysis of T cells suggests aspects of the microenvironment may regulate T cell phenotype:

Beginning of paragraph: *"First, to confirm and extend previous reports that aspects of the tissue microenvironment may regulate T cell phenotype in vivo, published scRNA-seq datasets were analyzed to compare CD8+ T cells located in tumors, adjacent normal tissues and blood for patients with non-small cell lung cancer (NSCLC), liver cancer, and colorectal cancer, representing diverse mechanical environments."*

End of paragraph: *"These results suggest that the tissue microenvironment may be playing a role in modulating T cell phenotype."*

The goal of the studies presented in the manuscript is therefore to investigate the extent to which the tissue mechanical properties could be contributing to these differences. To isolate the effects, we developed an in vitro ECM model with tunable mechanical properties and observed transcriptomic trends strikingly similar to our observations in both cancer and fibrosis patients (Figure 3, Extended Figure 5-6). While we do not wish to claim that the tissue ECM mechanical properties is the sole factor behind our in vivo observations, we believe that our findings indicate that distinct mechanical properties play a significant role in tuning T cell phenotype and function.

Fig1 a…missing x, y axis titles

We would like to thank the reviewer for pointing it out. We have added the missing axis titles as "UMAP 1" and "UMAP 2"

2. Rationale and succinct description of the CD3/CD28 dynabeads as a proxy for APCs for T cell activation should be described in results so that its clear how the T cells were activated before culture in slow/fast gels. It would also be interesting to additionally test CD3/28/137 combination that is used for expansion of effector/memory cells since fast relaxing conditions seem to naturally enrich the memory markers.

We would like to thank the reviewer for this comment. We edited the introduction with the following rationale:
"To control for initial T cell activation, T cells received the same initial stimulation regimen before they were cultured in matrices with different viscoelasticity. In vivo, T cells are primed when antigen presenting cells sample, process, and present antigen to T cells on their major histocompatibility complex (MHC) proteins. However, the phenotype of these antigen presenting cells themselves could be influenced by their mechanical environment, complicating one's ability to determine how matrix mechanical properties regulate T cell activation. Consequently, we used synthetic CD3/CD28 dynabeads, which is the industry standard for CAR-T cell activation and expansion for clinical use, and which can be easily separated from T cells via the use of magnets before the cells are seeded in collagen matrices."

We also performed additional experiments using CD3/28/137 and observed similar effects of ECM viscoelasticity as CD3/28 dynabeads. We have modified the manuscript text to reflect the additional experiments as follows:

"To understand the generalizability of ECM viscoelasticity in modulating T cell phenotypes in relation to mode of prior activation, T cells were either activated with CD3/CD28 or CD3/CD28/CD137 dynabeads, before embedding in collagen matrices, with the latter known to enhance memory T cell enrichment. Consistent with previous data, flow cytometry analyses showed an impact of ECM viscoelasticity in modulating T cell phenotype after CD3/CD28 activation. Umap, K-means, and PCA analyses confirmed these differences, with T cells harvested from slow-relaxing matrices being enriched in clusters with higher expression of activation markers: Clusters 1, 4, 8 (Extended Fig. 11a-d). Importantly, these phenotypic differences were maintained when the T cells were activated with CD3/CD28/CD137 dynabeads prior to matrix culture (Extended Fig. 11e-h)"

3. Since the T cells are activated prior to culture in fast/slow gels….it should be checked if the gels just lead to selective survival and proliferation of certain T cell subsets.

Live/dead cell staining can provide an idea.
Alternatively, the cells can be cultured in the gels first and then activated to confirm that the effect of the ECM viscoelasticity is on the T cell phenotype per se and not selective growth of subsets.

We would like to thank the reviewer for this comment. We did not see striking differences in live/dead staining, as T cell viabilities remained high in all the conditions (Extended Fig 3b). In addition, culturing T cells in vitro for any significant period without prior activation leads to cell death, whether or not the cells are cultured in collagen matrices.

To comprehensively address this comment and similar comments raised by reviewer 1, we performed single cell RNA and TCR sequencing of T cells cultured in fast relaxing and slow relaxing matrices, as well as plate-cultured T cells (without matrix culture). The T cells used for this study were from a different donor from the ones used in the initial RNA sequencing study. Our studies revealed that ECM viscoelasticity does not select for specific T cell clones that persist, but instead these phenotypes emerge as a function of viscoelasticity. We have updated the manuscript and figures with our findings (Figure 5 and Extended Figure 8). We summarize our findings here:

a. We investigated the profile of T cell receptor β (TRβ), a component of the TCR heterodimer. Similar frequency distributions were observed for the different TRβV genes as a function of ECM condition (Extended Fig. 8a). In addition, a Venn diagram of the TRβV genes showed that 96% of the genes were present in all the conditions (Extended Fig. 8b). Shannon entropy, which estimates the diversity within a specific population, showed similar TRβV diversity between T cells cultured in fast relaxing matrices, slow relaxing matrices, and plate-culture, as well as their parent T cell population (Pre-Gel) (Extended Fig. 8c). Importantly, when the Slow-Relaxing Module (SModule) generated from Fig 3 was applied to the T cells grouped by TRβV gene, the slow relaxing matrices had the highest expression across all TRβV genes (Extended Fig. 8d).
b. We next investigated the effects of ECM viscoelasticity on CDR3 profiles and the persistence of T cell clonotypes. First, it was observed that TCRβ CDR3 length distribution was similar across all the conditions, including in the parent T cell population (Fig. 5c). Assessment of TCR clones revealed that all the conditions had similar clonotypic diversity as estimated by Shannon entropy (Fig. 5d). The T cells generally had unique clonotypes, with only 3.7% of T cells being clonal (clones observed in >1 cell) (Fig. 5e). Dominant clones (clones observed in >3 cells) were not limited to specific conditions, but were shared across multiple conditions, with 5 clones being shared among all conditions (Fig. 5f). Importantly, T cells with shared TCRs derived from the various matrix conditions were transcriptomically different, with the T cells cultured in slow relaxing matrices having the highest expression of the SModule (Fig. 5g).
c. We hypothesized that if the matrix is acting by selecting specific T cell clones, whose phenotypes persist over time, then there should be a correlation between the transcriptomic profiles of those T cell clones before and after matrix culture. However, correlation analysis between individual T cell clones before and after their culture in collagen matrices showed weak correlation for the fast relaxing matrix condition (Pearson correlation=0.38) and no correlation for the slow relaxing matrix condition (Pearson correlation=-0.089) (Fig. 5h-i). Together, these findings suggest that ECM viscoelasticity directly modulates T cell phenotype, and does not select specific T cell clones which persist over time.

These exps are described later in Fig5…as acute vs chronic activation but only the protein expression of the AP 1 pathway components is analyzed, which seems to be operational in both slow and fast gels
It is important to analyze sc-RNA data from these conditions and test if the data are similar in 'acute' vs 'chronic' conditions.

Changes in the AP-1 pathway was observed as a function of matrix viscoelasticity for T cells chronically stimulated in the gels. Some of the AP-1 components showed consistent trends between acute and chronic stimulation (c-Jun, BATF and JunB) while others showed an inverse trend (c-Fos). We have modified the text to clarify the patterns of AP-1 expression for T cells chronically stimulated in fast and slow relaxing matrices. To better organize the manuscript, we have moved all the data on chronic stimulation to Extended Figure 14 (AP-1 pathway analysis, phenotyping and cytolysis).

"AP-1 protein analysis of T cells chronically stimulated in slow relaxing matrices maintained higher expression of c-Jun (Extended Fig. 14a-b), BATF, and JunB but expressed lower levels of c-Fos compared with cells in corresponding fast relaxing matrices (Extended Fig. 14b). c-Jun co-immunoprecipitation of cell lysates showed that T cells that received chronic stimulation in slow relaxing matrices had higher binding of JunB and BATF but not c-Fos (Extended Fig. 14c). Importantly, the amount of JunB and BATF bound to c-Jun increased markedly when the T cells were cultured in slow relaxing matrices with chronic stimulation, as compared to T cells in fast relaxing matrices (Extended Fig. 14c). In terms of the changes in the individual AP-1 proteins between acute and chronic stimulation, larger increases in BATF and JunB expression were found for slow relaxing matrices after chronic stimulation, while c-Fos, FosB, and c-Jun increased more with fast relaxing matrices (Extended Fig. 14d)."

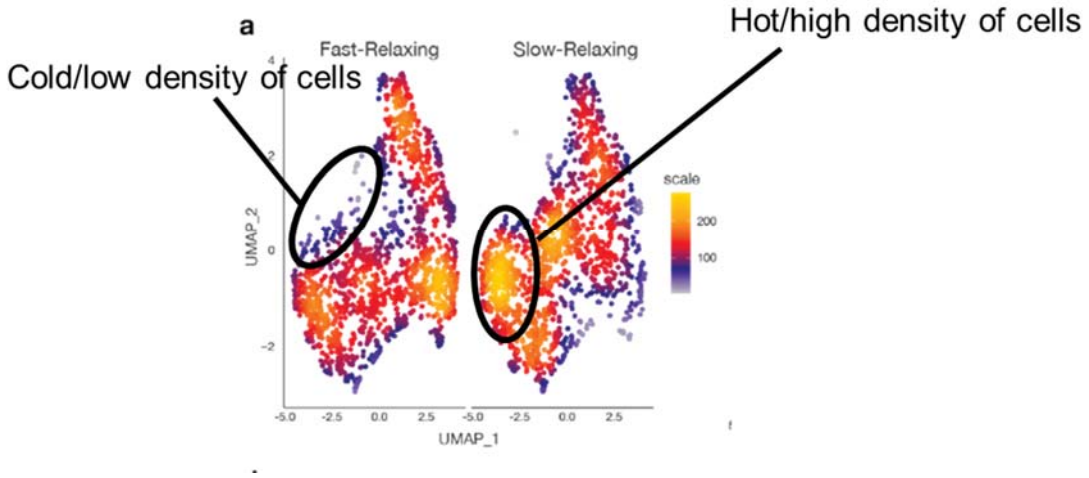
Unfortunately, we were unable to perform scRNA sequencing on T cells undergoing chronic stimulation due to budget constraints. However, the consistency between the AP-1 transcriptomic and protein expression profiles for the acute conditions suggests that a similar trend will be observed for the chronic conditions as well.

4. Figure 2- is the data in g and h marker expression from flow cytometry or gene expression? No introduction or details for the markers (including CD45Ra and CD62L) is provided which is needed to understand rationale.

We thank the reviewer for pointing out this confusion. The data is for flow cytometry. These markers are commonly used for T cell memory (CD45RA, CD62L, CCR7), T cell mediated apoptosis (Trail, TrailR2, Fas, AnnexinV), activation (CD25), and exhaustion (LAG3). These markers can be used to broadly understand the changes in T cell phenotype in various aspects. We have edited the manuscript text as follows:
"In particular, flow cytometry analysis of CD8+ T cells showed higher expression of activation and inhibitory markers in slow relaxing matrices, while T cells in fast relaxing matrices exhibited higher memory marker expression, such CD62L and CD45RA (Fig. 2f)."

5. In Figure 3a- this is single cell data so the cell clusters in the fast and slow gels should be defined and analyzed. What are the 3 colors in the UMAP?… In Fig e, the fast module seems to represented in both fast/slow gels ….how are the differential modules determined? The informatics and statistics need to be detailed here

We thank the reviewer for this comment. Figure 3a is a UMAP density plot. Each dot is a cell. Areas of the plot that are hot (red) means high density of cells, while areas that are cold (blue) means low density (please find annotations in the figure below). Representing the data this way makes it easier to better visualize differences between the two conditions.



Regarding the clustering/bioinformatic approach used, we utilized a technique called consensus non-negative matrix factorization (cNMF) [20] to group genes into expression programs in an unsupervised manner. Traditional clustering approaches tend to put a cell in a "box", without regard for continuity of expression profiles across the different clusters. This is especially problematic for T cells, where expression of many markers are continuous between the different T cell populations. cNMF addresses the limitations of traditional clustering approaches by inferring gene expression programs (functional gene modules) "directly from variation in single-cell expression profiles using matrix factorization" [20]. cNMF then estimates the relative contribution of each gene module in each cell, meaning that a given cell could have variable expression of multiple gene modules. This explains why cells in the slow gels seem to have some expression of the fast relaxing module, albeit at lower expression levels than T cells in fast gels.

The bioinformatics analysis pipeline and statistics are now detailed in the methods section of the manuscript as follows:
"cNMF was performed in Python version 3.8.8. First, SCT correct counts were imported, normalized and filtered for the top 3000 over-dispersed genes, after which factorization was performed from k=5 to k=21 with 200 NMF iterations for each k. After factorization, the optimal k was selected as a trade-off between stability and error (ie. highest stability and lowest error). The top 100 genes that characterize each usage/expression program for the selected k were then exported for downstream analysis.

The aggregate expression of the top 100 genes from each cNMF expression program was calculated for each cell in R using Seurat's AddModuleScore function. Expression programs that were significantly enriched in T cells cultured in slow or fast relaxing gels were determined using the Wilcoxon Rank Sum Test with FDR for p-value adjustment to estimate significance. Significant gene expression programs were selected as having a -log10 adjusted p-value>15. To estimate the condition in which a specific expression module is enriched, fold change was calculated as log2 (mean(slow relaxing[i])/mean(fast relaxing[i])), where i an expression

program. Finally, unique genes for expression programs enriched in slow or fast relaxing gels were separately combined. Pathway analysis was performed in Enrichr5 using the Elsevier Pathway Collection6. P-values were determined using the Fisher Exact Test and adjusted using FDR. The top pathway terms were selected for each condition, and the adjusted p-values for both conditions for that term were plotted against each other as bar plots."

6. To validate that the ECM viscosity changes are transduced through the AP1 pathway, modulation of this pathways (activation and inhibition) should be tested in the fast/slow gels.

We thank the reviewer for this comment. We have extended our studies to directly test the role of viscoelasticity on AP-1, and on T cells by blocking c-Jun function with the SP600125 Jun N-terminal kinase inhibitor[4]. We saw that SP600125 abrogated the effects of matrix viscoelasticity and led to increased expression of memory markers, supporting our claim that these changes are modulated via the AP-1 pathway. We have updated the manuscript text and figures with these findings (Fig 4d-g).

7. The CAR T data (Fig 6) is very interesting-howveer it has been carried out in 'acute' conditions only.
Due to the reservations expressed earlier about the selective growth potential…..the CAR T cells should be cultured in the fast/slow matrices first and then activated to discern the difference between ECM viscoelasticity

We thank the reviewer for this comment. We have also performed the CAR-T cytolysis in chronic conditions (Extended figure 14). We could not first culture CAR-Ts in fast/slow matrices before activation, as the T cells die in culture without activation. However, please refer to our response for comment 3 regarding findings from TCR sequencing of T cells cultured in fast and slow relaxing matrices.

The functional differences in tumor cell toxicity is exciting but has been carried out in a single tumor type-the raji cells are a cell line of lymphoblastic tumor…it would also be really interesting to test NSCLC, liver and colorectal cells lines that have differences in ECM viscoelasticity since the initial figure and data hypothesize that the tumor ECM differences will also affect the function of T cells.

We thank the reviewer for this comment. Unfortunately, we were unable to access the requested tumor cell lines. However, we have performed cytolysis with Pmel-1 T cells, which are TCR T cells that recognize gp100 on mouse B16-F10 melanoma tumor cells. We saw a similar trend as the Raji lymphoma cells (Extended figure 9g), suggesting that this observation is generalizable across different tumor types and species. We have updated the manuscript text as follows:
"We next explored T cell functionality as a function of viscoelasticity for TCR T cells using a different tumor type and in a different species to understand the generalizability of these observations. We first cultured mouse Pmel-1 T cells, which recognize the gp100 epitope on B16-F10 melanoma cells, in collagen matrices of different viscoelasticity, and subsequently co-cultured these T cells with B16-F10 tumor cells. Pmel-1 T cells cultured in slow relaxing matrices had the best killing relative to plate-culture and fast relaxing matrix conditions (Extended Fig. 13g)."

8. If possible, testing the function of CAR T cells generated in different matrices against tumors

in vivo would really underscore the clinical relevance of the study and the potential to use ECM matrices for the generation and modulation of CAR T and other therapeutic T cells.

We thank the reviewer for this comment. We have performed an in vivo lymphoma xenograft study with anti-CD19 CAR-T cells (Figure 7f-h). Similar to our in vitro cytolysis studies, CAR-T cells exposed to slow-relaxing matrices performed better in controlling tumour growth, resulting in significantly better mouse survival.

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Rebuttal 2

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

The authors have done outstanding work revising the manuscript. This has improved the quality of the manuscript and the scientific findings would be of high interest to the field. I do not have any further comments.

We would like to thank the reviewer for your thoughtful feedback throughout the review process. Your comments played a significant role in strengthening the manuscript, and we are glad that we have been able to satisfactorily address them.

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

Overall, this reviewer appreciates the significant effort the authors have made to perform additional experiments and clarify their findings in order to address the concerns of the three reviewers. The new data and clarifications have addressed a number of the raised concerns. However, it is this reviewer's opinion that a number of some concerns from all three reviewers were not adequately addressed and some of the new data is being over-interpreted.

Focusing on responses to this reviewer's comments, the characterization and presentation of viscoelastic behavior between the relaxing collagen gels vs. the crosslinked gels (termed fast vs. slow relaxing; this reviewer apologizes for the viscoelastic vs. elastic typo in the first review and hopes it did not create undo confusion) still lacks robust rigor. While the explanation defining the fundamentals of mechanics is an intriguing strategy, it is not convincing to a reviewer with extensive expertise in this area that the measures, data completeness, and language are rigorous. For instance, this reviewer is fully aware that the authors did not conduct creep experiments, but when the authors use terms such as "applied stress' for a displacement controlled relaxation experiments it can create confusion (which was part of the point about focusing the language used). Certainly stress is generated and there can be relaxation from the peak stress for a viscoelastic material, however, this is typically not a linear viscoelastic behavior for many biologic tissues and for collagen matrices.

We thank the reviewer for their comment and apologize for the previous mistakes and confusion caused by our language. We have modified the text as follows:

- We have modified our definition for viscoelasticity by replacing the phrase "applied stresses", with "applied strain". The definition has therefore been reworded as follows: *Stiffness describes a material's resistance to instantaneous deformation, whereas viscoelasticity, as exhibited by stress relaxation, is a distinct feature from stiffness that refers to a material's time-dependent mechanical response to applied strains*
- The legend for Figure 2b on stress relaxation has been modified by replacing the term "applied stress" with "applied strain". Consequently, the legend for Fig 2b reads as follows: *b. Shear Young's moduli (left) and time values for relaxation to 60% of initial stress for the applied strain (right) for 2 mg/ml (soft) and 4 mg/ml (stiff) collagen matrices*

Further confusion is added by the very limited presentation of data from both a shear relaxation test (as opposed to a more classic uniaxial stress relaxation test and where shear is not mentioned in the main text) and a classic oscillatory rheology test. However, the data in supplementary figure 1c is a greatly appreciated. Yet, the analysis is still quite limited (the nano-indentation data does not add greatly to the

analysis) and it is concerning that a more robust analysis of data and more rigorous discussion of viscoelastic behavior was not presented in the revision.

We thank the reviewer for their important comments regarding the mechanical testing performed. We have now provided further clarity in the Methods section. We have performed the following multiscale analyses to characterize our matrices, similar to previous papers that have characterized stress relaxation in nonlinear matrices[1–4]:

Shear rheology (This has also been updated in our Methods section):

- Oscillatory time sweep at 1 Hz, 1% strain to measure the storage modulus (G'), loss modulus (G"), and the loss angle (δ, delta) (Figure 2b-c).
- A shear stress relaxation test at 20% shear strain, 1 Hz. The strain was maintained at 20% for up to 12hrs while recording changes in shear stress. A humidity chamber was used at all times to prevent dehydration. From the stress relaxation measurements, we reported the stress relaxation profiles (ie. normalized stress vs time, Extended Fig 1c), and time values for relaxation to 60% of initial stress for the applied strain (Fig 2b).

Nanoindentation: Storage and loss moduli at surface approach frequency of 110Hz, oscillation amplitude of 500nm and contact phase change of 3 degrees. We believe this parameter is more representative of the uniaxial stress and strain profile cells may sense and respond to versus bulk indentation experiments.

We have also modified the text to specify areas where shear rheology was performed as follows:

- We have specifically mentioned that we performed stress relaxation in shear when we described results from shear mechanical measurements as follows: *Mechanical characterization of the matrices showed that while click crosslinking influenced matrix viscoelastic properties, as reflected in shear stress relaxation and loss angle of the matrices, it did not affect shear bulk stiffness values (Fig. 2b-c, Extended Fig. 1c).*
- The legend for Figure 2b has been modified to specify that shear rheology was performed as follows: *b. Shear Young's moduli (left) and time values for relaxation to 60% of initial stress for the applied strain (right) for 2 mg/ml (soft) and 4 mg/ml (stiff) collagen matrices*
- We have modified the methods on rheological characterization to specify that it is shear.

However, with all that said, this reviewer remains very excited about the work overall and concepts behind it. While this reviewer clearly has strong concerns about the testing and description of the base platform - that all conclusion derived from – it is also clear that the two gels are mechanically different. It may remain to be seen how the cells are sensing this difference, but for now the impact on T cell behavior is very impactful and interesting. As such, this reviewer believes the above points can be largely addressed to a reasonable degree with text edits (and more robust data analysis if available is welcomed) and that the manuscript could be acceptable for publication following these edits (and those noted below).

We are grateful that the reviewer is excited about the work overall and is open to their comments being addressed by text. We have attempted to do so as highlighted above. In addition, while the mechanical characterization parameters used have been historically reported for the characterization of gels[1–3], we have indicated in the text that further analysis may be performed in future studies. We have added the following text to the discussion to recognize this important point: *Further mechanical characterization and analyses could provide valuable additional insights into the viscoelastic behavior of these matrices*

Additional comments:

1) For the new data in Extended Figure 3. It is greatly appreciated (along with the more in depth data analyzing the gel structures in supp. Figure 1), however, it seems to show more variability than the authors are noting. The cluster and PCA analysis show some strong differences, suggesting the autors are over-interpreting the findings. Also,a more cell level analysis would be insightful.

We appreciate the reviewer's comments regarding Extended Figure 3. We acknowledge that the presence of the linkers did have some effects on the T cells. This is not unexpected, as very few molecules are inherently neutral to cells. However, as principal component analysis (PCA) shows, this effect is minimal, compared to the effects of crosslinking. Importantly, Col-Nb (+Linkers) was most different along principal component 1 (PC1), which explains over 85% of the variance. However, to address the reviewer's comment, we have modified the text as follows: *The presence of methyltetrazine crosslinkers had some, but minimal effect on T cells cultured in unmodified collagen matrices (Col (+Linkers)), but had significant effects on T cells cultured in modified collagen (Col-Nb (+Linkers)), as expected due to changes in matrix viscoelasticity from the resultant crosslinking in the latter (Extended Fig. 3c-e). Importantly, PCA analyses confirmed our observations, with Col-Nb (+Linkers) being most different along PC1 (Extended Fig. 3f).*

2) The new data in Figure 7 is also appreciated. However, Figure 7 is missing key controls. How do activated T cells that don't go into a 3D matrix behave? What if activated T cell that have never gone into a matrix out-perform the ones that are activated and then go into a matrix? This still would indicate very interesting differences from the matrices but may not indicate that going into 3D before infusion is a good clinical strategy. Likewise, for the tumor-only group it does not appear there was a control injection or introduction of non-specific T cells etc.

We thank the reviewer for the comments. The survival trends in our lymphoma xenograft study with anti-CD19 CAR-T cells (Figure 7f-h) cultured in fast relaxing and slow relaxing collagen matrices mirrored our in vitro cytolysis studies: CAR-T cells exposed to slow-relaxing matrices showed superior control of Raji tumours and resulted in significantly better mouse survival. Based on the extensive transcriptomic (Fig. 3f, Fig. 5, Extended Fig. 8), phenotypic analyses (Fig. 4, Extended Fig. 9, Extended Fig. 11) performed on plate-cultured T cells (ie. T cells that did not go into 3D matrices), our functional in vitro T cell cytolysis studies using both anti-CD19 CAR T cells and Pmel-1 TCR T cells that included plate-cultured T cells (Extended Fig 13), we would expect T cells cultured in slow-relaxing matrices to show superior control of mouse tumours than plate-cultured cells. We thus did not include plate-cultured T cells in the in vivo study to avoid unnecessary use of animals; the consistency between in vitro T cell cytolysis and the in vivo lymphoma xenograft study for cells cultured in the gels supports the predictive ability of the in vitro studies.

In addition, while we did not include a non-specific T cell control in our lymphoma xenograft study, we have performed a number of separate experiments using the same tumour model, where we compared the survival of mice injected with either Raji tumours only, or with Raji tumours and non-specific T cells[5] (we used a higher dose of non-specific T cells in that previous study than the CAR T cell dose we used in Fig 7). The figure below demonstrates no difference in survival between the two groups

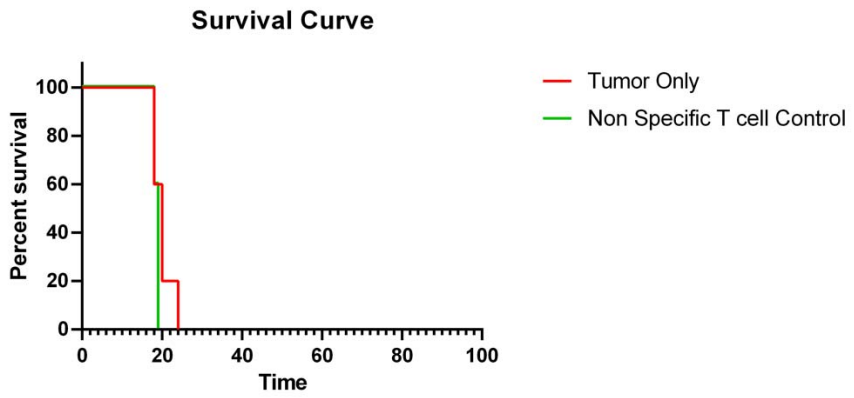

Figure: Kaplan-Meier survival curves of mice inoculated with luciferized Raji lymphoma. Mice either received only tumor inoculation (red) or tumor inoculation and T cells that were not transduced with CAR vectors (green). N=5 per condition. P = 0.8643

3) (minor) Along the lines of other mechanics issues, the figure listed showing the comparison to the 70 kPa gummy bear highlights the reviewers point as the other metrics are largely from inappropriate mechanical testing schemes such as indentation (oddly the 70 kPa for gummy bears comes from a classic stress strain test, unlike muscle which also has a hundreds of kPa to MPa modulus when tested in the correct configuration, e.g. uniaxial stress-strain test). Unfortunately, a disregard for an extensive body of tissue mechanics literature with enormous consensus data is quite problematic in the cancer mechanobiology field.

We agree that it would be beneficial for the field to develop a more streamlined framework for mechanical characterization of substrates, and have added this point to our discussion, "Developing frameworks across the field for mechanical testing of different substrates could serve as guidelines for the future design and iterations of these matrices." Along those lines, it is important to acknowledge that different stiffness regimes/substrate types may require different forms of mechanical characterization and instrumentation. For instance, a major issue with uniaxial testing for soft gels/tissues is mechanical failure due to clamping[6,7]. In addition, localized mechanical characterization approaches such as nanoindentation is important for determining "local mesoscale properties" [8], and cannot be achieved by uniaxial testing.

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

The revised manuscript by Adu-Berchie et al., from the David Mooney lab addresses an interesting aspect of T cell engineering, which has gained importance in the recent years due to its high clinical efficacy and hence significance in cancer therapeutics. The manuscript explores the role of matrix stiffness and viscoelasticity as a potential modulator of T cell phenotype and function in the context of different tumor types.

The authors have satisfactorily addressed all concerns that were raised-clarifying methods and text and adding new data. Added new experiments especially the in vivo studies, inhibitor studies as well as inclusion of additional tumor cell lines have significantly strengthened the study which in my opinion is now suitable for publication.

We are grateful to the reviewer for your time and thoughtfulness. We are happy that we have been able to address your comments satisfactorily. We believe that this manuscript has been strengthened because of your feedback.