

# Tumour-intrinsic features shape T cell differentiation through precursor to symptomatic multiple myeloma

Corresponding Author: Dr Kwee Yong

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**This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.**

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Review comments:

In the manuscript Foster et al., the authors aimed to identify changes in T cell subsets driven by MM progression from precursor states and understand how these alterations to T cell differentiation influence disease progression. They demonstrate using single cell RNA and TCR sequencing methods that altered T cells in MM patients possess features of terminal memory differentiation, not exhaustion, and resemble T cell skewing observed in immune ageing. This is correlated with disease stage and driven by tumor intrinsic features. Since T cells were found to not be exhausted, this may explain why checkpoint inhibitors are largely unsuccessful in MM. The authors also identify Treg loss as a novel biomarker for SMM progression, contradictory to the reported increase of immunosuppressive Tregs. The degree of T cell characterization and immune dysfunction through disease stages is extensive, providing a framework for future studies that is immensely valuable to the field. The data analysis is excellent, and the interpretation and conclusions are well-supported.

1. MAIT cell abundance is repeatedly found to be different between healthy and disease. Can the authors comment on this cell type and possible function/absence in disease?
2. The authors discuss the "accumulation of TCRs possessing similar CDR3 sequences indicates responses against common antigens" and were not found to be viral reactive. Is there a way to show/model that the increased non-viral TCR repertoire are tumor antigen-specific (antitumor immunity)? Is tumor MHC-I expression increase a good enough correlate? More evidence to support this association?
3. Have the authors identified any shared tumor neoantigens among patients? If tumor-reactive T cells are not exhausted, why can they not control tumor growth (propose mechanism of tumor escape)?
4. The authors conclude overall Treg loss "may reflect early reshaping of the BM microenvironment towards a state favoring progression." This is contradictory to reports of increased Tregs contribute to immunosuppression and favor tumor progression. Could the authors discuss how Treg loss may contribute to active MM progression mechanistically?
5. Were senescent T cells (characterized by CD28-, CD57+, KLRG-1+, CD160+) not found? TEMRA cells can exhibit cellular senescence. Include more references to T cell senescence in MM?

Minor comments:

Line 61: typo "increase h" risk

Line 119: "show diverse" or "diversify"

Line 191: "BM T-cell composition was strikingly similar in patients and controls" refers to Fig.3A

Line 249: ...unique feature "of" T cell differentiation in myeloma.

Line 370: "frequency of CDR3 clusters among expanded TCRs rose post-ASCT" refers to fig.6C?

Figure 5D: typo: graph x-axis "% tumour stress"

Supplemental Figures

Line 87: typo: "pver" donor

Line 300: "exaggerated T-cell ageing similarly correlated with paraprotein" refers to fig. S8B or C?

Reviewer #2

(Remarks to the Author)

In this manuscript, Foster et al. performed a complex and valuable work aiming to understand the T-cell differentiation in multiple myeloma and its precursor stages in response to tumor-intrinsic features by integrating a large collection of single-cell data, including healthy samples, MGUS, SMM, and MM from both bone marrow and peripheral blood. Through accurate sample integration and annotation they thoroughly annotate T-cells, showing a T-cell skewing that moves from naive and early T-cell subsets towards terminal memory cytotoxic and was associated with disease stages and age independently. They further characterize T cells clonality and the relation with T cell skewing further differentiating this tumor associated clonality with viral specific clones. By analyzing also tumor plasma cells authors establish a correlation between clonality and specific T cell populations and MRD positivity.

There is no question this manuscript is a valuable resource and also provides some interesting hypothesis for understanding the interaction between the immune microenvironment and MM. However, there are a number of limitations that need to be addressed

Main

- Integration of scRNA-seq datasets: When presenting the datasets used in the study, authors referred to 12 public + UCL studies (if I'm not wrong, sometimes referred to as "This study", "Foster 2024" or "Foster 2025"), however, there are inconsistent numbers between Fig.1, Fig.S1A, B, Table S1 and S6. Seems that Botta 2023 study is not included in the integrated dataset or used independently. Please, clarify and correct the descriptions and figures accordingly. Authors also reach some conclusions based on the cell type composition analysis in the integrated dataset. Due to the divergencies in the sorting strategies followed between studies, these approaches are biologically meaningless, as the differences observed may point to technical biases. Importantly, plasma cells represent only 6.6% of the integrated dataset, a consequence of the different sorting strategies applied, where several removed the plasma cell fraction, and others enriched for the T-cell fraction.

- Authors did a complete annotation of the different T cell phenotypes. Also, they deeply annotated the CD4+T-reg cells and the Invariant, identifying gdT cells, MAIT cells, etc. Why is this deep annotation not integrated into the general annotation of the dataset?

- According to the study, BM of myeloma patients is not enriched in exhausted T-cells which is unlike other reported studies so several comments are raised: 1) Why do authors focus on the comparison of CD8+Tex with CD8+Tem-CD69+ cells? What about the other CD8+Tem cells?; 2) Authors stated: "Importantly, we distinguish CD8+Tex from PDCD1-expressing CD8+Tem.CD69+." However, the increased expression of PDCD1 in CD8+Tem.CD69+ cells is not evident (Fig.2F, G, and Fig.S3B). Could the authors clarify that?; 3) Similar to Fig.2B, it will be nice to see the distribution of cell populations per disease group. The way to show the distribution in Fig.S5A, although informative, provides different messages; 4) Have the authors tried to subcluster the CD8+Tem.CD69+ cells? Could the subclustering reveal CD8+Tem.CD69+ subclusters related to T-cell exhaustion? Or within the overall CD8+Tem cells, could you identify a subcluster with an exhausted phenotype? 5) When comparing T-cell composition between marrow and peripheral blood, the sample donor was included as a random effect in the analysis. If the analysis is performed only with paired marrow and PB samples, are the conclusions the same?

- Authors stated: "BM T-cell composition was strikingly similar in patients and controls"; however, to support that, a representation similar to Fig.2B must be provided for the T-cell phenotypes. Fig.S5A shows relevant information, but does not support the stated message.

- In the T-cell composition analysis of controls with each myeloma disease stage (Fig.3B and Fig.S5B), why is the number of individuals per group different between Fig.3B and Fig.S5B?

- The authors define the T-cell skewing based on the PCA of T-cell phenotypes' abundances, showing association with disease (SMM and MM) and age independently (Fig.3C, Fig.S5H, and Fig3.E). However, the correlation between MM age and T-cell skewing was not significant (Fig.3F,  $p=0.27$ ). Moreover, the age included in the regression model was binarized (Fig.3E). Why did the authors do that? How was the threshold (62 years old) defined? Is the age still significant if the variable introduced in the model is continuous instead of categorical?

- The authors performed linear regression models to calculate the "exaggerated T-cell aging" and "T-cell years" (Fig.3G). How good are the defined models? Could the authors show the linear regression model performance? Or include the residuals for the non-cancer donors in the plots? To note, the differences between real age and predicted age are surprisingly high. Model predicts patients' age higher than 100? Could you explain how you can get a residual of 80 or >100?

- Within the characterization of T-cells in disease marrow, authors stated: "PC1 values and the abundance of terminal memory subsets in the BM showed a strong correlation with PB (fig.3H), indicating a similarity to systemic T-cell alterations seen with ageing (8)." As initially this section was focused on bone marrow data, now I am wondering how PC1 (T-cell skewing) was computed, in order to allow the comparative analysis between PB and BM samples. Are the T-cell skewing derived only from BM samples or both, marrow and PB?

This needs to be clarified and should align with the title of the section.

- Regarding features of antigen specific clones why restrict the general analysis to CD8-expressing memory clones? Have you checked the CD4+T memory cells?

- In Fig.5G, the result of cell-to-cell interaction analysis is shown, providing nice results. As the pathways identified in Fig.S7F are patient-specific, it would be interesting to explore what happens if this analysis is performed per patient. Detailed information on the analysis should be provided in the methods section.

- When exploring the implications in the response to immunotherapy, specifically TCE therapy, why are only 3 patients used? And only patients who respond? What about those that not respond? Behave differently?

- Regarding TCE therapy, authors stated that, "Interestingly, T-cells bearing these TCRs highly expressed the non-viral specificity signature pre-treatment (fig.5I), suggesting this phenotype contains TCR clones with the capability to respond to immunotherapy and may be tumour-reactive.", pointing out that having these clones with increased non-viral specificity signature can help to fight against the tumor (tumour-reactive). However, within the ASCT cohort, the ASCT-expanded clones, also associated with non-viral signature, with high clonality associated with MDR+. If there are tumor-reactive clones expanded, how do the authors explain the association with MRD+? Is it expected?

- Authors reference Fig.S5D to compare SMM and MM, pointing to a CD4+Treg reduction in MM; however, this reduction is not identified in Fig.S5D, only CD4+Tem is highlighted. Could you comment on that? How do you identify CD4+Treg?

- Interestingly, CD4+Treg loss is presented as a biomarker of SMM risk. Although "Multivariate analysis showed CD4+Treg abundance predicted progression independently of existing SMM risk models (Fig. 7G)", how much predictive power are we winning with the CD4+ Treg information?? Is this multivariate model better or significantly more powerful than the one performed only with the Mayo or IMWG risk models?

Minor

- Define "decreased" in Fig.S1B.
- Include in Table S1 the information regarding sorting, sequencing strategy, and tissue.
- "B" is missed in Figure S3.
- Fig.3C; check the legend. A variance of 24.27% is indicated in the legend, differing from the one shown in the plot and text (21.9%). What do the blue and red represent in the figure? Define in the legend.
- In Fig.S6B, if 279 clusters have been defined, why are only 248 shown according to the figure legend?
- Check the reference of figures, in line 273, Fig.4H refers to Fig.4G?; in line 274 Fig.4I refers to Fig.4H?
- In line 300, authors refer to Fig.S8D, please check that is the correct reference.
- Fig.5B shows a  $p=0.053$  but in the text says  $p<0.05$ .
- Check the reference of figures, in line 370, Fig.4D refers to Fig.4C?
- Clonality: In some plots shown as  $\log_{10}(\text{Simpson's diversity Index})$ , in others, as the Simpson's diversity Index. Please specify the value used in the legends and/or methods sections.
- Really interesting the identification of CD4+T-reg loss as a putative biomarker of SMM risk, however, the authors should show if the addition of this information improve significantly the prediction of existing SMM risk models.

Reviewer #3

(Remarks to the Author)

The manuscript by Foster et al. presents an ambitious and comprehensive analysis of T-cell differentiation across the spectrum of multiple myeloma (MM) and its precursor states (MGUS and SMM). The authors integrate more than one million single cells from 259 individuals across 12 datasets, including their own newly generated scRNA-seq and scTCR-seq data, to provide an in-depth characterization of T-cell states and dynamics. They uncover a pattern of skewed differentiation not attributable to classical exhaustion, but instead driven by antigen-specific terminal memory features.

The manuscript is well written, the analyses are rigorous, and the figures clearly communicate key findings despite the large volume of data. Public release of the dataset adds further value. This study significantly contributes to the understanding of immune surveillance and dysfunction in MM and its progression.

Among the important findings presented, the most noteworthy are:

1. Absence of classical exhaustion: Contrary to prior assumptions, the bone marrow of MM patients does not exhibit widespread enrichment of exhausted CD8<sup>+</sup> T cells (Tex) (Except for one patient described as outlier). Instead, a more abundant population (CD8<sup>+</sup>Tem.CD69<sup>+</sup>) appears to be activated rather than exhausted. Neither subset is associated with disease progression, suggesting immune dysfunction in MM may not follow classical exhaustion pathways.
2. T-cell skewing mirrors immune aging: The most prominent change in T-cell differentiation during disease evolution is the enrichment of terminal memory CD4<sup>+</sup> and CD8<sup>+</sup> subsets (Temra, Tte), termed "T-cell skewing." This pattern strongly resembles immune aging and is more pronounced in SMM and MM compared to MGUS and healthy controls.
3. TCR repertoire changes suggest antigen-driven responses: Expanded TCR clones in MM show clonal enrichment, terminal memory phenotypes, and lack viral-specific signatures. These data suggest a tumor-driven, antigen-specific T-cell response, especially given the correlation between T-cell skewing, paraprotein levels, and TCR clonality.
4. Clinical correlations and prognostic implications: T-cell skewing is associated with tumor burden (serum paraprotein levels) and MRD positivity after ASCT, but it does not predict progression from SMM to MM. In contrast, CD4<sup>+</sup>Treg loss emerges as a novel and independent biomarker of progression risk.

While the results are promising, further validation and clarification of certain aspects would strengthen the study. I look forward to reviewing a revised version.

Suggestions for Improvement:

1. Survival Analysis Beyond CoMMpass: Survival analysis is currently limited to the CoMMpass cohort. Given the size of the integrated dataset and available clinical metadata, a survival analysis incorporating T-cell skewing or CD4<sup>+</sup>Treg abundance in their own cohort would significantly increase the translational value of the findings.
2. Correlation with Cytogenetics: While the authors note the inability to analyze tumor genomic classification, the lack of correlation between key T-cell findings and cytogenetic abnormalities (e.g., t(4;14), del17p, gain1q) is a limitation. If feasible, integrating cytogenetic risk information from a subset of patients or datasets would provide valuable context.
3. Beyond Correlation. Causation and Risk Modeling: The multivariate analysis demonstrating that CD4<sup>+</sup>Treg loss predicts SMM progression independently of standard risk models is compelling. Extending similar modeling to T-cell skewing, particularly in the context of post-ASCT outcomes or disease aggressiveness, could strengthen the argument for a causal or predictive role.
4. Clarify the functional role of CD8<sup>+</sup>Tem.CD69<sup>+</sup> cells in myeloma:  
While the manuscript clearly distinguishes CD8<sup>+</sup>Tem.CD69<sup>+</sup> from exhausted T cells, their functional significance remains somewhat speculative. Since these cells are abundant and express partial exhaustion markers, further clarification, using approaches like by functional assays, cytokine profiling, or external dataset validation, could help determine whether they contribute to anti-tumor immunity, immune suppression, or bystander activation. This would help to clarify the concept of "pseudo-exhausted" CD8<sup>+</sup> T cells in the marrow.

Reviewer #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have responded appropriately to all critiques, strengthening their manuscript. I have no further suggestions or concerns. Leif Bergsagel.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)

The authors have answered my comments adequately and I have no further issues

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)

I commend the authors on their thorough responses to the prior critiques. I now find the manuscript acceptable for publication.

Arun Wiita

(Remarks on code availability)

Reviewer #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

(Remarks on code availability)

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## Point-by point response to reviewers

### Reviewer #1 (Remarks to the Author):

Please note, we include a list of references specific to this rebuttal at the end of the document.

#### Review comments:

In the manuscript Foster et al., the authors aimed to identify changes in T cell subsets driven by MM progression from precursor states and understand how these alterations to T cell differentiation influence disease progression. They demonstrate using single cell RNA and TCR sequencing methods that altered T cells in MM patients possess features of terminal memory differentiation, not exhaustion, and resemble T cell skewing observed in immune ageing. This is correlated with disease stage and driven by tumor intrinsic features. Since T cells were found to not be exhausted, this may explain why checkpoint inhibitors are largely unsuccessful in MM. The authors also identify Treg loss as a novel biomarker for SMM progression, contradictory to the reported increase of immunosuppressive Tregs. The degree of T cell characterization and immune dysfunction through disease stages is extensive, providing a framework for future studies that is immensely valuable to the field. The data analysis is excellent, and the interpretation and conclusions are well-supported.

We thank the reviewer for their generous and insightful comments, and we are glad they found our study to be effective and informative.

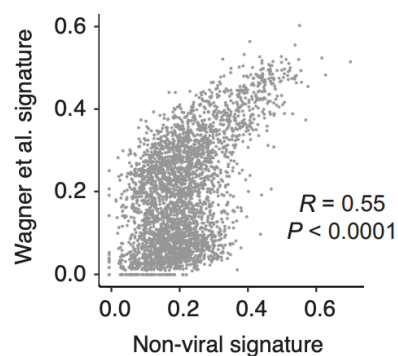
1. MAIT cell abundance is repeatedly found to be different between healthy and disease. Can the authors comment on this cell type and possible function/absence in disease?

We find a myeloma-associated loss of naïve T-cells subsets and MAIT cells in our data (Fig.S5C). From this, we infer that MAIT loss represents an additional component of the T-cell immune aging-like differentiation which we found in the marrow and blood of patients relative to controls (Fig.3). Supporting this hypothesis, several studies have described a reduction of MAIT cell abundance in the peripheral blood with advancing age (Walker et al., 2014; van der Geest et al., 2018) (similar to naïve T cells), suggesting MAIT loss may also a feature of T-cell aging. We also note that the MAIT cluster with the most inflammatory transcriptional profile, MAIT17 cells (MAIT.3; Fig.S4B) was not enriched in disease (Fig.S5G), suggesting that inflammatory MAIT cells may not play an active role in progression. However, MAIT cells were relatively rare in our dataset (<3% cells) precluding further analysis of this subset.

2. The authors discuss the “accumulation of TCRs possessing similar CDR3 sequences indicates responses against common antigens” and were not found to be viral reactive. Is there a way to show/model that the increased non-viral TCR repertoire are tumor antigen-specific (antitumor immunity)? Is tumor MHC-I expression increase a good enough correlate? More evidence to support this association?

We screened patient TCR repertoires for shared tumour antigen-reactive TCRs (from VDJdb, IEDB and CEDAR accessed 06/2023, N=13 unique human cancer epitopes, N = 5261 TCR-epitope pairs). We did find matching TCRs in our patient dataset (N = 20). However, these consisted of single alpha or beta chain matches (unlike the higher-confidence paired chain matches we made to viral reactive-TCRs) and all these TCRs possess additional annotated reactivities against at least one viral antigen. This suggests these TCRs may be commonly observed across patients due to their frequent probability of generation (Chernigovskaya et al., 2025), versus possessing a shared reactivity. We additionally speculated the poor coverage of tumour antigens, none of them notably myeloma-specific (with the majority of annotated tumour epitopes being the melanoma antigens MART1 and PMEL, 4034/5261, 77%) led to the failure to annotate tumour antigens in our data. For this reason, we did not report this analysis.

To explore the role of tumour-reactivity in the T-cell dynamics we describe, we take advantage of a recent pre-print which has reported a gene signature of CD8+ myeloma-reactive T-cells (Wagner et al., 2025). This phenotype largely overlaps with the cells we describe as terminal memory cells (expressing *GZMB*, *ZNF683*, *ZEB2*) and possessed a significant positive correlation with our reported non-viral signature ( $R=0.55$ ,  $P<0.001$ , Pearson correlation), which we report in fig.S8H (inset below, lines 341-343 in manuscript). This supports our hypothesis that myeloma-associated T-cell differentiation represents the activity of tumour-reactive clones.



*Fig.S8H. Dot plot showing the correlation between the expression of the non-viral gene signature and the Tumor-reactive Features in T cells gene signature from Wagner et al. (2025). R and P-values were calculated by Pearson correlation*

Finally, while we agree MHC-I expression at the RNA level is only a proxy for immunogenicity, we point to several other studies in solid tumours that have connected MHC-I expression at the RNA level to proxies of immunogenicity such as T-cell infiltration (immune “heat”) and the response to immunotherapy (Dhatchinamoorthy et al., 2021; Yang et al., 2023).

3. Have the authors identified any shared tumor neoantigens among patients? If tumor-reactive T cells are not exhausted, why can they not control tumor growth (propose mechanism of tumor escape)?

Regarding shared neoantigens, genome sequencing was unfortunately unavailable for the majority of patients in our single-cell atlas, precluding this likely informative analysis.

We would like to suggest several credible tumour-extrinsic and -intrinsic features which may impede the ability of T-cells to control tumour growth in the absence of exhaustion. Principally, while the T-cell subsets we find enriched in SMM and MM are not phenotypically exhausted (Fig.3A-B), they do possess hallmarks of TEMRA-like differentiation including the expression of T-bet (*TBX21*) and *ZEB2* (Fig.S3B) (Kay Chung et al., 2021). TEMRA cells possess blunted effector capacity relative to less differentiated effector memory T-cells. Therefore, even in the absence of canonical exhaustion, a loss of effector function may underpin myeloma associated T-cell differentiation, contributing to immune escape. Alternatively, other immune cell types may interact with non-exhausted T-cells to curtail their ability to control tumour growth, such as immunosuppressive myeloid populations (Costa et al., 2021). Regarding tumour-intrinsic features, the high level of genomic sub-clonality seen in myeloma cells at diagnosis (Dutta et al., 2019) may present too wide a range of antigenic targets for T-cells. This means that while some tumour sub-clones are effectively targeted by non-exhausted T-cells, other clones escape and continue to grow, leading to tumour escape. Further studies can be built on our findings to better explore this mechanism.

We have included these points in our Discussion section (lines 486-492).

4. The authors conclude overall Treg loss “may reflect early reshaping of the BM microenvironment towards a state favoring progression.” This is contradictory to reports of increased Tregs contribute to immunosuppression and favor tumor progression. Could the authors discuss how Treg loss may contribute to active MM progression mechanistically?

We agree these results appear at odds with previous work studying Tregs in the MM microenvironment. However, CD4<sup>+</sup>Treg are a known constituent cell type of the homeostatic bone marrow niche where they support haematopoiesis and normal B-lymphopoiesis (Méndez-Ferrer et al., 2020; Camacho et al., 2020; Pierini et al., 2017). Therefore, CD4<sup>+</sup>Treg loss may reflect the reshaping of the marrow microenvironment and disruption of the haemopoietic niche, with a healthy homeostatic state giving way to one favouring tumour growth. Additionally, we only report Treg loss relative to untreated precursor patients (Fig.7), where the microenvironment is likely most similar to healthy marrow. On the other hand, an accumulation of immunosuppressive Tregs may be a feature of late-stage disease or treated patients, where their presence promotes therapeutic resistance.

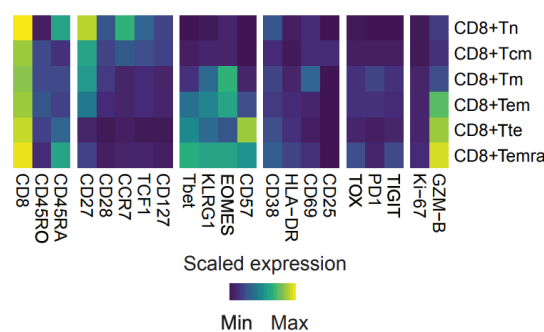
5. Were senescent T cells (characterized by CD28<sup>-</sup>, CD57<sup>+</sup>, KLRG-1<sup>+</sup>, CD160<sup>+</sup>) not found? TEMRA cells can exhibit cellular senescence. Include more references to T cell senescence in MM?

We apologise for the confusion regarding T-cell phenotype terminology. The terminally differentiated *GZMB*<sup>+</sup> CD4<sup>+</sup> and CD8<sup>+</sup> subsets did possess the features of cellular senescence at the RNA level: *CD28*<sup>-</sup>, *KLRG1*<sup>+</sup>, *CD160*<sup>+</sup>. To further illustrate this point, we



have performed a GSEA using a senescence gene signatures (*SenMayo*) (Saul et al., 2022) and found them to be significantly enriched in terminally differentiated subsets (CD8+Tte:  $P=0.03$ , NES=1.6; CD8+Temra:  $P=0.03$ , NES=1.). We have reworded our description of this phenotype in the results section to highlight the senescent and TEMRA features of these subsets in our Results (lines 138-139 in manuscript) and Discussion sections (lines 486-492).

Furthermore, we have performed additional CD8+T-cell phenotyping at the protein level by performing a clustering analysis of our CyTOF data (Fig.S8J, inset below). This analysis revealed the presence of CD8+ subsets in MM patients defined as CD28-KLRG1+ and either CD45RA-CD57<sup>hi</sup> or CD45RA+CD57<sup>lo</sup>, which we term CD8+Tte and CD8+Temra, respectively, as in our scRNA-seq data.



*Fig.S8J. Heatmap showing the scaled expression of T-cell marker proteins in CD8+ T-cell clusters identified by Cytometry by Time-of-Flight*

#### Reviewer #2 (Remarks to the Author):

Please note, we include a list of references specific to this rebuttal at the end of the document.

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There is no question this manuscript is a valuable resource and also provides some interesting hypothesis for understanding the interaction between the immune microenvironment and MM. However, there are a number of limitations that need to be addressed

We are glad this reviewer found our manuscript to be interesting, and we extend our sincere thanks for their insightful feedback.

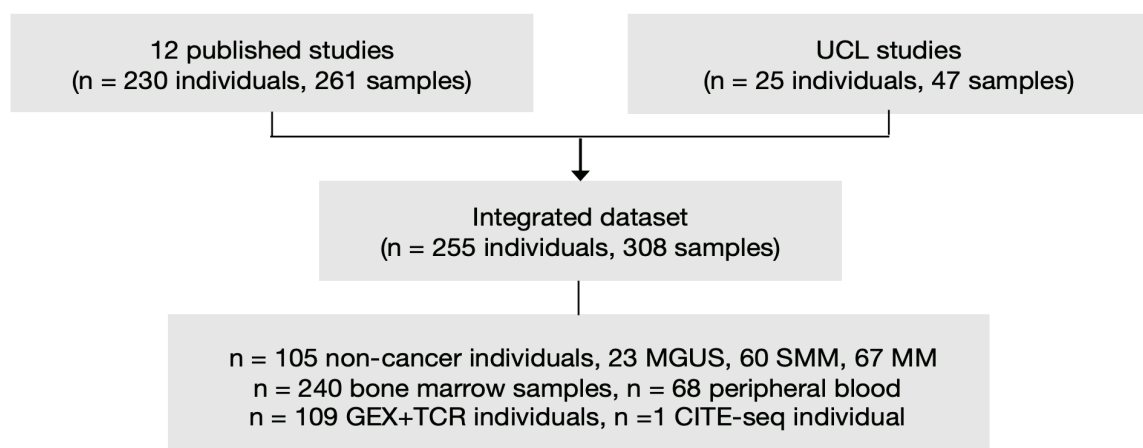
## Main

- Integration of scRNA-seq datasets: When presenting the datasets used in the study, authors referred to 12 public + UCL studies (if I'm not wrong, sometimes referred to as "This study", "Foster 2024" or "Foster 2025"), however, there are inconsistent numbers between Fig.1, Fig.S1A, B, **Table S1 and S6**. Seems that Botta 2023 study is not included in the integrated dataset or used independently. Please, clarify and correct the descriptions and figures accordingly.

We apologise for the confusion regarding the number and naming of individual datasets used in this study. We have updated Figures S1, S2, S3 and S5 and the Zenodo repository (<https://zenodo.org/records/17418275>) to more accurately describe the cells generated in this manuscript solely as "This study".

We re-analyse data from 12 published studies in this manuscript: Oetjen et al. (2018), Bailur et al. (2019), Granja et al. (2019), Zavidij et al. (2020), Kfoury et al. (2021), Zheng et al. (2021), Liu et al. (2021), Stephenson et al. (2021), Sklavenitis-Pistofidis et al. (2022), Botta et al. (2023), Conde et al. (2023), and Maura et al. (2023). We additionally analysed data from Friedrich et al., (2023; Fig.S8I) – however, as this study not included in our overall integrated dataset, we omitted it from these calculations.

This publicly-available data comprised 261 samples from 230 patients. We combined these data with 47 samples from 25 patients we generated ourselves. Together, this totalled 1,079,979 single cells from 308 samples from 255 individuals (n=105 non-cancer control, n=23 MGUS, n=60 SMM, n=67 MM). Of these 308 samples, 240 were derived from the bone marrow and 68 from the peripheral blood. Additionally, 109 patients had scTCR-seq data and 1 had CITE-seq. We have made these changes into most recent Zenodo dataset version (<https://zenodo.org/records/17418275>) have updated the text and figures accordingly (Fig.1, Fig.S1 inset below, lines 91-96 in manuscript).



*Fig.S1A. Sample collection for scRNA-seq cohort.*

These calculations include the data from Botta et al. (2023), which was analysed by scANVI re-projection (Fig.S4G) and omitted from the original (v4) Zenodo repository. This dataset (with predicted cluster label annotations) is now available on the Zenodo resource (<https://zenodo.org/records/17418275>).

Please note, the actual number of cells, patients, and samples in the Zenodo repository is less than listed here, as data from Sklavenitis-Pistofidis et al. (n=253,239 cells, n=73 samples, n=51 individuals) are omitted as per our data sharing agreement with these authors and must be acquired through direct contact with them.

Authors also reach some conclusions based on the cell type composition analysis in the integrated dataset. Due to the divergencies in the sorting strategies followed between studies, these approaches are biologically meaningless, as the differences observed may point to technical biases. Importantly, plasma cells represent only 6.6% of the integrated dataset, a consequence of the different sorting strategies applied, where several removed the plasma cell fraction, and others enriched for the T-cell fraction.

We agree that different sorting strategies will make comparison of cell type composition across studies invalid for biological insight. We chose to describe cell type composition across studies in the results to help provide an unbiased overview of the dataset, particularly for readers who may be interested in utilising our scRNA-seq resource. We have updated the results text to reflect that this comparison is purely for descriptive purposes, and not meant to reflect biological insight (lines 112-113, “the use of different sorting strategies across studies precluded in-depth analysis of overall immune composition”). Furthermore, we were careful to only make biological inferences using T-cell cluster composition as a proportion of T-cells, which we calculated through *in silico* T-cell selection (Fig.S2C-E) as is widely performed in immunological studies including other large scRNA-seq atlas studies (Chu et al., 2023; Zheng et al., 2021).

- Authors did a complete annotation of the different T cell phenotypes. Also, they deeply annotated the CD4+T-reg cells and the Invariant, identifying gdT cells, MAIT cells, etc. Why is this deep annotation not integrated into the general annotation of the dataset?

We have updated the uploaded dataset to include these cell type labels (<https://zenodo.org/records/17418275>). We chose to report the phenotype and abundance of these rarer T-cell subsets in separate supplemental figures (Fig.S4A, Fig.S5G) to enhance clarity and allow for deeper interpretation of all T-cell subsets in Fig.2.

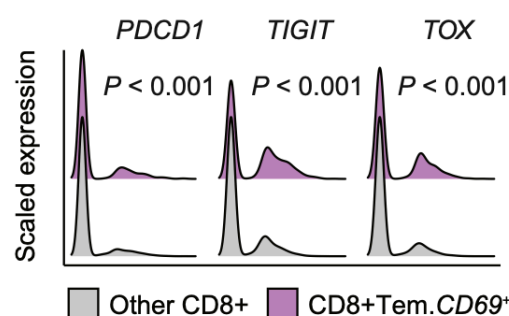
- According to the study, BM of myeloma patients is not enriched in exhausted T-cells which is unlike other reported studies so several comments are raised: 1) Why do authors focus on the comparison of CD8+Tex with CD8+Tem-CD69+ cells? What about the other CD8+Tem cells?;

We focused the comparison of CD8+Tex with CD8+Tem.CD69<sup>+</sup> as both expressed exhaustion-related genes (*TIGIT* and *TOX* and, to a lesser, extent *PDCD1* – please see below) relative to other CD8<sup>+</sup> subsets. We inferred from this that CD8+Tem.CD69<sup>+</sup> cells are the prevalent subset termed “exhausted” in previous myeloma studies. Thus, we chose to focus on this subset, in order to show that they are actually distinct from cells with a phenotypic profile more closely resembling exhausted T-cell (CD8+Tex).

Fig.2F-G and Fig.S3B compares CD8+Tex with other CD8<sup>+</sup> subsets, revealing the enrichment of exhaustion-associated genes in the CD8+Tex cluster. Finally, we would like to point to a recent publication which similarly fails to identify an enrichment of phenotypically exhausted T-cells in myeloma (Shasha et al., 2025). This would suggest our findings are shared with other emerging studies and we include this point in our Discussion (lines 455-456).

2) Authors stated: “Importantly, we distinguish CD8+Tex from PDCD1-expressing CD8+Tem.CD69<sup>+</sup>.” However, the increased expression of PDCD1 in CD8+Tem.CD69<sup>+</sup> cells is not evident (Fig.2F, G, and Fig.S3B). Could the authors clarify that?;

We apologise for the lack of clarity here. *PDCD1* was most highly expressed in CD8+Tex. *PDCD1* was also expressed in CD69+CD8+Tem at higher levels than other CD8<sup>+</sup> subsets, excluding CD8+Tex. However, this was not sufficiently evident in Fig.2G due to the much higher absolute expression of *PDCD1* in CD8+Tex. Therefore, we have plotted the expression with CD8+Tex omitted to better highlight this difference in the new Fig.S3F (inset below), referred to in line 161. Additionally, marker gene testing showed *PDCD1* was significantly enriched in CD69+CD8+Tem relative to other clusters (*adjusted P*<0.001, unpaired Wilcoxon). We include this finding in the results section to further enhance clarity line 160).



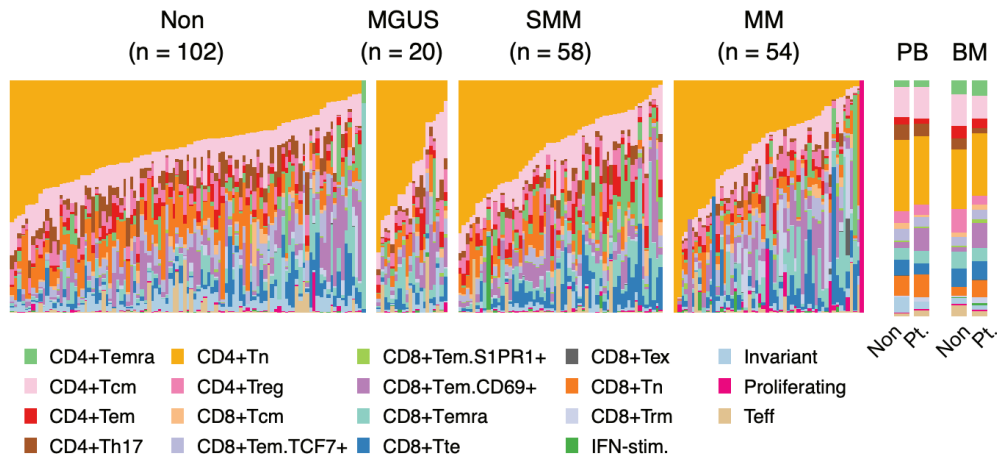
**Fig.S3F. Ridge plot comparing exhaustion-associated gene expression CD8+Tem.CD69<sup>+</sup> and other CD8<sup>+</sup> clusters.**

3) Similar to Fig.2B, it will be nice to see the distribution of cell populations per disease group. The way to show the distribution in Fig.S5A, although informative, provides different messages;

- Authors stated: “BM T-cell composition was strikingly similar in patients and controls”; however, to support that, a representation similar to Fig.2B must be provided for the T-

cell phenotypes. Fig.S5A shows relevant information, but does not support the stated message.

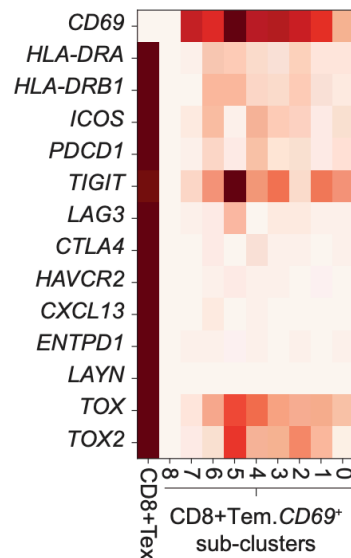
We agree such a figure would be informative and have provided this in fig.S5A.



*Fig.S5A. Bar chart comparing T-cell cluster composition in non-cancer controls (Non), MGUS, SMM and MM patients (left) and in the peripheral blood (PB) and bone marrow (BM) of all controls and patients (Pt.; right).*

4) Have the authors tried to subcluster the CD8+Tem.CD69+ cells? Could the subclustering reveal CD8+Tem.CD69+ subclusters related to T-cell exhaustion? Or within the overall CD8+Tem cells, could you identify a subcluster with an exhausted phenotype?

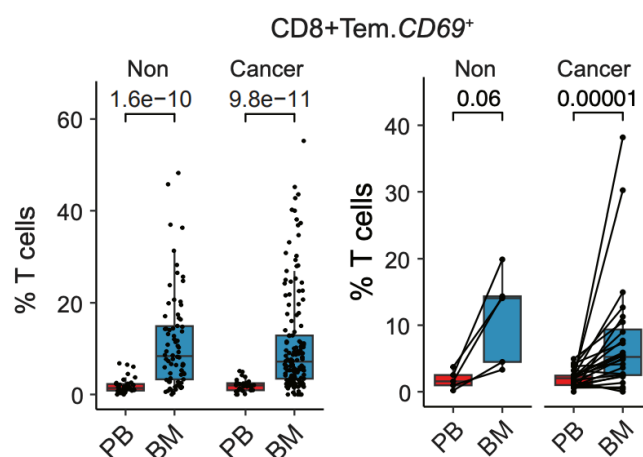
This is a compelling suggestion which we agree would help advance our findings. We isolated, re-integrated and re-clustered CD8+Tem.CD69+ cells (Fig.S3G, inset below). We refer to this new analysis in the manuscript lines 163-164: "A dedicated subclustering of CD8+Tem.CD69+ did not identify a subset of these cells with features of CD8+Tex". This suggests the exhausted phenotype is unique to the CD8+Tex cluster, and not to be identified within CD8+Tem.CD69+ cells.



*Fig.S3G. Heatmap showing scaled average RNA expression of exhaustion-associated markers in CD8+Tem.CD69+ sub-clusters and CD8+Tex.*

5) When comparing T-cell composition between marrow and peripheral blood, the sample donor was included as a random effect in the analysis. If the analysis is performed only with paired marrow and PB samples, are the conclusions the same?

This is a good suggestion. We have repeated our marrow and blood T-cell composition comparison using paired samples (Fig.S4F, inset below). These results are similar to our random effect testing, notably showing CD69+CD8+Tem are enriched in the marrow in both controls ( $P=0.06$ , paired Wilcoxon) and patients ( $P<0.001$ , paired Wilcoxon).



*Box plot showing the abundance of CD8+Tem.CD69+ in PB and BM samples from all controls and patients (left, P-value calculated by unpaired Wilcoxon test) and controls and patients with paired PB and BM samples (right, P-value calculated by paired Wilcoxon test).*

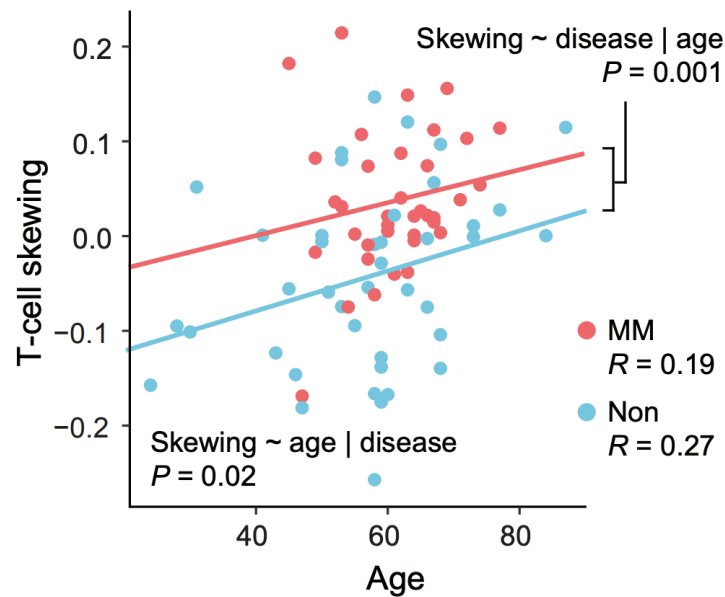
- In the T-cell composition analysis of controls with each myeloma disease stage (Fig.3B

and Fig.S5B), why is the number of individuals per group different between Fig.3B and Fig.S5B?

The reported patient numbers differ between figures because the boxplots in Fig.3B show the abundance of T-cell subsets among all patients in the cohort, whereas Fig.S5C reports the results of a differential abundance analysis analysing solely patients who had available age data (which was used in the linear model to calculate the reported P-values). The lack of age information for all patients in the cohort is an unfortunate limitation but perhaps unavoidable for a large meta-analysis such as this (we reached out to all original authors for age data, but it was not always available). We have updated the Fig.S5 legend to reflect this difference for enhanced clarity: “Only individuals with age information are included in this comparison”.

- The authors define the T-cell skewing based on the PCA of T-cell phenotypes' abundances, showing association with disease (SMM and MM) and age independently (Fig.3C, Fig.S5H, and Fig3.E). However, the correlation between MM age and T-cell skewing was not significant (Fig.3F,  $p=0.27$ ).

We apologise for the confusion regarding Fig.3F. We have redesigned this figure to better-communicate our findings (inset below). This figure serves as a visualisation for the effects modelled statistically in Fig.3E. We report the presence of similar age versus skewing slopes in controls (Non) and MM patients. This suggests an equivalent effect of age on T-cell skewing in both controls (Non) and MM patients. We quantify this by calculating the individual  $R$  values ( $R=0.27$  in Non,  $R=0.19$  in MM) and the significance of the age versus skewing association independent of disease stage ( $P=0.02$ , Non & MM only; in the full cohort  $R=0.29$  and  $P<0.002$ , Fig.S5I). However, the two slopes possess different y-intercept values. This suggests that, independent of age, MM patients possess a higher degree of T-cell skewing relative to controls (or MM patients tend to have enhanced T-cell skewing across a range of ages). We quantify this statistically in the linear model in Fig.3E ( $P<0.001$ ) and the same model for controls and MM only in Fig.3F ( $P=0.001$ ). Taken together, this shows the joint, independent effects of age and disease stage on T-cell skewing.



*Fig.3F. Dot plot showing the absolute difference between T-cell skewing in controls and MM patients across the range of ages. Inset P-values indicate the significance of the association between T-cell skewing and age independent of disease (age | disease) and disease independent of age (disease | age). P-values derived from linear regression. R value calculated by Pearson correlation.*

We have updated the text to include this interpretive detail (lines 225-235): “T-cell skewing was greatest (meaning an enrichment of terminal memory clusters) in SMM and MM relative to controls independent of age ( $P < 0.004$  and  $P < 0.001$ , respectively, linear regression; fig.3E), demonstrating this metric captured the major alterations to T-cells in myeloma. Conversely, T-cell skewing was associated with age independent of patient group ( $P = 0.013$ , linear regression,  $R = 0.29$ , Pearson correlation; fig.3E and F; fig.S5I).”

We also update the legend for fig.3F for enhanced clarity (lines 1355-1359): “Dot plot showing the absolute difference between T-cell skewing in controls and MM patients across the range of ages. Inset P-values indicate the significance of the association between T-cell skewing and age independent of disease (age | disease) and disease independent of age (disease | age).”

Moreover, the age included in the regression model was binarized (Fig.3E). Why did the authors do that? How was the threshold (62 years old) defined? Is the age still significant if the variable introduced in the model is continuous instead of categorical?

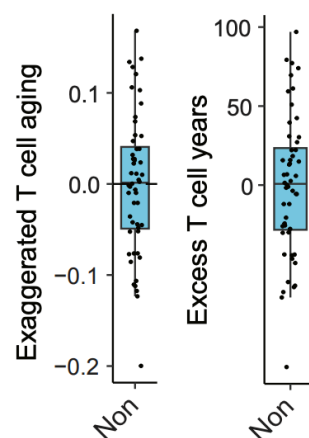
We binarized age by the median value in the cohort (62 years) so as to reduce the influence of outliers of non-linearity on the association. However, we repeated our modelling with age as a continuous variable. As in our original median-binarized age model, we found statistically significant independent associations between T-cell skewing and both age and disease relative to controls in both SMM (disease  $P < 0.001$ , age  $P = 0.01$ , linear model) and MM (disease  $P < 0.001$ , age  $P = 0.02$ , linear model). We include



the results of this test in our updated methods section (lines 656-656): “age values were binarized to above and below the median (62 years) but results remained significant when modelling age as a continuous variable in both SMM (disease  $P < 0.001$ , age  $P = 0.01$ , linear model) and MM (disease  $P < 0.001$ , age  $P = 0.02$ , linear model)”.

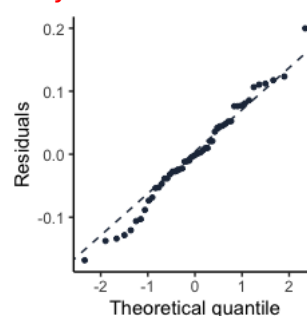
- The authors performed linear regression models to calculate the “exaggerated T-cell aging” and “T-cell years” (Fig.3G). How good are the defined models? Could the authors show the linear regression model performance? Or include the residuals for the non-cancer donors in the plots?

We thank the reviewer for these insightful comments on our linear modelling. The inclusion of non-cancer control residuals was an instructive suggestion, and we provide this in a new Fig.S5J (inset below). We chose to plot the non-cancer donor residuals on a separate plot so as to not complicate interpretation of Fig.3G). This analysis reveals a wide distribution of values in control samples residuals. This suggests additional unmodelled sources of variation in T-cell skewing, highly likely given this analysis ignores known correlates of immune aging (such as history of infection). However, the average values in patient groups are higher than the average in controls (excess T-cell years: SMM vs controls 25.1 years, MM vs controls 38.1 years). Therefore, there remains an effect of disease on exaggerated T-cell aging and excess T-cell years.



*Fig.S5J. Box plot showing exaggerated T-cell ageing and excess T-cell years (see Methods) in non-cancer control patients.*

We assessed our *T-cell skewing ~ age* model (used in Fig.3G) fit by analysing the normality of residuals. Residuals were normally distributed as assessed by a Shapiro-Wilk test



( $P=0.89$ ) and Kolmogorov-Smirnov test ( $P=0.97$ ). We also confirmed this with a Q-Q plot (inset below). From this, we infer the suitability of our model. We include this additional data in our methods section (lines 677-679): “the residuals of this model (Fig.S5J) were normality distributed as assessed by a Shapiro-Wilk test ( $P=0.89$ ) and Kolmogorov-Smirnov test ( $P=0.97$ )”.

To note, the differences between real age and predicted age are surprisingly high. Model predicts patients' age higher than 100? Could you explain how you can get a residual of 80 or >100?

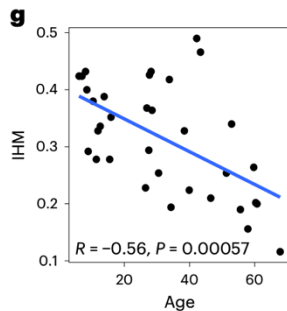
As noted, there are large differences between real and predicted T-cell age (>100 years, Fig.3G). We calculated “excess T-cell years” as an interpretable metric for accelerated immune aging. However, we agree that >100 years of immune aging may not be accurate. The inclusion of control residuals (Fig.S5J) helps contextualise these data and shows a high degree of variation around the mean, likely due to other unmodelled variables (such as history of infection or ethnicity, unfortunately such data was not available). However, other investigators have conducted similar immune aging analyses in other disease settings and found equivalent results.

Alpert et al. (2019) compared individuals with and without cardiovascular disease and found age- and gender-adjusted “IMM-AGE” values of in excess of 50 and up to 100 (Alpert et al. 2019, Extended Data Fig. 10c), where IMM-AGE is an immune-based metric of immune aging directly re-scaled to age *in years*.

[FIGURE REDACTED]

**c.** Age- and gender-adjusted IMM-AGE score of individuals stratified based on cardiovascular disease (dots); bold lines denote mean values ( $P=0.0023$ ,  $n=2,292$ , two-tailed t-test). Taken unedited from Alpert et al. (2019) Extended Data Figure 10.

A second study by Sparks et al. (2024) calculated an immune health metric (IHM) where a reduction of an IHC value by 0.1 roughly corresponded to aging 20 years in controls (Sparks et al. 2024: Fig.3g). Patients with X-linked chronic granulomatous disease (X-CGD) possessed extreme reductions of IHM values of up to 0.3 relative to the average IHM in controls (Sparks et al. 2024: Fig.3e), corresponding to roughly 60 years of accelerated immune aging.



[FIGURE REDACTED]

**g.** Scatterplot with trendline showing the age dependence of the IHM in healthy individuals only (Pearson correlation and P values shown;  $n = 34$  healthy participants in the training set with serum protein, whole blood transcriptomic and CBC/TBNK data). **e.** IHM scores of individual participants grouped by condition. Unadjusted P values from two-sided Wilcoxon test comparing each disease to the healthy group: \*  $P < 0.0001$ . Taken unedited from Sparks et al. (2024) Fig. 3.

From this, we conclude that the current approaches to estimate immune age in health and disease employed by us and others will predict extreme degrees of immune aging up to 50-100 years. Future work, with more specific immune metrics and greater clinical annotation, may derive more precise measures. Please note both metrics presented by these articles require data types not available in our scRNA-seq atlas, hence we were unable to calculate these metrics for comparison with our own.

We also tentatively speculate that, if accurate, excess T-cell years of several decades may explain the loss of functional capacity leading to tumour immune escape and progression.

- Within the characterization of T-cells in disease marrow, authors stated: “PC1 values and the abundance of terminal memory subsets in the BM showed a strong correlation with PB (fig.3H), indicating a similarity to systemic T-cell alterations seen with ageing (8).” As initially this section was focused on bone marrow data, now I am wondering how PC1 (T-cell skewing) was computed, in order to allow the comparative analysis between PB and BM samples. Are the T-cell skewing derived only from BM samples or both, marrow and PB? This needs to be clarified and should align with the title of the section.

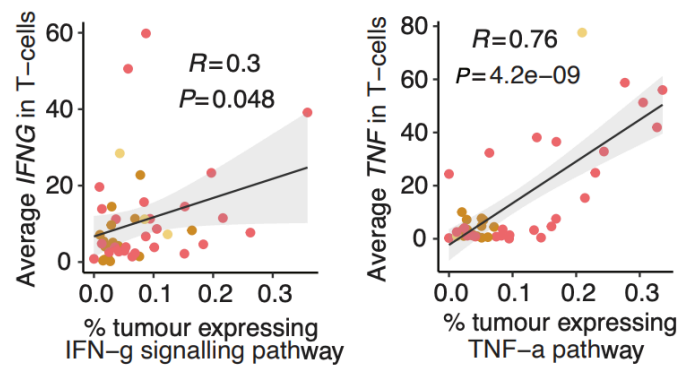
We apologised for omitting this from our methodology section. We initially calculated PCs on scaled normalised bone marrow T-cell cluster abundance. This is reported in Fig.3C and Fig.S5H. We have updated the methods section to include our methodology for this step: “For the comparison of T-cell skewing with the peripheral blood (Fig.3H), we first re-scaled normalised peripheral blood T-cell cluster abundance to the same centre and range of the scaled BM T-cell abundance matrix. Next, we multiplied this scaled PB matrix by the pre-calculated BM PCA feature loadings via feature (cluster)-wise matrix multiplication, yielding the PC1 values for the input PB matrix” (lines 671-675). We report these PB PC1 values in Fig.3H.

- Regarding features of antigen specific clones why restrict the general analysis to CD8-expressing memory clones? Have you checked the CD4+T memory cells?

We share this reviewer’s curiosity about the specificity of CD4+ T-cells. However, the vast majority of annotated TCR specificities are against MHC-I epitopes, thus CD8+T-cell TCRs. In the subset of the VDJdb we utilised, only 1,297 of 62,091 (2.1%) of TCRs annotated as viral-reactive were against MHC-II epitopes. Therefore, we reasoned we were far less likely to find shared CD4+ specifics and omitted this analysis in favour of CD8+ T-cells.

- In Fig.5G, the result of cell-to-cell interaction analysis is shown, providing nice results. As the pathways identified in Fig.S7F are patient-specific, it would be interesting to explore what happens if this analysis is performed per patient. Detailed information on the analysis should be provided in the methods section.

This is interesting suggestion and we have pursued this line of reasoning with the following analysis. Noting their node centrality in the inter-cellular signalling network, we focused on TNF- $\alpha$  and IFN- $\gamma$ -mediated pathways. We asked if the expression of these effector molecules by Teff was correlated with the expression of associated pathways in tumour cells on a *per-patient basis*. This analysis revealed the expression of these effector cytokine in T-cells was significantly correlated with the association downstream pathway activity in tumour cells (fig.S8G; IFN- $\gamma$ :  $R=0.3$ ,  $P<0.05$ ; TNF- $\alpha$ :  $R=0.76$ ,  $P<0.001$ , Pearson correlation). This suggests that the functional profile of effector T-cells is directly related to tumour transcriptional state in a subset of patients. We include this analysis in our discussion section (lines 331-334): “Patients with high T-cell effector cytokine expression possessed the highest corresponding pathway activity in tumour cells (IFN- $\gamma$ :  $R=0.3$ ,  $P<0.05$ ; TNF- $\alpha$ :  $R=0.76$ ,  $P<0.001$ , Pearson correlation; fig.S8G), mechanistically linking these processes”.



*Fig.S8G. Dot plots showing the correlation between indicated effector molecules (IFNG and TNF) in T-cells and the abundance of cancer cells highly expressing downstream signalling pathways for each effector molecule.*

- When exploring the implications in the response to immunotherapy, specifically TCE therapy, why are only 3 patients used? And only patients who respond? What about those that not respond? Behave differently?

For the TCE analysis, we simply selected clinically responding patients with a sufficient number of T-cells present in pre-treatment samples that subsequently expanded following TCE therapy. We adopted this approach based on the assumption that T-cell expansion in clinical responding patients likely involves (at least in part) TCR tumour-reactivity, as was argued by Friedrich et al. (2023). This allows us to connect our non-viral signature to the phenotype of putative tumour-reactive T-cells. Regarding responders and non-responders, we discussed this analysis among co-authors and agreed that this comparison, whilst interesting, was not the aim of our project. Hence, we chose not to pursue this. However, our scRNA-seq resource and non-viral signature may prove helpful in investigating this area in the future.

- Regarding TCE therapy, authors stated that, “Interestingly, T-cells bearing these TCRs highly expressed the non-viral specificity signature pre-treatment (fig.5I), suggesting this phenotype contains TCR clones with the capability to respond to immunotherapy and may be tumour-reactive.”, pointing out that having these clones with increased non-viral specificity signature can help to fight against the tumor (tumour-reactive). However, within the ASCT cohort, the ASCT-expanded clones, also associated with non-viral signature, with high clonality associated with MDR+. If there are tumor-reactive clones expanded, how do the authors explain the association with MRD+? Is it expected?

We are pleased this reviewer shares our interest in this compelling paradoxical finding. We have discussed this at length internally, and share our opinions below:

Firstly, we would suggest that TCE therapy is unique in that it relies (in part) on endogenous T-cell immunity and specificity, as showed by Friedrich et al. (2023) and

Wagner et al. (2025). Therefore, a high abundance of endogenous tumour-reactive T-cells is likely to be associated with superior outcome in this treatment modality.

However, a high number of tumour-reactive T-cells, whilst superficially beneficial, may be indicative of negative patient features such higher tumour burden or higher tumour mutational burden (giving rise to neoantigens, thus tumour-reactive T-cells). Therefore, there is likely to be a balance of positive and negative clinical outcomes associated with such T-cells, contextually dependent on treatment modality and time of sampling (such as pre- or post-therapy).

In our analysis of patient outcome post-ASCT, we find the clonal expansion of non-viral signature-expressing T-cells is associated with MRD+. As this is day 100 post-treatment, the presence of these cells may be indicative of ongoing T-cell responses insufficient to clear residual tumour cells (hence, MRD+). It is also possible the high levels of clonal expansion we see post-ASCT represents an excessive narrowing of the TCR repertoire against a small set of tumour antigens (“original antigenic sin”), meaning these T-cells would be unable to control tumour subclones that emerge post-therapy. The protective function of these T-cells may not be sufficient to eradicate tumour, and longer-term clinical outcomes may be dependent on post-ASCT therapy to further enhance T cell function with maintenance agents that stimulate T cell function such as IMiDs and/or CD38 monoclonal antibodies such as is now standard of care. Additionally, post-ASCT responses deepen with time in some patients, in whom such expanded clones may indeed be functional and eradicate tumour.

Further studies of different treatments in the context of consolidation and maintenance therapy are required to clarify these points. However, we believe our work makes the first step towards understanding how frequency and expansion behaviour of tumour-specific T cells relates to outcomes of different types of therapies in myeloma.

- Authors reference Fig.S5D to compare SMM and MM, pointing to a CD4+Treg reduction in MM; however, this reduction is not identified in Fig.S5D, only CD4+Tem is highlighted. Could you comment on that? How do you identify CD4+Treg?

We apologise for insufficiently explaining this result. Fig.S5E shows the corrected *P*-values for the comparison of T-cell composition between SMM and MM. In this analysis, the difference in CD4+Treg abundance was not significant following *P*-value correction (corrected *P* > 0.1). However, the uncorrected *P*-value was 0.03, which we reported when subsequently analysing CD4+Treg abundance in more detail in Fig.7. We have updated the results text to better reflect this difference in reported *P*-values (lines 414-415): “*We observed a reduction in the abundance of CD4+Treg in MM relative to SMM in the scRNA-seq (uncorrected P=0.03)*”.

- Interestingly, CD4+Treg loss is presented as a biomarker of SMM risk. Although “Multivariate analysis showed CD4+Treg abundance predicted progression independently of existing SMM risk models (Fig. 7G)”, how much predictive power are we winning with the CD4+ Treg information?? Is this multivariate model better or

significantly more powerful than the one performed only with the Mayo or IMWG risk models?

- Really interesting the identification of CD4<sup>+</sup>T-reg loss as a putative biomarker of SMM risk, however, the authors should show if the addition of this information improve significantly the prediction of existing SMM risk models.

This is valid concern, and we agree an extended analysis including statistical power and an assessment of predictive ability would enhance this analysis. However, we are unable to sufficiently answer this point due to lack of data from the relevant cohorts. We simply wished to highlight initial observations that provide an insight into T cell biology in the development of MM and connect these findings to clinical outcomes as a “proof of principle” for future of immune-based disease monitoring. Subsequent work in larger immunologically annotated cohorts will be required to fully address this important point.

Include in Table S1 the information regarding sorting, sequencing strategy, and tissue.

As table S1 shows clinical metadata at the level of individual donors, this information is not directly applicable here. However, this metadata is directly available in the data object shared on Zenodo (<https://zenodo.org/records/17418275>).

### **Reviewer #3 (Remarks to the Author):**

Please note, we include a list of references specific to this rebuttal at the end of the document.

The manuscript by Foster et al. presents an ambitious and comprehensive analysis of T-cell differentiation across the spectrum of multiple myeloma (MM) and its precursor states (MGUS and SMM). The authors integrate more than one million single cells from 259 individuals across 12 datasets, including their own newly generated scRNA-seq and scTCR-seq data, to provide an in-depth characterization of T-cell states and dynamics. They uncover a pattern of skewed differentiation not attributable to classical exhaustion, but instead driven by antigen-specific terminal memory features. The manuscript is well written, the analyses are rigorous, and the figures clearly communicate key findings despite the large volume of data. Public release of the dataset adds further value. This study significantly contributes to the understanding of immune surveillance and dysfunction in MM and its progression.

Among the important findings presented, the most noteworthy are:

1. Absence of classical exhaustion: Contrary to prior assumptions, the bone marrow of MM patients does not exhibit widespread enrichment of exhausted CD8<sup>+</sup> T cells (Tex) (Except for one patient described as outlier). Instead, a more abundant population (CD8<sup>+</sup>Tem.CD69<sup>+</sup>) appears to be activated rather than exhausted. Neither subset is associated with disease progression, suggesting immune dysfunction in MM may not follow classical exhaustion pathways.
2. T-cell skewing mirrors immune aging: The most prominent change in T-cell

differentiation during disease evolution is the enrichment of terminal memory CD4<sup>+</sup> and CD8<sup>+</sup> subsets (Temra, Tte), termed "T-cell skewing." This pattern strongly resembles immune aging and is more pronounced in SMM and MM compared to MGUS and healthy controls.

3. TCR repertoire changes suggest antigen-driven responses: Expanded TCR clones in MM show clonal enrichment, terminal memory phenotypes, and lack viral-specific signatures. These data suggest a tumor-driven, antigen-specific T-cell response, especially given the correlation between T-cell skewing, paraprotein levels, and TCR clonality.

4. Clinical correlations and prognostic implications: T-cell skewing is associated with tumor burden (serum paraprotein levels) and MRD positivity after ASCT, but it does not predict progression from SMM to MM. In contrast, CD4<sup>+</sup>Treg loss emerges as a novel and independent biomarker of progression risk.

While the results are promising, further validation and clarification of certain aspects would strengthen the study. I look forward to reviewing a revised version.

We thank the reviewer for their positive comments and helpful suggestions that we feel have greatly advanced our manuscript.

#### Suggestions for Improvement:

1. Survival Analysis Beyond CoMMpass: Survival analysis is currently limited to the CoMMpass cohort. Given the size of the integrated dataset and available clinical metadata, a survival analysis incorporating T-cell skewing or CD4Treg abundance in their own cohort would significantly increase the translational value of the findings.

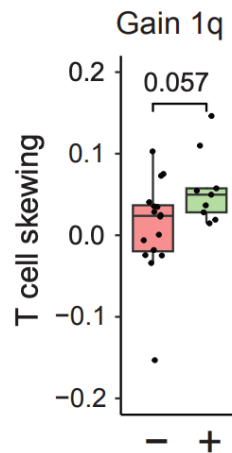
Unfortunately, outcome data was not available for majority of the scRNA-seq studies we analysed. Outcome data was available for Maura et al. (2023) but of the patients present in our scRNA-seq, only 1/16 progressed. Additionally, our scRNA-seq cohort is derived from numerous centres and countries-of-origin, which would have created an extremely clinically diverse cohort in which to analyse outcome. Taken together, this precluded further survival analysis in our scRNA-seq cohort. We hope future scRNA-seq datasets will include more complete clinical annotation to enhance future meta-analysis such as this.

2. Correlation with Cytogenetics: While the authors note the inability to analyze tumor genomic classification, the lack of correlation between key T-cell findings and cytogenetic abnormalities (e.g., t(4;14), del17p, gain1q) is a limitation. If feasible, integrating cytogenetic risk information from a subset of patients or datasets would provide valuable context.

We share this reviewer's curiosity regarding an association between T-cell differentiation and tumour genomic features. Due to incomplete clinical annotation in published datasets, the only tumour genomic features with sufficient data across studies were 1q gain (positive n=10, negative n=19) and t(4;14) (positive n=6, negative n=17). For del17p, positive n=2, negative n=10. t(4;14) was not significantly associated with T-cell skewing (P=0.45, Wilcoxon test) but 1q gain showed a trend for enhanced T-cell skewing (Fig.S7G;



$P=0.06$ , Wilcoxon test), potentially suggesting this adverse prognostic factor may also influence T-cell differentiation. Future studies with more complete clinical data or matched genomic sequencing will help answer this question. We included this analysis in our results (lines 321-325).

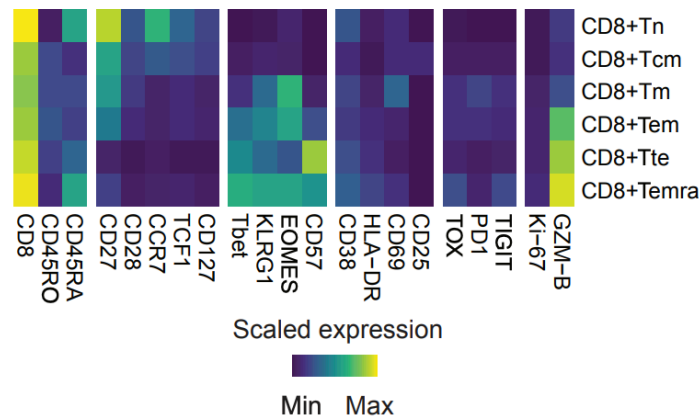


*Fig.S7G. Box plot showing T-cell skewing in patients with confirmed 1q gain positivity (+) and negativity (-). For (C), (D), and (G) P-values derived by Wilcoxon test.*

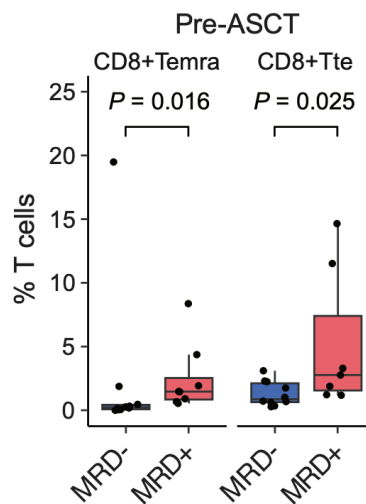
3. Beyond Correlation. Causation and Risk Modeling: The multivariate analysis demonstrating that CD4<sup>+</sup>Treg loss predicts SMM progression independently of standard risk models is compelling. Extending similar modeling to T-cell skewing, particularly in the context of post-ASCT outcomes or disease aggressiveness, could strengthen the argument for a causal or predictive role.

Following the suggestion of this reviewer, we sought to strengthen the clinical associated we describe between T-cell skewing and depth of response following ASCT in newly-diagnosed patients, defined as MRD negative response.

Following this, we asked if T-cell skewing in *pre-treatment samples* may have the ability to predict outcome following ASCT. We carried out CyTOF analysis of pre-treatment bone marrow samples in newly-diagnosed MM patients and acquired information on MRD status at day 100 post-ASCT. To examine the same CD8<sup>+</sup>T-cell phenotypes we identified in scRNA-seq, we performed additionally clustering analysis, identifying 6 CD8<sup>+</sup>T-cell clusters equivalent to the phenotype in our scRNA-seq data (Fig.S8J, inset below). When we correlate pre-treatment CD8<sup>+</sup>T-cell subset abundance with subsequent MRD at day 100 post-ASCT, we found both CD8<sup>+</sup>Temra (CD27-CD28-GZMB+Tbet+CD45RA+) and CD8<sup>+</sup>Tte (CD27-CD28-GZMB+CD57+) were enriched in MRD+ patients (Fig.6I, inset below; Temra  $P=0.02$ , Tte  $P=0.03$ , unpaired Wilcoxon test). Therefore, T-cell skewing in newly diagnosis patient pre-treatment is also associated with incomplete tumour eradication.



*Fig.S8I. Heatmap showing the scaled expression of T-cell marker proteins in CD8+ T-cell clusters identified by CytoFlow by Time-of-Flight (CyTOF)*

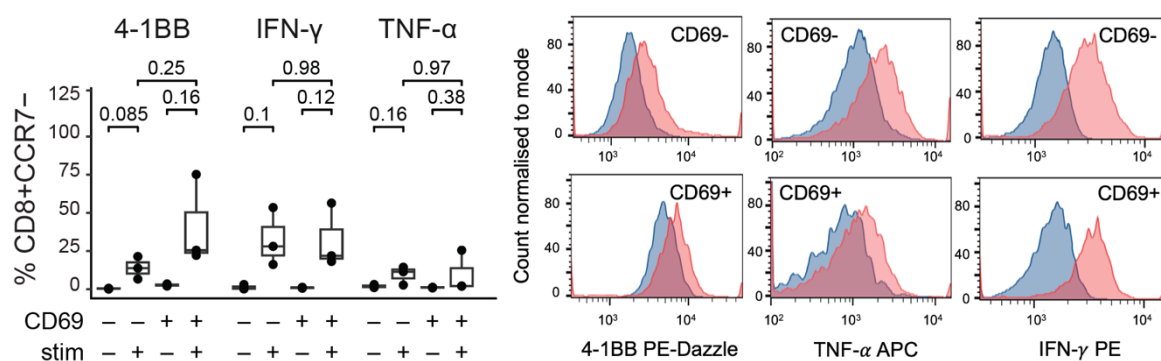


*Fig.6I. Box plot comparing CD8+T-cell CyTOF cluster (see Methods) abundance in pre-treatment samples from patients who achieved MRD+ and MRD- at D100 post-ASCT. For (B), (C), (F) and (H) P-values derived by Wilcoxon test.*

We include these findings in the Results section (lines 388-393). Together, these results further support the association between T-cell differentiation state and patient response to treatment and demonstrates the clinical utility of immune-based disease assessment in myeloma.

4. Clarify the functional role of CD8Tem.CD69 cells in myeloma: While the manuscript clearly distinguishes CD8Tem.CD69 from exhausted T cells, their functional significance remains somewhat speculative. Since these cells are abundant and express partial exhaustion markers, further clarification, using approaches like by functional assays, cytokine profiling, or external dataset validation, could help determine whether they contribute to anti-tumour immunity, immune suppression, or bystander activation. This would help to clarify the concept of “pseudo-exhausted” CD8 T cells in the marrow.

We appreciate this reviewer's interest in the CD8 Tem.CD69+ subset. As suggested, we explored whether the expression of exhaustion-associated markers *TIGIT* and *TOX* (fig.2G) in this subset reflected a loss of functional capacity. To assess this, we isolated CD69+ and CD69- fractions of CD8+ memory (CCR7-) CD3+T-cells from the bone marrow of MM patients (n=3) and activated cells in vitro with anti-CD3/anti-CD28 antibodies. After overnight stimulation, CD69+ cells demonstrated no significant differences in expression of activation markers and effector cytokines (fig.S4C, inset below). Therefore, CD69+ cells have a similar functional profile to other CD8+ memory T-cells, reflecting their "pseudo-exhausted" phenotype. We include this analysis in the Results (lines 167-171), Methods (lines 823-839) and Discussion (line 460) sections.



Left, box plots comparing the expression of activation markers and effector cytokines in CD69+ and CD69- fractions of MM patient BM CD8+ memory (CCR7-) T-cells under conditions of no stimulation (stim-) and after overnight in vitro activation with anti-CD3/anti-CD28 antibodies (stim+). A set of three independent experiments is shown. P-value calculated by paired Wilcoxon test. Right, representative histograms from one experiment showing the expression of 4-1BB, TNF-α, and IFN-γ in stimulated (red) and unstimulated (blue) CD69- (upper) and CD69+ (lower) cells.

To summarise, we provide evidence that CD8 Tem.CD69+ are a marrow-enriched populations (in both health and myeloma, Fig.S4E-F) which are not enriched in tumour-specific TCRs (Fig.S6F). The dominant T-cell differentiation axis we describe (T-cell skewing) which was significantly related to disease severity (Fig.3E) and paraprotein (Fig.5A-B) did not involve CD8 Tem.CD69+. This suggests CD8 Tem.CD69+ may predominantly be a bystander population, and not directly involved in the anti-myeloma immune response.

Reviewer #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

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