nature portfolio

Peer Review File

Multi-omic Profiling Reveals Age-Related Immune Dynamics in Healthy Adults

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Any redactions in this file are there to maintain patient confidentiality, the confidentiality of unpublished data, or to remove third-party material.

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

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)
Gong et al. use a multi-omics systems biology approach to determine the impact of aging on immune status during homeostasis, chronic infection (CMV), and vaccination (influenza). For this purpose, they collected blood samples from two independent cohorts of 96 and 234 subjects containing healthy adults aged 25-90. They then use single-cell RNA-seq to identify 71 different immune cell subsets and profile their transcriptome, reaching a total of >16 million single cells; they use a highly multiplexed proximity extension approach to profile the plasma levels of thousands of protein markers; and they use ELISA and Hemagglutination Inhibition Assays to determine the vaccine-induced antibody response in a subset of subjects from the smaller cohort. Using this dataset, the authors first explore the impact of age on the immune system and find age-related transcriptional changes in T cells independent from systemic inflammation and CMV infection. Interestingly, the authors don't see a systemic increase in inflammation-associated markers, such as IL-6, TNF, or IL-1b, typically associated with aging. Next, the authors determine the impact of aging on vaccine-induced immunity. Using their multi-omics approach, they make a string of observations painting a picture of an altered adaptive immune cell compartment leading to altered vaccine responses in older subjects:

- reduced levels of vaccine-induced functional antibody responses to a recall vaccine strain in older subjects
- no change in vaccine-induced antibody response to neoantigen vaccine strain
- reduced expansion of distinct memory B cell subsets after vaccination
- altered transcriptional profiles in B cells and plasmablasts in older vs younger adults
- differences in the expression of class-switched antibody genes in B cell subsets between older and younger adults
- reduced activity of T follicular helper cells in older adults that is accompanied by a global increase of a Th2 phenotype in those cells

Based on these findings, the authors conclude that a shift in the T cell compartment leads to suboptimal T-cell help for B cells during memory reactivation and hence reduced antibody production.

Overall, the manuscript is well-written and provides an extensive multi-omics dataset detailing peripheral immune cell and protein marker dynamics in a cohort of more than 300 subjects, encompassing over 16 million single-cell transcriptomic data points. The authors use this comprehensive dataset to explore a significant and timely question: How does aging influence the immune system, particularly in response to vaccination?

While the dataset's size and depth are indeed impressive, and the authors offer valuable insights, it is essential to view these findings in the context of existing literature. Previous work, such as that by Nakaya et al. (Immunity, 2015, PMID: 26682988) and Kumar et al. (Science Advances, 2024, PMID: 39331702), has already identified key age-related immune alterations, including reduced antibody responses and B cell activation. Additionally, previous studies (Mansfield, Clin Exp Immunol, 2012, PMID 23039889; Uciechowski, Exp Gerontology, 2008, PMID: 18166287) have highlighted Th2 skewing phenomena in the elderly. Thus, while the current study adds new details, such as the molecular regulation of Th2 skewing and detailed changes in the B cell compartment, it remains debatable whether these findings represent a significant

advancement or a nuanced extension of existing knowledge.

The most notable contribution of this study may be the dataset itself. The authors present an impressive effort in deploying single-cell RNA sequencing on such a large scale, utilizing rigorous wet-lab and computational approaches. The statistical analyses are appropriate and well-described, adding robustness to the study's findings. However, it is worth noting that while the overall sample size is substantial, the subset of vaccinated individuals (68 subjects) is comparable to, but smaller than, that in some prior studies, such as Nakaya et al. (around 140 vaccinated subjects in two cohorts), and significantly smaller than the cohort used by Kumar et al. (234 vaccinated subjects). This difference in sample size has implications for the study's power to address complex questions about immune response heterogeneity, a topic Kumar et al. explored extensively but is not fully addressed in the present manuscript.

The authors have commendably made their dataset publicly accessible, which is a valuable contribution to the scientific community. Nevertheless, the manuscript's conclusions, while well-grounded in the presented results, remain primarily descriptive. The proposed hypothesis about how Th2-skewed T cell compartments negatively impact vaccination, although intriguing, requires more functional and mechanistic exploration to establish broader relevance and novelty.

In summary, the authors have provided a high-quality and detailed dataset that will serve as an essential resource. However, the study's novelty is perhaps somewhat limited, and the authors would benefit from addressing mechanistic questions and delving deeper into immune response heterogeneity to more clearly differentiate their findings from prior research.

Major Points:

1. The global skewing of the T-cell compartment toward a Th2 phenotype is a critical and intriguing finding. However, the authors rely primarily on transcriptional and epigenetic analyses, without detecting differences in plasma protein levels of key Th2 cytokines. To strengthen this conclusion, it would be beneficial to perform functional assays measuring activation-induced cytokine production, including IFNγ, IL-4, IL-13, IL-5, IL-17, IL-21, IL-10, and IL-2, in both older and younger adults. These assays would provide robust functional validation of the Th2 shift. Additionally, measuring B-cell growth factors (IL-21, IL-10, and IL-2) could offer insights into the potential of these T cells to support B cell responses. Including both vaccine-specific and antigen-independent stimuli would further clarify whether the observed skewing is relevant to vaccine-induced responses and overall immune function.

2. The conclusion that older adults' CD4 T cells have a reduced capacity to assist B cells is compelling but currently based on transcriptomic data alone. The lack of differences in the frequency of circulating Tfh cells at day 7 post-vaccination adds complexity to this finding. A co-culture assay using sorted memory CD4 T cells and naive/memory B cells to measure total IgG production (as demonstrated in Morita et al., Immunity, 2011, PMID: 21215658) would provide functional evidence of reduced B cell help and significantly enhance the study's impact.

3. The observed changes in B cell populations are described globally, without specific data on antigen-specific B cells. Including analyses, such as flow cytometry using an influenza hemagglutinin probe, on a subset of samples would substantiate whether these transcriptional changes are occurring within the vaccine-specific B cell population, strengthening the manuscript's relevance to influenza vaccination.

4. The antibody response data currently focus on two influenza B strains, although the vaccine includes multiple strains, notably more prevalent influenza A strains. Since the authors mention the use of MSD-based assays measuring seven strains, providing a comprehensive analysis, especially of the influenza A strains, would offer a more holistic understanding of age-related impacts on vaccine efficacy.

5. The transcriptional differences in B cell subsets suggest alterations in class-switching behavior. To ascertain the functional relevance, conducting Ig-subtype-specific ELISA for vaccine-targeted influenza strains would be informative. This could reveal if the transcriptional changes translate into altered antibody class distribution.

6. The manuscript currently presents antibody data at day 7 post-vaccination, a time when responses are peaking. However, immune response kinetics may differ with age, potentially affecting the analysis. Including data from day 90 post-vaccination would address whether older adults have a delayed or reduced response and provide insights into the longevity of immunity, which is crucial for understanding age-related differences.

7. The study reports notable age-related differences in B cell subsets and their transcriptional profiles. To better contextualize these findings, correlating these parameters with hemagglutination inhibition (HAI) titers could offer an understanding of which of these differences translate how into protective immunity.

8. A recent study (Kumar, Science Advances, 2024) has highlighted significant heterogeneity in vaccine responses among older adults. Given the depth of the current study, exploring inter-individual variability could reveal important insights and enhance the manuscript's impact. Leveraging the study's unique dataset to address this would be a valuable addition.

Minor Points:

1. On page 6, the manuscript mentions that participants received the same vaccine but does not specify the vaccine type. Including details on whether the vaccine was inactivated, high-dose, or adjuvanted, and whether it was trivalent or tetravalent, would provide essential context, given the varying efficacy of different formulations in older populations.

2. The proposed model suggests that reduced T cell help affects responses to recall antigens but not to neoantigens. Given that T cells can recognize a broad range of influenza epitopes and often exhibit cross-reactivity, the authors should provide a rationale for this selective impact. A discussion on the potential for pre-existing cross-reactive T cells, even in neoantigen settings, would be helpful.

3. The reduction in Type 2-polarized B cell expansion during vaccination in older adults appears to contradict the observed Th2 skewing in the T cell compartment. The authors should elaborate on how these findings align or provide potential mechanisms for this observation.

(Remarks on code availability)

Referee #2

(Remarks to the Author)
In this study, Gong et al examined peripheral blood plasma and leukocytes in 2 separate cohorts. The first comprised healthy young 37 (25-35 yrs) and older (55-65 yrs) adults over 2 years, at multiple timepoints both before and after annual influenza vaccination. In the second cohort they studied a cross-sectional cohort of healthy adults with a wider age range especially in the older end of the age spectrum. They used multiple platforms for these analyses including scRNA-seq, high-dimensional plasma proteomics and spectral flow cytometry. They have created a valuable resource for future researchers including scRNA-seq datasets of >16 million PBMCs, and have identified longitudinal changes in 71 distinct immune cell subsets before and after vaccination in different age groups. However the current interpretation of their data and the perceived highlights are limited.

Comments:

1) There have been previous studies that have searched for and identified age associated changes in the immune system and many of these studies have been cited in the current manuscript e.g. Alpert et al 2019, Sayed et al 2021, Sparks et al 2024 and others). Each of these separate studies claim to have identified unique age-related immune signatures. Where is the overlap with the current work? How are the immune ageing characteristics shared or different in these studies? And ultimately for scientists or clinicians who want to use this data as a metric of immune ageing and/or immune competency, who do we believe? For these elaborate datasets to be useful, there has to be some consensus on what changes during ageing and whether they are beneficial or detrimental. Although beyond the scope of the current study, as not every young or old individual has the same level of physical competence, it would have been good to know how the results obtained are linked to metrics of frailty.

2) The current work is laudable in that that a huge amount of data has been collected, analysed and integrated, but it does not provide any definitive answers. One of the key observations stated in the abstract is that "We find that T cells earlier in differentiation accumulate distinct age-related transcriptional changes more than other immune cells, independent from systemic inflammation and chronic perturbation". However this has been shown to some extent previously (Pulko V et al.2016, Human memory T cells with a naive phenotype accumulate with aging and respond to persistent viruses. Nat. Immunol. 17, 966–975). It is noted however that systemic inflammation and chronic perturbation was not investigated in the Pulko study. Moreover another conclusion in the abstract that "...impaired memory B cell responses to vaccination are linked to a gradual GATA3/Th2-polarization in T cell subsets within older adults is not totally novel. End-stage TEMRA T cells that accumulate during ageing have been shown to express GATA3 (Callender LA et al GATA3 induces mitochondrial biogenesis in primary human CD4+ T cells during DNA damage. Nat Commun. 2021 ;12:3379). While it is appreciated that the vaccination arm of the studies was labour intensive an laborious, the key questions of what immune or plasma signatures are associated with good or bad vaccine responsiveness, especially in the older group, is not forthcoming. Nevertheless the extensive volume of data that has been generated and deposited is a valuable resource for future investigators.

3) Their data to some extent contradicts previous findings including no increase in inflammatory proteins over time (including IL-11 that was published in Nature recently) and no impact of previous CMV infection on the proteome over time. This raises the questions of when exactly inflammation and CMV infection induce their effects on the proteome and on leukocyte subsets and why does this effect not change over time especially as CMV induces lifelong persistent infection? This has not addressed. If not inflammation and/or CMV infection, what maintains these changes over time? What is the extent of proliferative activity in the different leukocyte populations over time, especially in the T cell subsets?

4) It was good to see that vaccination induced antibody production and B cell function overall (but not plasma cells) was reduced during ageing. The extensive datasets that have been generated will be a great resource for further investigations of B cell function before and after vaccination during ageing.

5) In the current clinical climate that is moving to the use of senolytic drugs in the treatment of ageing-related disorders, it would be good to know which leukocyte population acquire senescent characteristics during ageing and whether this affects the outcome of vaccination, especially in the older subjects. It is possible that the close interrogation of their datasets will

identify gene signatures associated with cellular senescence in the different populations studied by ScRNAseq to address this point.

6) One problem with the interpretation of the results that is present in the current study but also in all previous studies identifying age associated signatures is that it has only been possible to investigate blood leukocytes, for obvious ethical and ethical considerations, that applies to all human work. Is what is being observed reflect immunity globally? For example the data on Tfh/B cell interactions, the blood is not where these interactions take place. It is known that circulating leukocytes generally decrease with age while those in tissues actually increase.

Overall, the current interpretation of the datasets is very superficial given the volume involved, but the value of this work lies in the future when this data becomes available to other researchers. However the inescapable caveat is that as leukocytes age, non-lymphoid cells in every organ also ages. This will lead to altered lymphoid/non-lymphoid cell interactions during immune responses. Ultimately, although extremely difficult, the most valuable insights into understanding age associated changes and strategies for enhancing immunity will come from unravelling these altered interactions.

(Remarks on code availability)
I am not able to comment on the data collation, standardization and statistical analysis as this is beyond my expertise

Referee #3

(Remarks to the Author)
Gong et al. reported a multi-omic profiling study (scRNA-seq, proteomics, flow cytometry) of human blood immune cells, analyzing data from approximately 300 healthy adults and 96 adults followed over two years. Through single-cell RNA sequencing of over 16 million PBMCs, they found that T cells at earlier stages of differentiation accumulate distinct age-related transcriptional changes more than other immune cells, independent of systemic inflammation and chronic perturbation. Additionally, they observed that impaired memory B cell responses to influenza vaccination in older adults are associated with gradual GATA3/Th2 polarization in T cell subsets. The authors have made this extensive data resource available for further data mining and exploration.
1. Given the 60–80 years of human adult life, multiple visits over a longer follow-up period would be ideal for a human longitudinal study. Practically, a minimum of 5 to 10 years would be needed to observe the impact of aging. In this context, a two-year follow-up in relatively younger age groups (>65 years old in the 'old' group) would be more appropriately considered a repeat measurement rather than a longitudinal study. Additionally, the age range of the 'old' group (55–65 years) does not align with the current standard definition of older adults, which is generally >70 years. Therefore, in my opinion, the title may be overstated. A mostly cross-sectional analysis of immune cell aging with over 300 donors is still an impressive amount of work.

2. The findings on the dynamics of “up- and down-regulated genes within immune cells in adults over 65 years of age, which may differ from those occurring between ages 25 to 65,” are intriguing. It would be valuable if the authors could perform the same analysis on published data, such as Terekhova et al. (Immunity, 2023), to confirm these findings.

3. The authors concluded that the reduced transcriptional propensity to support memory B cells with age is based on a general decrease in CD40LG expression in central memory (CM) CD4 T cells, rather than a decrease in CD40 expression in core memory B cells. However, the highly heterogenous nature of these subsets, this finding does not necessarily apply to flu-specific CM CD4 T cells and flu-specific core memory B cells, which represent only a fraction of the total CM CD4 T cells and core memory B cells. If the authors have data on antigen-specific CM CD4 T cells or core memory B cells, it will substantially strengthen this conclusion.

4. Figure 1: It is essential to include a filter based on gene expression percentage (>60–70%) within the subset, in addition to log2fc and p.adj, for identifying DEGs. The expression percentage-filtered DEGs and related analyses should be presented consistently throughout the results.

5. Extended Figure 4: The age-associated transcriptional changes in human T cell subsets, as well as shared transcriptional changes across different T cell subsets, were reported by Lu et al. 2022 (DOI: 10.1038/s41467-022-32869-x) and should be cited.

6. In several places within the Results section, the authors mention previous studies without providing citations. Please add references wherever prior studies are referenced.

7. Based on Figure 6, the authors concluded that there was no age-related alteration in the vaccine-specific expansion of plasma cells. However, since the oldest donors in the 'old' group were 65 years, it remains unclear if such alterations might exist in individuals older than 65. The authors should discuss this limitation and suggest directions for future studies.

(Remarks on code availability)

Referee #4

(Remarks to the Author)
The manuscript provides a detailed analysis of age-related immune dynamics using longitudinal and cross-sectional

cohorts, focusing on T and B cell transcriptional and compositional changes. The major findings include identifying age-associated transcriptional changes in naïve and central memory T cells, highlighting a GATA3/Th2-polarized state; impaired memory B cell responses in older adults, marked by reduced class switching and functional antibody production after influenza vaccination; a distinct immune landscape shaped by CMV infection, largely independent of aging; and the development of the Human Immune Health Atlas, a valuable resource for immune research.

While the study presents an impressive dataset that will undoubtedly serve as a significant resource for the scientific community, several findings lack novelty. Nonetheless, the manuscript is of high quality and deserves rapid publication. I would, however, suggest aligning the findings more closely with existing literature and emphasizing their novel insights.

The age-related GATA3/Th2 polarization was previously reported by Terekhova et al. (2023). This manuscript acknowledges their findings in the discussion: “Our findings on the age-related increase in a Th2-like state in central memory CD4 T cells align with recent literature finding elevated CCR4+ memory T cells in advanced age (Terekhova et al. 2023), however, we find this GATA3/Th2 polarization is not specific to one T cell type but is found in many T cell subsets, suggesting a mechanism where age-related elevation in GATA3 activity impedes T-bet activation in older adults (Dai et al. 2024; Naradikian et al. 2016).” Terekhova et al. linked GATA3/Th2 polarization to external cytokine signaling (e.g., IL-4/IL-13) and used trajectory analysis to map T cell progression. Here, the authors note no significant increase in circulating Th2-polarizing cytokines (IL-4, IL-5, IL-13). It would be valuable to investigate upstream drivers of GATA3 activity and distinguish between intrinsic and extrinsic mechanisms. Trajectory analysis could model T cell developmental transitions leading to GATA3-mediated Th2 polarization. Additional experiments, such as stimulating naïve T cells from young donors with IL-4 or IL-13 or co-culturing naïve CD4+ T cells with autologous or heterologous APCs, could help disentangle intrinsic T cell changes from APC-driven signals. GATA3 mRNA and protein levels could then be assessed using qPCR and flow cytometry, while downstream cytokines (IL-4, IL-13) could be analyzed with ELISA.

The manuscript identifies impaired memory B cell responses in older adults, marked by reduced class switching and antibody production after influenza vaccination. While supported by the data, these findings are not particularly novel, as similar results have been reported in several studies on age-related vaccine responses. Relevant references include:
• Nakaya HI et al. Systems Analysis of Immunity to Influenza Vaccination across Multiple Years and in Diverse Populations Reveals Shared Molecular Signatures. Immunity. 2015 doi: 10.1016/j.immuni.2015.11.012
• Furman D et al. Apoptosis and other immune biomarkers predict influenza vaccine responsiveness. Mol Syst Biol. 2013 Apr 16;9:659. doi: 10.1038/msb.2013.15.
• Frasca D et al. Unique biomarkers for B-cell function predict the serum response to pandemic H1N1 influenza vaccine. Int Immunol. 2012 Mar;24(3):175-82. doi: 10.1093/intimm/dxr123.
• Tsang JS et al. Global analyses of human immune variation reveal baseline predictors of postvaccination responses. Cell. 2014 Apr 10;157(2):499-513. doi: 10.1016/j.cell.2014.03.031.

Experiments with aged Cd4cre Gata3f/f KO mice could provide mechanistic insights into the authors' proposed findings regarding antibody production.

3. The manuscript highlights the decrease of ZBTB38, a marker gene of adaptive NK cells, in CMV+ older adults. The authors could validate this finding using datasets from related studies, such as:
• Aino-Elina Häkkinenet al. Single-Cell Transcriptomics Reveals Adaptive NK Cell and Antigen-Specific T Cell Responses Shaped by CMV Viremia Post-Hematopoietic Stem Cell Transplant. Blood 2023; 142 (Supplement 1): 4786. doi: https://doi.org/10.1182/blood-2023-179714
• Kar R et al. Single-cell transcriptomic and T cell antigen receptor analysis of human cytomegalovirus (hCMV)-specific memory T cells reveals effectors and pre-effectors of CD8+- and CD4+-cytotoxic T cells. Immunology. 2024 Jul;172(3):420-439. doi: 10.1111/imm.13783.

4. The manuscript could benefit from refining the classification of participants into age groups by incorporating biological age metrics, which better reflect physiological differences than the current arbitrary cutoff of 55 years (in the first cohort). Leveraging established age-related transcriptomic and their own proteomic markers, a composite biological age score could be developed and applied to the cohorts. This approach would allow grouping participants based on biological age, potentially revealing variability in immune responses and vaccine efficacy.

5. The statistical analysis in the manuscript could benefit from greater clarity and rigor. For instance, the "delta changes" in Figure 1 are not well defined, making it difficult to interpret their biological significance beyond statistical differences. Additionally, some statements, such as the "continuous increase" in the age metric (Figure 2F) and the "trending decrease" in KLRC2 expression, lack statistical evidence or clear descriptions of the methods used to assess these trends. Phrases like "trending decrease" are imprecise and would be strengthened by specific statistical tests and p-values.

6. The findings regarding the impact of CMV infection on immune subsets are intriguing, but the robustness of the statistical significance could be further strengthened with an empirical test. To ensure that the observed differences are not simply due to random variability, the authors could perform a permutation analysis. By randomly assigning individuals to CMV+ and CMV- groups multiple times and repeating the differential analysis, they could estimate the likelihood of identifying the same or similar subsets purely by chance. This approach would provide a robust empirical baseline for assessing the true significance of the results. Additionally, combining the confirmation from Figure 5A with the findings in Figure 3A could help reinforce the conclusions by providing a more comprehensive validation of the differential effects observed.

(Remarks on code availability)

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)
I would like to commend the authors for the extensive and thoughtful revisions they have undertaken in response to the previous review. The additional analyses and data substantially strengthen the manuscript, providing important mechanistic insights and novel findings that enhance the scientific value of the work. The revised version successfully addresses the majority of my prior concerns.

I have only a few remaining points for the authors to consider, which I believe would further clarify and enrich the manuscript:

1. Extended Data Figure 8H and I: The re-analysis of the publicly available dataset to examine CD40 expression in vaccine-specific B cells from young and older adults is appreciated and well executed. However, one of the key conclusions of the manuscript is a skewing toward IgG2 subclass usage in highly boosted responses in older individuals. Given the availability of antigen-specific B cell transcriptomic data in this dataset, I encourage the authors to take this analysis one step further by assessing the expression ratio of IGHG2 to IGHG3 in these cells. This would provide valuable support (or nuance) to the claim of subclass skewing and directly test whether this phenomenon can be detected in the independent dataset.

2. Th2 skewing upon repeated exposure: The observation that the reduced antibody responses in older adults are most pronounced for the highly boosted B/Yamagata strain is intriguing. It would benefit the reader if the authors could expand briefly on their interpretation of this result. Specifically, what are the potential immunological mechanisms or contextual cues that might drive a Th2-biased response and the associated antibody response changes following repeated exposure to the same antigen? Speculation on how this might influence the quality and durability of protection in older adults would also be welcome. The authors should also explain how a Th2-skewing affects only the antibody response to one strain. Given that T cells are often cross-reactive and respond to additional antigens within the vaccine, besides HA and NA, one would expect the Th2-related impact to be spread across all strains in the influenza vaccine.

3. Recent supporting literature: A recent study employing human tonsil organoids (PMID: 39986275) highlights the role of Th1 responses in supporting inactivated influenza vaccine (IIV)-induced immunity. This study complements the authors' findings and could usefully be integrated into the discussion to provide additional context and support for the importance of T cell polarization in effective vaccine responses.

In summary, the manuscript is much improved and is nearing readiness for publication. The above points are intended to strengthen the clarity and impact of the findings and are not intended as major revisions.

(Remarks on code availability)

Referee #2

(Remarks to the Author)

(Remarks on code availability)

Referee #3

(Remarks to the Author)
This revision has addressed most of my questions. However, the current differential expression analysis uses a minimal 10% gene expression cutoff, which is quite low. It is difficult to interpret a change observed in only 10% of cells as representative of the entire population. If the cutoff were increased to 50% for DEGs that are increased expression in a cell population, how many of the DEGs would remain valid (e.g. Figure 1F-G and other similar findings)? Would applying a more stringent cutoff improve the consistency of DEG identification across datasets?

(Remarks on code availability)

Referee #4

(Remarks to the Author)
While the revisions considerably strengthen the manuscript, particularly through the addition of functional assays and

expanded antibody analysis, the work remains largely descriptive and incrementally novel by Nature standards. The resource value is undeniable, and the RNA Age Metric is potentially impactful. However, further mechanistic insights or causal experimentation would be necessary to elevate its significance. I do recommend publication as a resource paper, rather than as a report of conceptual breakthroughs.

(Remarks on code availability)

Version 2:

Reviewer comments:

Referee #1

(Remarks to the Author)
I want to thank the authors for providing additional analyses and thoughts. All my concerns are addressed.

(Remarks on code availability)

Referee #3

(Remarks to the Author)
I would like to thank the authors for providing the rationale for using a 10% gene expression cutoff through literature review and comparative data analysis. Establishing an appropriate balance between sensitivity and specificity is always challenging. Given the sparsity of the data (30–40% detection rate) and the heterogeneity of defined cell subpopulations, applying a 10% cutoff is reasonable as an initial step for identifying DEGs. However, it remains difficult to determine how many of the findings represent true versus false positives if the 10% cutoff is used as the basis for biological conclusions. Employing a higher cutoff (20–30%) would likely yield more reproducible results.

(Remarks on code availability)

Response to Reviewers

We thank the reviewers for their thoughtful comments on our manuscript. We have carefully considered all comments and in order to enhance the novelty and impact of our findings, we have 1) added multiple new analyses on internal and external datasets examining metrics of immune aging, 2) expanded our studies on influenza-specific vaccine responses, including the addition of Day 90 samples, examination of influenza-specific IgG isotypes and HA-specific B cell gene expression for further validation, and 3) functionally investigated the observed Th2-like skewing in the memory T cell compartment with age.

From these revisions, we now provide a new, unified model of immune aging in which broad Th2/Gata3-mediated T cell re-programming with age promotes reduced effector B cell activation, altered IgG class-switching and impaired antibody functionality in settings of repetitive, acute antigen exposure. Additionally, we demonstrate that the age-related transcriptional state of peripheral immune cells translates into functional dysregulation that includes higher spontaneous production of IL-4 from memory T cells, independent of overt systemic inflammation or underlying chronic CMV infection - further highlighting the importance of considering age in the design of vaccination strategies, cell-based therapeutics and clinical immune-focused treatments.

We believe the additional experiments and data analyses done here raise the broad interest and strengthen the impact of this manuscript to merit publication in *Nature*. We also think that providing this resource to the community will ultimately facilitate many new discoveries beyond our initial focus. Below is a detailed point-by-point response to the referees' comments.

Referee #1:

Gong et al. use a multi-omics systems biology approach to determine the impact of aging on immune status during homeostasis, chronic infection (CMV), and vaccination (influenza). For this purpose, they collected blood samples from two independent cohorts of 96 and 234 subjects containing healthy adults aged 25-90. They then use single-cell RNA-seq to identify 71 different immune cell subsets and profile their transcriptome, reaching a total of >16 million single cells; they use a highly multiplexed proximity extension approach to profile the plasma levels of thousands of protein markers; and they use ELISA and Hemagglutination Inhibition Assays to determine the vaccine-induced antibody response in a subset of subjects from the smaller cohort. Using this dataset, the authors first explore the impact of age on the immune system and find age-related transcriptional changes in T cells independent from systemic inflammation and CMV infection. Interestingly, the authors don't see a systemic increase in inflammation-associated markers, such as IL-6, TNF, or IL-1b, typically associated with aging. Next, the authors determine the impact of aging on vaccine-induced immunity. Using their multi-omics approach, they make a string of observations painting a picture of an altered adaptive immune cell compartment leading to altered vaccine responses in older subjects:

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Based on these findings, the authors conclude that a shift in the T cell compartment leads to suboptimal T-cell help for B cells during memory reactivation and hence reduced antibody production.

Overall, the manuscript is well-written and provides an extensive multi-omics dataset detailing peripheral immune cell and protein marker dynamics in a cohort of more than 300 subjects, encompassing over 16 million single-cell transcriptomic data points. The authors use this comprehensive dataset to explore a significant and timely question: How does aging influence the immune system, particularly in response to vaccination?

While the dataset's size and depth are indeed impressive, and the authors offer valuable insights, it is essential to view these findings in the context of existing literature. Previous work, such as that by Nakaya et al. (Immunity, 2015, PMID: 26682988) and Kumar et al. (Science Advances, 2024, PMID: 39331702), has already identified key age-related immune alterations, including reduced antibody responses and B cell activation. Additionally, previous studies (Mansfield, Clin Exp Immunol, 2012, PMID 23039889; Uciechowski, Exp Gerontology, 2008, PMID: 18166287) have highlighted Th2 skewing phenomena in the elderly. Thus, while the current study adds new details, such as the molecular regulation of Th2 skewing and detailed changes in the B cell compartment, it remains debatable whether these findings represent a significant advancement or a nuanced extension of existing knowledge.

The most notable contribution of this study may be the dataset itself. The authors present an impressive effort in deploying single-cell RNA sequencing on such a large scale, utilizing rigorous wet-lab and computational approaches. The statistical analyses are appropriate and well-described, adding robustness to the study's findings. However, it is worth noting that while the overall sample size is substantial, the subset of vaccinated individuals (68 subjects) is comparable to, but smaller than, that in some prior studies, such as Nakaya et al. (around 140 vaccinated subjects in two cohorts), and significantly smaller than the cohort used by Kumar et al. (234 vaccinated subjects). This difference in sample size has implications for the study's power to address complex questions about immune response heterogeneity, a topic Kumar et al. explored extensively but is not fully addressed in the present manuscript.

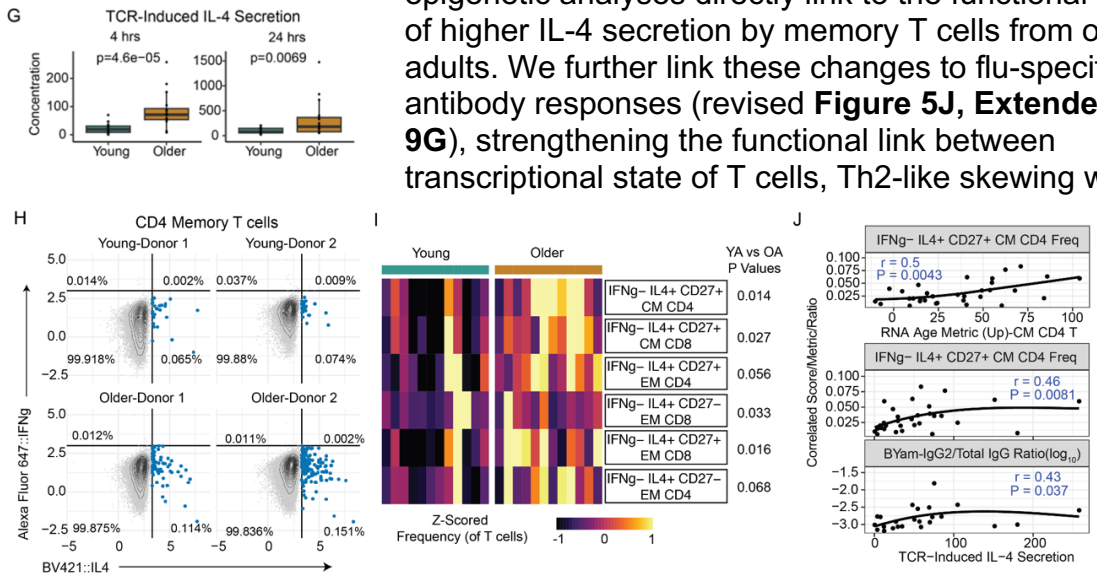
The authors have commendably made their dataset publicly accessible, which is a valuable contribution to the scientific community. Nevertheless, the manuscript's conclusions, while well-grounded in the presented results, remain primarily descriptive. The proposed hypothesis about how Th2-skewed T cell compartments negatively impact vaccination, although intriguing, requires more functional and mechanistic exploration to establish broader relevance and novelty.

In summary, the authors have provided a high-quality and detailed dataset that will serve as an essential resource. However, the study's novelty is perhaps somewhat limited, and the authors would benefit from addressing mechanistic questions and delving deeper into immune response heterogeneity to more clearly differentiate their findings from prior research.

Major Points:

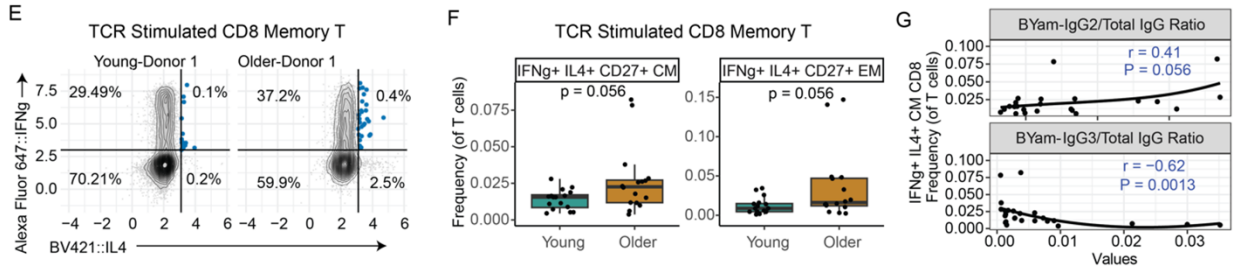
1. The global skewing of the T-cell compartment toward a Th2 phenotype is a critical and intriguing finding. However, the authors rely primarily on transcriptional and epigenetic analyses, without detecting differences in plasma protein levels of key Th2 cytokines. To strengthen this conclusion, it would be beneficial to perform functional assays measuring activation-induced cytokine production, including IFNγ, IL-4, IL-13, IL-5, IL-17, IL-21, IL-10, and IL-2, in both older and younger adults. These assays would provide robust functional validation of the Th2 shift. Additionally, measuring B-cell growth factors (IL-21, IL-10, and IL-2) could offer insights into the potential of these T cells to support B cell responses. Including both vaccine-specific and antigen-independent stimuli would further clarify whether the observed skewing is relevant to vaccine-induced responses and overall immune function.

We agree with the reviewer that demonstrating potential Th2-like skewing of T cells with age at a more physiological level is of importance. To address this question, we performed functional assays on T cells from young and older adults, both resting and with T cell receptor stimulation, measuring Th2 and Th1 cytokine secretion via a 7-plex MSD assay (including IL-4 and IFNg). We further examined which specific subsets of CD4 and CD8 T cells are generating IL-4 and/or IFNg via intracellular flow cytometry pre- and post-stimulation. From these experiments, we found that 1) T cells from older adults secrete higher levels of IL-4 post-TCR stimulation, (revised **Figure 5G**) and 2) multiple memory CD4 and CD8 T cell subsets have a higher spontaneous IL-4 production in older adults that correlates with CM CD4 RNA Age Metrics. (revised **Figure 5H, I**) Moreover, an expansion of IFNg+IL4+ CD27+ memory CD8 T cells post-TCR stimulation is observed in older adults compared with young adults (p=0.056). (**Extended Figure 9E, 9F**) Thus, we now demonstrate that our transcriptional and epigenetic analyses directly link to the functional outcome of higher IL-4 secretion by memory T cells from older adults. We further link these changes to flu-specific antibody responses (revised **Figure 5J, Extended Figure 9G**), strengthening the functional link between transcriptional state of T cells, Th2-like skewing with age.



Revised Figure 5. G. *Concentration of IL-4 (pg/mL) secreted by aCD3/aCD28 T cell receptor (TCR) stimulation of T cells isolated from young (n=16) and older (n=16) adults at 4 and 24hrs.*

H. *Representative IL-4 and IFNg expression by memory CD4 T cells determined by intracellular flow cytometry and* ***I.*** *heatmap of IFNG$^-$IL-4$^+$ memory T cell subset frequencies from young (n=12) and older (n=12) adults rested for 4hrs without TCR stimulation. P-values were calculated using Wilcoxon rank sum test.* ***J.*** *Spearman correlations of IFNG$^-$IL-4$^+$ CM CD4 T cells with RNA Age Metric in CM CD4 T cells and IL-4 secretion post-TCR stimulation with frequencies of IFNG$^-$IL-4$^+$ CM CD4 T cells and normalized B/Phuket-specific IgG2 levels at day 7 post-vaccination.*

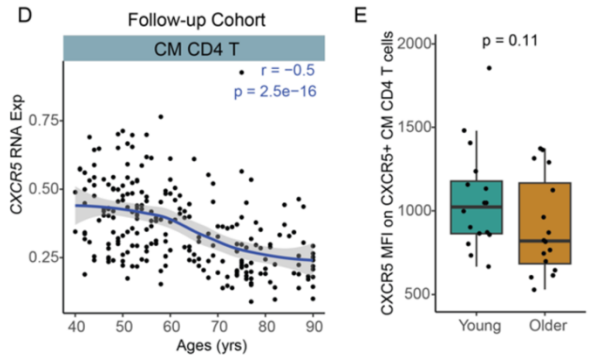


Extended Figure 9. E. *Representative IL-4 and IFNg expression in aCD3/aCD28-stimulated (4 hours) memory CD8 T cells with age determined by intracellular flow cytometry.* ***F.*** *Percentage of IFNG$^+$IL-4$^+$ CD8 memory T cell subsets from young and older adults after 4 hrs of aCD3/aCD28 stimulation. P-values were calculated using Wilcoxon rank sum test.* ***G.*** *Spearman correlations of frequencies of IFNG$^+$IL-4$^+$ CD27+ CM CD8 T cells post-stimulation with normalized B/Phuket-specific IgG2 and IgG3 levels on day 7 post-vaccination.*

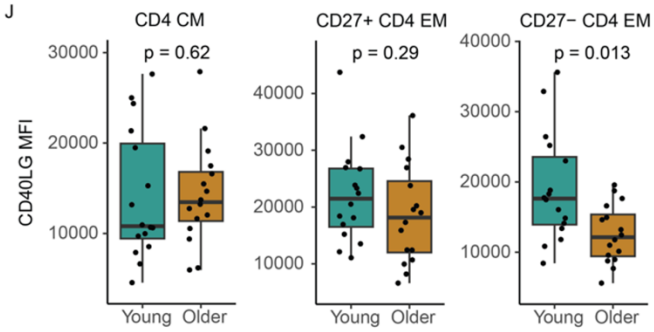
2. The conclusion that older adults' CD4 T cells have a reduced capacity to assist B cells is compelling but currently based on transcriptomic data alone. The lack of differences in the frequency of circulating Tfh cells at day 7 post-vaccination adds complexity to this finding. A co-culture assay using sorted memory CD4 T cells and naive/memory B cells to measure total IgG production (as demonstrated in Morita et al., Immunity, 2011, PMID: 21215658) would provide functional evidence of reduced B cell help and significantly enhance the study's impact.

We agree that understanding how transcriptional changes translate into protein expression and downstream functional interactions is critical - but also not a trivial task. Due to Institutional Biosafety regulations, we had difficulties getting approval to use the suggested reagents (i.e., SEB) from Morita et.al. Thus, we took an alternative approach to demonstrate the potential for memory CD4 T cells to provide B cell help using flow cytometry to evaluate surface protein expression of CXCR5 on memory CD4 T cells. We assessed median fluorescent intensity not percentage, as this is a more direct correlation with our RNA expression results. **(Extended Figure 8E**) From these results, we find that CXCR5 expression levels in memory CD4 T cells trends lower in older adults (n=16) compared with young adults (n=16). (p=0.11) Moreover, in order to assess how lower *CD40LG* gene expression at baseline may translate into protein expression, we assessed CD40LG protein expression on memory CD4 T cells post-TCR stimulation, as CD40LG is not readily detectable without stimulation. (**Extended Figure 8J**) We found that some but not all subsets of memory CD4 T cells exhibit reductions in CD40LG protein. As activation can change the surface phenotype of T cell subsets, we cannot directly correlate these results with our baseline data; however, this

data collectively suggests there may be a subtle reduction in direct helper capacity of memory CD4 prior to advanced age.



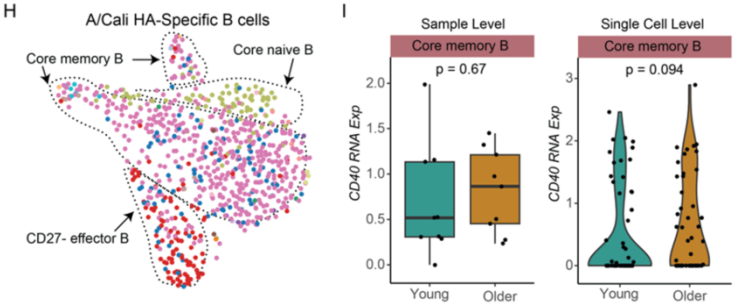
Extended Figure 8. *D. CXCR5 expression in CM CD4 T cells across age in our follow-up cohort. Regression line shown with 95% confidence intervals. E. CXCR5 protein expression (median fluorescence intensity) on CXCR5⁺ CM CD4 T cell subsets at baseline in young (n=16) and older (n=16) adults determined by spectral flow cytometry.*



Extended Figure 8. ***J****. CD40 ligand (CD40LG) surface expression on anti-CD3/anti-CD28-stimulated (24 hours) memory CD4 T cell subsets in young (n=16) and older (n=16) adults determined by flow cytometry.*

3. The observed changes in B cell populations are described globally, without specific data on antigen-specific B cells. Including analyses, such as flow cytometry using an influenza hemagglutinin probe, on a subset of samples would substantiate whether these transcriptional changes are occurring within the vaccine-specific B cell population, strengthening the manuscript's relevance to influenza vaccination.

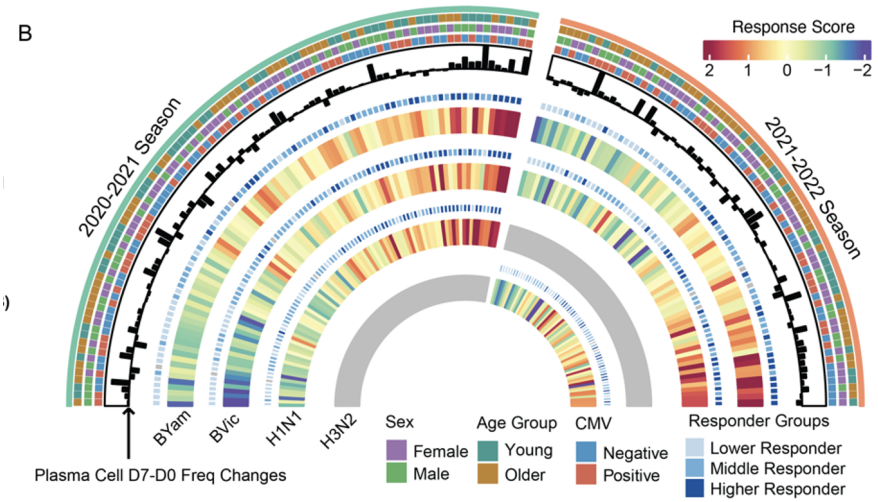
Utilizing an externally available dataset of influenza (A/California) HA-specific B cells (*GSE167823*), we examined *CD40* RNA expression on flu-specific core memory B cells between young and older adults and, as previously seen at a global level, found no significant difference in expression. These data are now shown in **Extended Figure 8H, 8I**. While the HA antigen used here is not B/Phuket-specific, A/California is another highly boosted flu antigen that was present in flu vaccines from 2010-2017, thus is likely reflective of similarly repetitive B/Phuket responses.



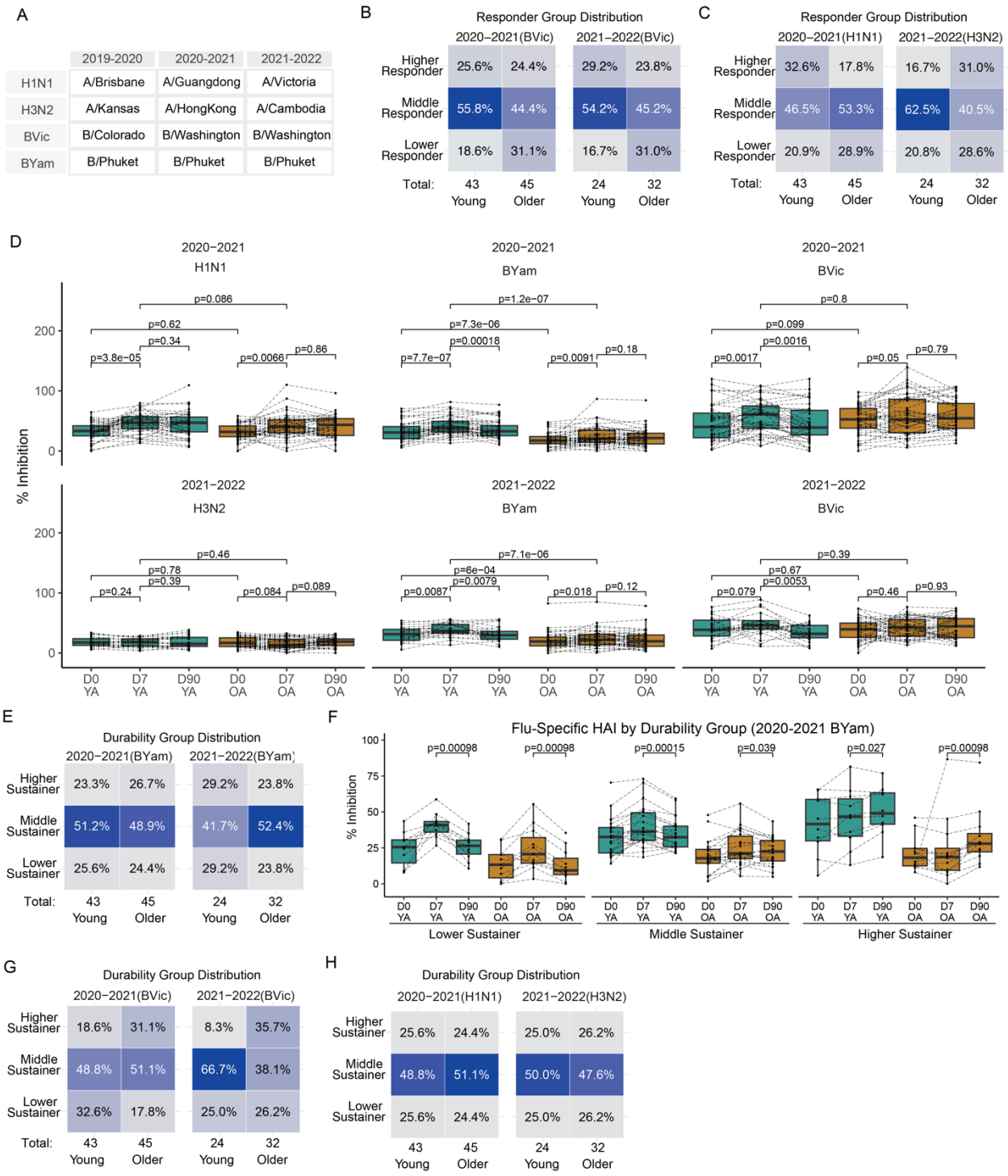
Extended Figure 8. H. *UMAP of A/Cali HA-specific B cells from young and older adults, labelled with our Human Immune Health Atlas B cell subsets.* ***I.*** *Comparison of CD40 expression on HA-specific Core memory B cells from young (n=9) and older (n=9) adults. (GEO GSE167823)*

4. The antibody response data currently focus on two influenza B strains, although the vaccine includes multiple strains, notably more prevalent influenza A strains. Since the authors mention the use of MSD-based assays measuring seven strains, providing a comprehensive analysis, especially of the influenza A strains, would offer a more holistic understanding of age-related impacts on vaccine efficacy.

The influenza vaccine strains vary season by season. We have extensively updated our influenza antibody response data to include information on the multiple strain-specific responses including influenza A strains relevant to the vaccine years that our study participants were enrolled. (Revised **Figure 4B, Extended Figure 5**)



Revised Figure 4. B. *Variation in HAI responses to season influenza strains, comparing day 0 and day 7 post-vaccination in the 2020-2021 flu season.*

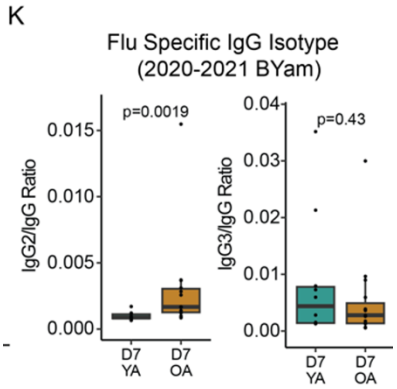


Extended Figure 5. Influenza-specific HAI responses in young and older adults. A *Overview of specific influenza A and B strains contained with seasonal influenza vaccines during our study collection period.* ***B-C.*** *Distribution of response groups across age groups of* ***B.*** *BVic, and* ***C.*** *H1N1 and H3N2 strains in two flu seasons, determined by Day 0 to Day 7 comparisons. 'Higher' represents the top 25%, 'Middle' the middle 50%, and 'Lower' the bottom 25% based on response score cutoffs.* ***D.*** *Mean percent inhibition of flu hemagglutinin (HA) antigen as determined by the HAI assay for H1N1, BYam and BVic in 2020-2021 season in*

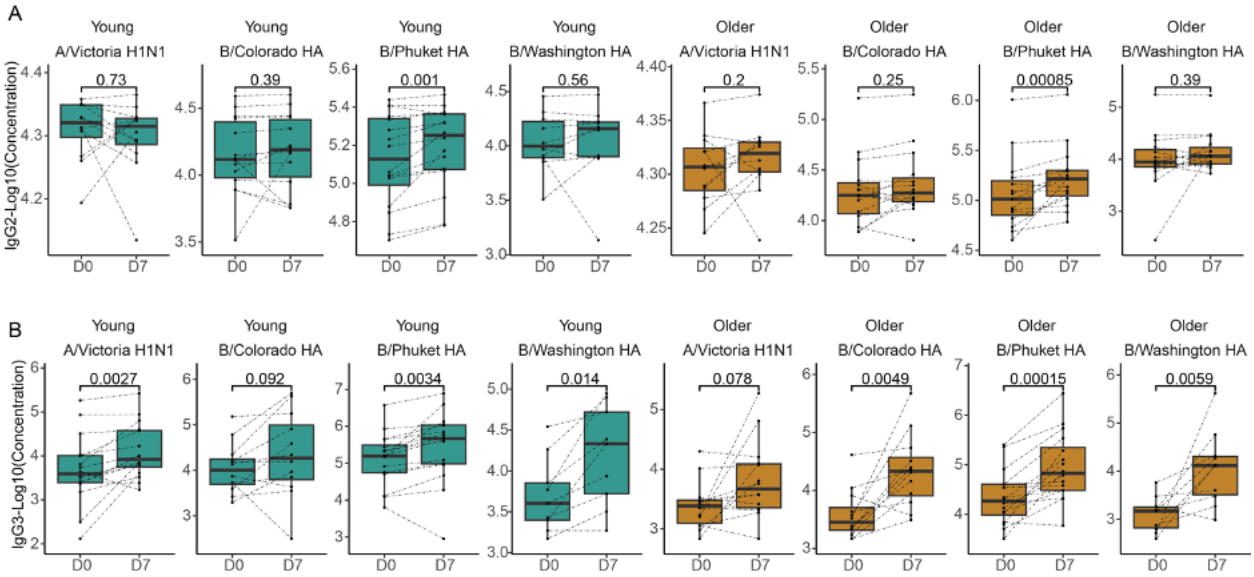
young and older adults at days 0, 7 and 90. P-values were calculated using paired Wilcoxon if it is within age group and non-paired Wilcoxon for cross-group comparison. ***E.*** *Distribution of durability groups across age groups of BYam strain in two flu seasons, determined by Day 7 to Day 90 comparisons.* ***F.*** *Percent inhibition of BYam determined by HAI for both young and older adults at days 0 7 and 90, separated by durability group. P-values were calculated using Wilcoxon's signed-rank test (paired).* ***G-H.*** *Distribution of durability groups across age groups of* ***G.*** *BVic, and* ***H.*** *H1N1 and H3N2 strains in two flu seasons, determined by Day 7 to Day 90 comparisons. 'High' represents the top 25%, 'Middle' the middle 50%, and 'Low' the bottom 25% based on durability score cutoffs.*

5. The transcriptional differences in B cell subsets suggest alterations in class-switching behavior. To ascertain the functional relevance, conducting Ig-subtype-specific ELISA for vaccine-targeted influenza strains would be informative. This could reveal if the transcriptional changes translate into altered antibody class distribution.

This was an intriguing question. To interrogate this, our team developed a multi-plex MSD assay to detect levels of flu-specific IgG2 and IgG3 from the corresponding strains in our flu-specific total IgG assays. These two isotypes were selected as it has been nicely demonstrated that IL-4 changes the ratio of these two IgG isotypes in human B cells *in vitro*. (Avery et al. 2008) From these new analyses, we found significantly increased flu-specific IgG2 in older adults, corresponding to elevated IgG2 responses specific to B/Phuket HA. (revised **Figure 4K, Extended Figure 7**) This elevation in IgG2 is also consistent with increased *IGHG2* and decreased *IGHG3* expression in CD27- effector B cells in older adults we previously described. (**Figure 4H**) Thus, in line with our transcriptional differences indicative of altered class-switching, we have now directly demonstrated altered class switching in flu-specific antibody responses in older adults, with unique class-switching patterns specific to a highly-boosted antigen.



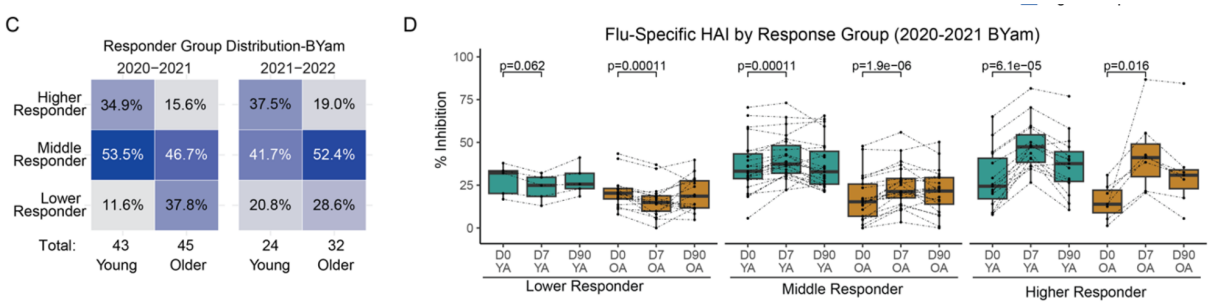
Revised Figure 4. K. *B/Phuket-specific IgG2 and IgG3 antibody levels normalized to B/Phuket-specific total IgG levels in plasma compared to expression at Day 0 and 7 post-vaccination for both young (n=10) and older (n=14-15) adult cohorts.*



Extended Figure 7. Influenza-specific IgG2 and IgG3 responses in young and older adults. A*. Flu-specific IgG2 and* ***B****. flu-specific IgG3 levels against multiple influenza strains at Day 0 and Day 7 post-influenza vaccination. Young adult responses are shown in teal. Older adult responses are shown in bronze. P-vals determined by Wilcoxon's signed-rank test (paired).*

6. The manuscript currently presents antibody data at day 7 post-vaccination, a time when responses are peaking. However, immune response kinetics may differ with age, potentially affecting the analysis. Including data from day 90 post-vaccination would address whether older adults have a delayed or reduced response and provide insights into the longevity of immunity, which is crucial for understanding age-related differences.

We added more detailed analyses of our influenza data including Day 90 HAI into the manuscript, looking at both response (D0 to D7 comparisons) and durability (D7 to D90 comparisons) for multiple strains. (see Revised **Figure 4, Extended Figure 5** [see above]) From these more extensive analyses, we find that older adults display reduced capacity of initial antibody response to B/Phuket (BYam) (**Figure 4C-D**), however they have similar durability compared with young adults.

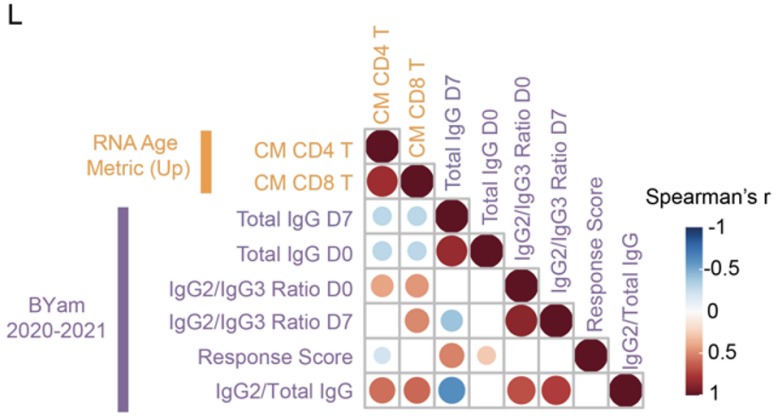


Revised Figure 4. C. *Distribution of response groups across age groups of Byam (B/Phuket) for two separate flu years. 'High' represents the top 25%, 'Middle' the middle 50%, and 'Low' the*

bottom 25% based on response score cutoffs. ***D.*** *Mean percent inhibition of flu hemagglutinin (HA) antigen as determined by the HAI assay (**C, E**) for both young and older adults at days 0 7 and 90, separated by responder group. P-values were calculated using Wilcoxon's signed-rank test (paired).*

7. The study reports notable age-related differences in B cell subsets and their transcriptional profiles. To better contextualize these findings, correlating these parameters with hemagglutination inhibition (HAI) titers could offer an understanding of which of these differences translate how into protective immunity.

In order to understand how B cell responses correlate with age-related transcriptional responses at baseline, we compared influenza-specific HAI responders ("Response Score") to influenza-specific total IgG, IgG isotype expression and RNA Age metric for CM T cell subsets. (revised **Figure 4L**) Notably, we find correlations between HAI responses and CM CD4 RNA Age metric and total IgG levels but not IgG isotype expression. The correlation between influenza-specific total IgG levels is in line with decreasing levels of IgG in older adults specifically for Phuket antigen as well as mechanistically, as changes in IgG isotype should not impact the outcomes of the HAI assays. Thus, we now demonstrate a number of novel associations between T cell transcriptional states, B cell class-switching and functional antibody responses.



Revised Figure 4. L. *Spearman correlations comparing influenza-specific responses with RNA age metric for CM T cell subsets.*

8. A recent study (Kumar, Science Advances, 2024) has highlighted significant heterogeneity in vaccine responses among older adults. Given the depth of the current study, exploring inter-individual variability could reveal important insights and enhance the manuscript's impact. Leveraging the study's unique dataset to address this would be a valuable addition.

We have more deeply analyzed our influenza vaccine response data as described above, including individual heterogeneity across multiple influenza strains (**Figure 4B**), comparison of Response (D0 - D7) and Durability (D7-D90) across multiple influenza strains (**Figure 4, Extended Figure 5**) and influenza-specific IgG isotype analyses. (revised **Figure 4K**) We believe these analyses provide further, interesting insight into the variation in vaccine responses in healthy adults.

Minor Points:

1. On page 6, the manuscript mentions that participants received the same vaccine but does not specify the vaccine type. Including details on whether the vaccine was inactivated, high-dose, or adjuvanted, and whether it was trivalent or tetravalent, would provide essential context, given the varying efficacy of different formulations in older populations.

Donors received the following inactivated, standard-dose, non-adjuvanted quadrivalent influenza vaccinations based on the corresponding flu season; FLUARIX Quadrivalent in 2019-2020 and 2021-2022 and Fluzone in 2020-2021. Basic influenza vaccination information has been added into the **Methods** and **Results** sections.

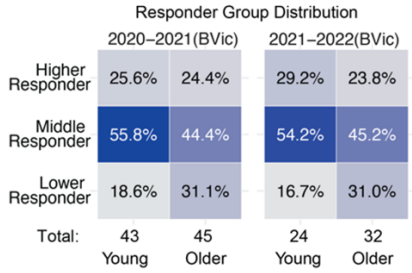
2. The proposed model suggests that reduced T cell help affects responses to recall antigens but not to neoantigens. Given that T cells can recognize a broad range of influenza epitopes and often exhibit cross-reactivity, the authors should provide a rationale for this selective impact. A discussion on the potential for pre-existing cross-reactive T cells, even in neoantigen settings, would be helpful.

In our re-analyses of the influenza-specific results, we interestingly found a more nuisanced finding - where it is not just a factor of recall responses but specific to B/Phuket (eg. B/Washington doesn't show this effect in 2021-2022 in which it was now a recall antigen (**Extended Figure 5B**)). The B/Phuket antigen has been present in influenza vaccines since 2015, thus is a long-term repetitive, acute exposure. This type of exposure is most similar to the recent study tracking memory T cells over a decade in mice with repeat, acute vaccine exposures (Soerens, Nature, 2023) Here, they show slow transcriptional and epigenetic re-programming in a similar fashion to what we observe. While additional long-term vaccination studies in humans are out of the scope of this work, we think that it would be interesting to see whether this is a common feature of repeat acute exposures. We have added these concepts into our Discussion.

A

	2019-2020	2020-2021	2021-2022
H1N1	A/Brisbane	A/Guangdong	A/Victoria
H3N2	A/Kansas	A/HongKong	A/Cambodia
BVic	B/Colorado	B/Washington	B/Washington
BYam	B/Phuket	B/Phuket	B/Phuket

B



Extended Figure 5. A. *Overview of specific influenza A and B strains contained with seasonal influenza vaccines during our study collection period.* ***B.*** *Distribution of response groups across age groups of BVic (B/Washington) in two flu seasons, determined by Day 0 to Day 7 comparisons. 'Higher' represents the top 25%, 'Middle' the middle 50%, and 'Lower' the bottom 25% based on response score cutoffs.*

3. The reduction in Type 2-polarized B cell expansion during vaccination in older adults appears to contradict the observed Th2 skewing in the T cell compartment. The authors

should elaborate on how these findings align or provide potential mechanisms for this observation.

Type-2 polarized B cells classically arise from allergic responses (PMID: 38324637), which is usually an independent response from that elicited by vaccination. Indeed, the lack of systemically elevated Th2 cytokines suggests that the Th2-like skewing in memory T cells is IL-4 independent. Notably, recent literature has highlighted GATA3 expression and early Th2-like priming independent of IL-4 production. (PMID: 16172258, 16467870) Moreover, higher tonic mTOR signaling has been associated with elevated Th2 polarization in mice (PMID: 31067469) and is also a recently defined feature of human T cell aging (PMID: 40249803). We have added these aspects to the **Discussion**.

Referee #2:

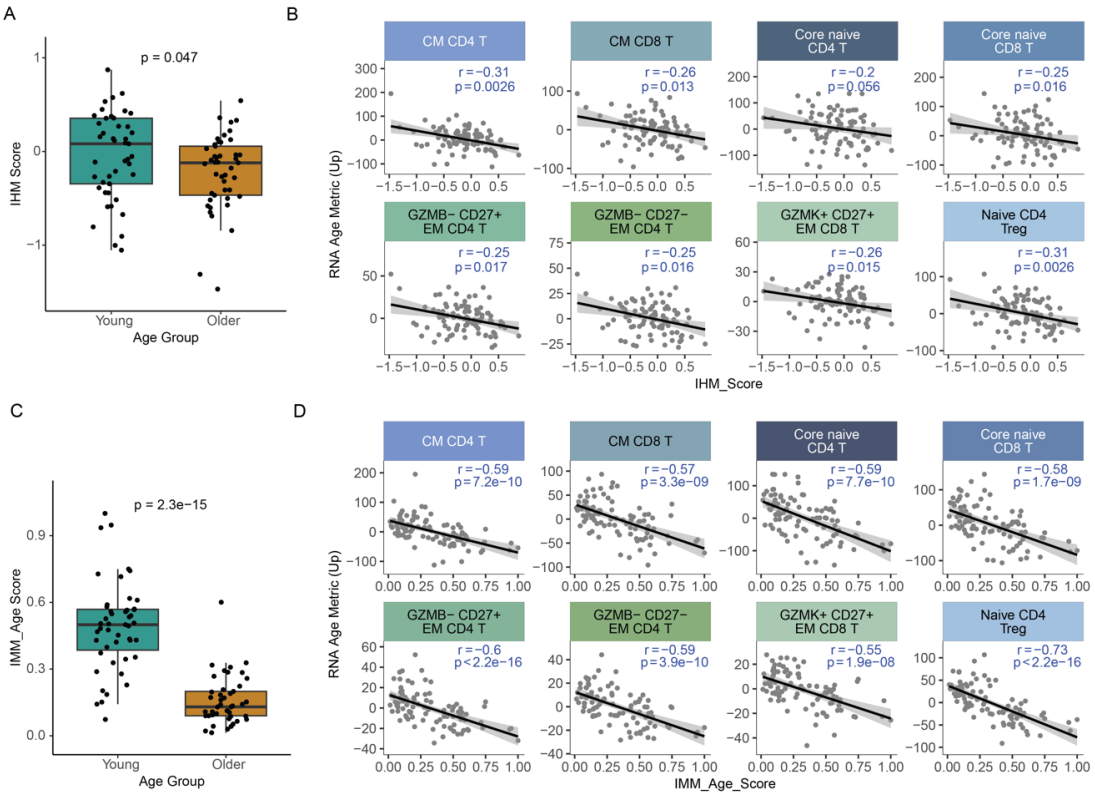
In this study, Gong et al examined peripheral blood plasma and leukocytes in 2 separate cohorts. The first comprised healthy young 37 (25-35 yrs) and older (55-65 yrs) adults over 2 years, at multiple timepoints both before and after annual influenza vaccination. In the second cohort they studied a cross-sectional cohort of healthy adults with a wider age range especially in the older end of the age spectrum. They used multiple platforms for these analyses including scRNA-seq, high-dimensional plasma proteomics and spectral flow cytometry. They have created a valuable resource for future researchers including scRNA-seq datasets of >16 million PBMCs, and have identified longitudinal changes in 71 distinct immune cell subsets before and after vaccination in different age groups. However the current interpretation of their data and the perceived highlights are limited.

Comments:
1) There have been previous studies that have searched for and identified age associated changes in the immune system and many of these studies have been cited in the current manuscript e.g. Alpert et al 2019, Sayed et al 2021, Sparks et al 2024 and others). Each of these separate studies claim to have identified unique age-related immune signatures. Where is the overlap with the current work? How are the immune ageing characteristics shared or different in these studies? And ultimately for scientists or clinicians who want to use this data as a metric of immune ageing and/or immune competency, who do we believe? For these elaborate datasets to be useful, there has to be some consensus on what changes during ageing and whether they are beneficial or detrimental. Although beyond the scope of the current study, as not every young or old individual has the same level of physical competence, it would have been good to know how the results obtained are linked to metrics of frailty.

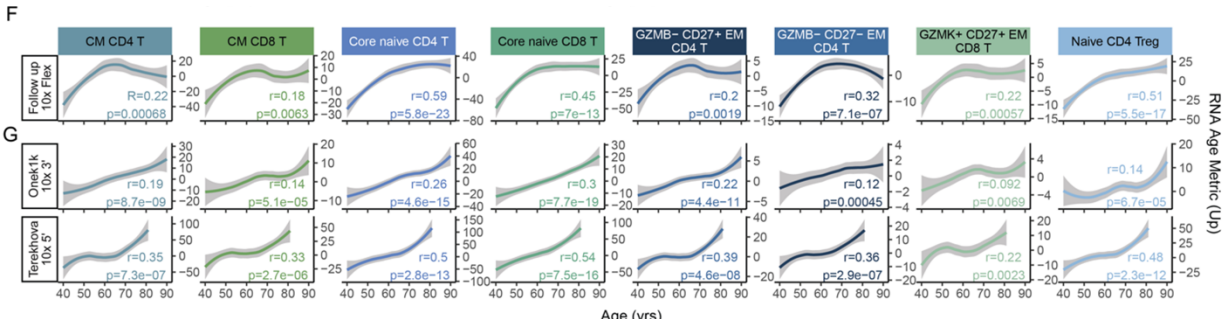
Ultimately, our goal here was to establish a common signature indicative of the overall transcriptional state of a cell - that could be used to track cellular states more broadly over time and with age (instead of on a gene-by-gene level) – and provide more mechanistic insight into immune dysregulation with age. However, developing metrics of immune health is important when thinking about long-term clinical screening. Thus, we

compared our RNA Age Metrics to two Immune Health Signatures, 1) IMM-AGE that is based on cellular frequencies and 2) IHM that is based on whole blood bulk RNA-seq transcriptional signatures. We found that our age-related transcriptional profile metric correlated with both IMM-AGE and IHM. (**Extended Figure 4**) The weaker association with IHM may be due to the fact that whole blood contains additional cell types, such as neutrophils, as well as being a mixed signature of both composition and cellular states (i.e. changes in the RNA expression profiles of cells).

To better assess the commonality of age-related changes in transcriptional programming of T cell subsets across different populations, we determined RNA Age Metrics across age in two additional immune health cohorts in combination with our follow-up cohort *(Terekhova et al. 2023; Yazar et al. 2022)* - that totals more than 1300+ people across 18-90 year of age. (revised **Figure 2F-G**) This demonstrates that aging impacts T cell subsets in a common fashion across healthy people, although the kinetics of these changes at the extremes of age are more variability across cohorts. We agree with the reviewer that future studies to better understand the cause of this variability in advanced age, such as frailty, BMI, chronic disease, etc, would help provide clarity in this regard.



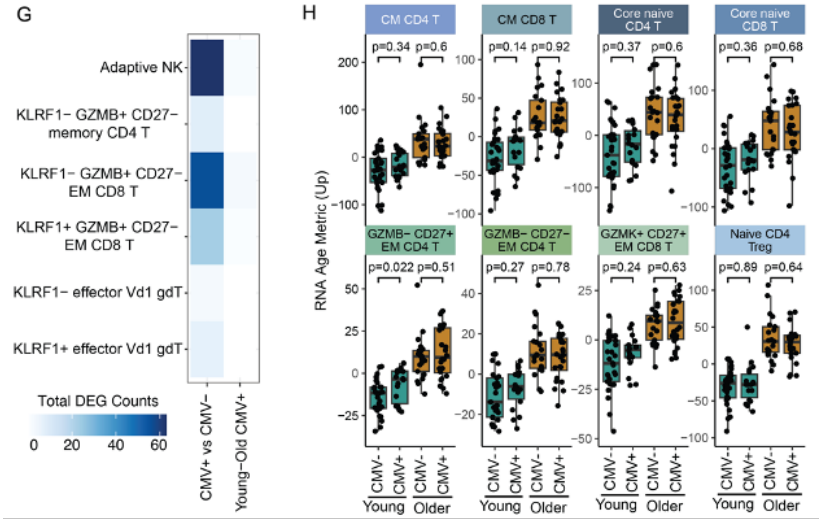
Extended Figure 4. Comparison of RNA Age Metric to Imm-Age and IHM metrics. A. *Comparison of IHM score determined from whole blood RNA-seq for young and older adults.* ***B.*** *Spearman correlation between IHM and RNA Age metric (up) in age-susceptible T cell subsets* ***C.*** *Comparison of Imm-AGE score determined from cell frequencies from scRNA-seq data for young and older adults.* ***D.*** *Spearman correlation between Imm-AGE and RNA Age metric (up) in age-susceptible T cell subsets.*



Revised Figure 2. F-G. *The average RNA Age Metric of upregulated genes for the top age-impacted immune cell subset, shown across age in* ***F.*** *our follow-up cohorts and* ***G.*** *two external immune aging datasets. (Terekhova et al. 2023; Yazar et al. 2022) Regression line shown with 95% confidence intervals in gray with Spearman correlations.*

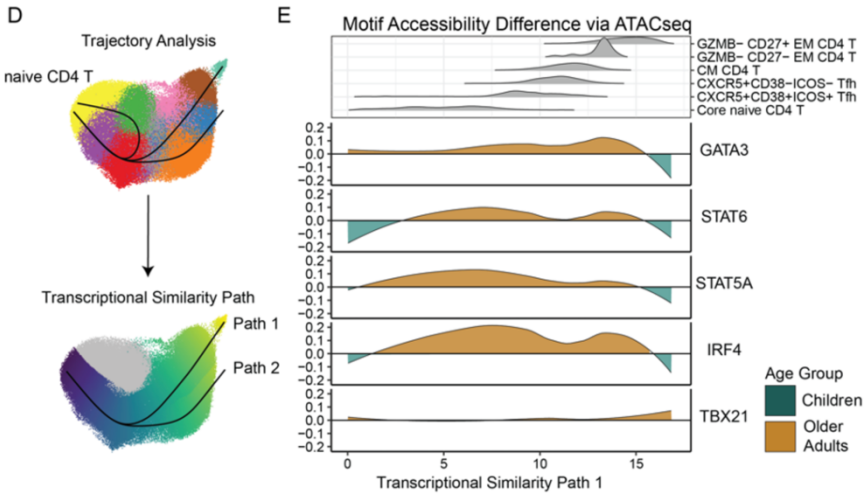
2) The current work is laudable in that a huge amount of data has been collected, analysed and integrated, but it does not provide any definitive answers. One of the key observations stated in the abstract is that "We find that T cells earlier in differentiation accumulate distinct age-related transcriptional changes more than other immune cells, independent from systemic inflammation and chronic perturbation". However this has been shown to some extent previously (Pulko V et al.2016, Human memory T cells with a naive phenotype accumulate with aging and respond to persistent viruses. Nat. Immunol. 17, 966–975). It is noted however that systemic inflammation and chronic perturbation was not investigated in the Pulko study. Moreover another conclusion in the abstract that "...impaired memory B cell responses to vaccination are linked to a gradual GATA3/Th2-polarization in T cell subsets within older adults is not totally novel. End-stage TEMRA T cells that accumulate during ageing have been shown to express GATA3 (Callender LA et al GATA3 induces mitochondrial biogenesis in primary human CD4+ T cells during DNA damage. Nat Commun. 2021 ;12:3379). While it is appreciated that the vaccination arm of the studies was labour intensive an laborious, the key questions of what immune or plasma signatures are associated with good or bad vaccine responsiveness, especially in the older group, is not forthcoming. Nevertheless the extensive volume of data that has been generated and deposited is a valuable resource for future investigators.

We thank the reviewer for their positive comments on the data generated for this manuscript. We have made extensive revisions to the manuscript that we hope address a number of the points raised here. Firstly, we agree disentangling age effects from those mediated by chronic CMV infection is critically important - as CMV infection is a large confounder in many aging studies. Here, we further dissect the transcriptome of immune cell subsets to distinctly demonstrate that transcriptional reprogramming of T cells with age is independent from those induced by CMV. (revised **Figure 3H**) This is the same for the circulating proteome, where CMV does not demonstrate any major changes in our cohorts. We also find that terminal T cell subsets (GZMB+ subsets), which are clearly CMV-responding cells, have little to no changes in their transcriptional programming with age in CMV+ people <65 yrs of age. (revised **Figure 3G**) Instead, their transcriptional programming is highly altered by CMV-positivity.

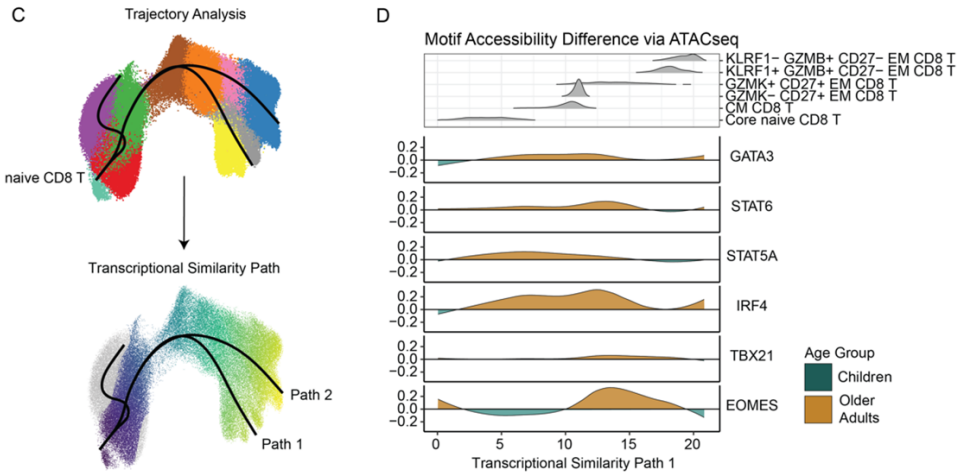


Revised Figure 3. G. *Heatmap of mean number of differential genes from CMV+ vs CMV- individuals as well as young vs older CMV+ adults.* ***H.*** *RNA age metric (upregulated genes) in age-susceptible T cell subsets split by age and CMV infection status. P-values were calculated using Wilcoxon rank sum test. Box plots are median with quartiles shown.*

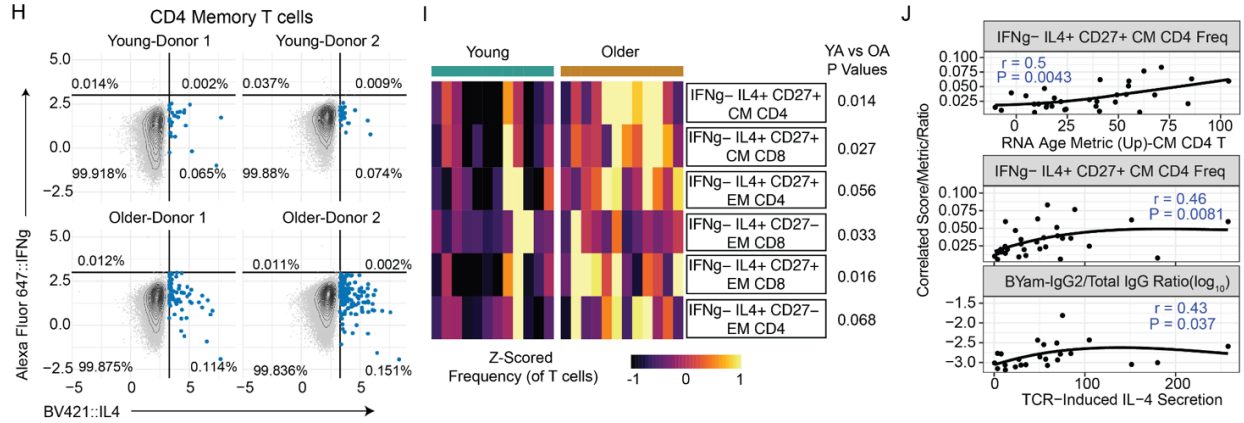
Our original findings on elevated GATA3 activity was observed within less terminal differentiated T cell subsets - thus independent from higher levels of GATA3 observed in TEMRAs with age in Callender, Nat Comms, 2021. To better clarify our findings, we utilized trajectory analysis to compare GATA3 and other Th2-related TF activities (based on scATAC-seq Chromvar analyses) across all CD4 and CD8 T cell subsets categorizing them based on transcriptional similarity. (Revised **Figure 5D-E**; **Extended Figure 9C-D**) From these analyses, we see that the age-related GATA3/TH2-like polarization occurs broadly across the naive and memory T cell compartments but less so in the more terminal GZMB+ subsets. This Th2-like skewing was functional confirmed using intracellular flow cytometry in which we found higher spontaneous IL-4 production by memory T cell subsets in older adults that associated with RNA Age Metrics. (Revised **Figure 5H-J**) Notably, GATA3 expression can be modulated by features previously linked with age including DNA damage, TCR signaling strength and IL-2 responses (Callender et al. 2021; Al-Aghbar et al. 2024; Yamane, Zhu, and Paul 2005; H. Zhang et al. 2022) – suggesting this age-related dysregulation is likely occurring in an IL-4 independent manner broadly across the T cell compartment. This has been added into the **Discussion**.



Revised Figure 5. D. *RNA-based trajectory analysis of CD4 T cells from T cell TEA-seq dataset.* ***E.*** *Th1 (TBX21)- and Th2 (GATA3, STAT6, STAT5B, IRF4)-related transcription factor (TF) motif accessibility differences based on Chromvar scATAC-seq analysis across CD4 T cells from children (n=8) and older adults (n=8). Teal indicates lower accessibility in older adults, whereas bronze indicates higher accessibility in older adults compared with children.*



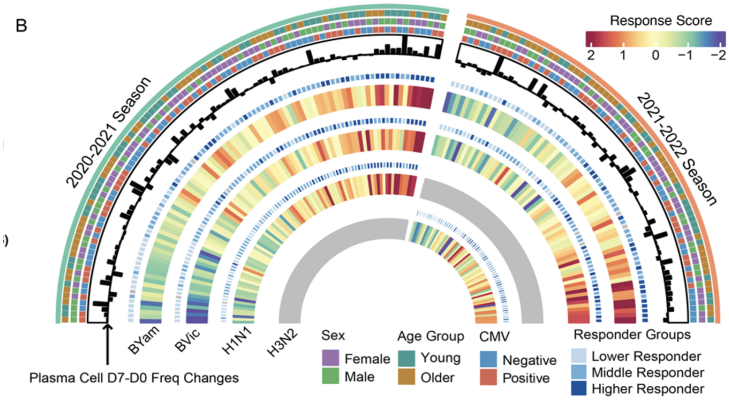
Extended Figure 9.C. *RNA-based trajectory analysis of CD8T cells from T cell TEA-seq dataset.* ***D.*** *Th1 (TBX21)- and Th2 (GATA3, STAT6, STAT5B, IRF4)-related transcription factor (TF) motif accessibility differences based on Chromvar analysis of TEA-seq data across CD8 T cells from children (n=8) and older adults (n=8). Teal indicates lower accessibility in older adults, whereas bronze indicates higher accessibility in older adults compared with children.*



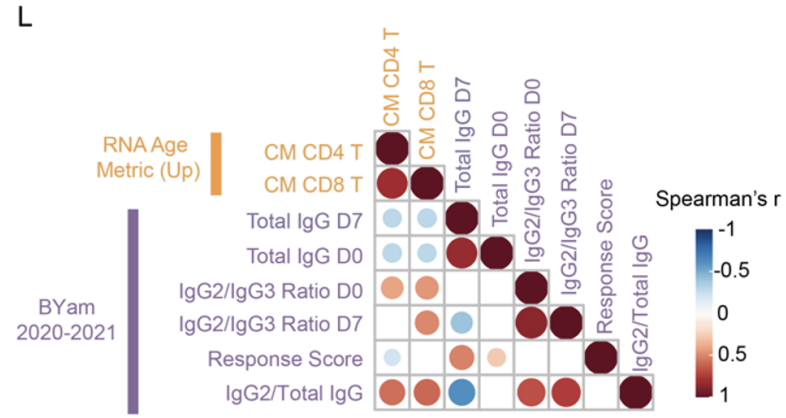
Revised Figure 5. H. *Representative IL-4 and IFNg expression by memory CD4 T cells determined by intracellular flow cytometry and* ***I.*** *heatmap of IFNG$^-$IL-4$^+$ memory T cell subset frequencies from young (n=12) and older (n=12) adults rested for 4hrs without TCR stimulation. P-values were calculated using Wilcoxon rank sum test.* ***J.*** *Spearman correlations of IFNG$^-$IL-4$^+$ CM CD4 T cells with RNA Age Metric in CM CD4 T cells and IL-4 secretion post-TCR stimulation with frequencies of IFNG$^-$IL-4$^+$ CM CD4 T cells and normalized B/Phuket-specific IgG2 levels at day 7 post-vaccination.*

In order to understand how B cell responses correlate with age-related transcriptional responses at baseline, we first determine low, medium and high responders across all influenza strains (Revised **Figure 4B**) then compared influenza-specific HAI responders (“Response Score”) to influenza-specific total IgG, IgG isotype expression and RNA Age metric for CM T cell subsets. (revised **Figure 4L**) Notably, we find correlations between HAI Response Scores with CM CD4 RNA Age metric and flu-specific total IgG levels but not flu-specific IgG isotype expression. The correlation between flu-specific total IgG levels is in line with decreasing levels of IgG in older adults specifically for Phuket

antigen as well as technically, in that IgG class-switching should not directly impact HAI assays. Moreover, we reveal a novel association between flu-specific IgG class-switching in response to a highly boosted antigen (B/Phuket HA) and the age-related transcriptional state of CM T cells (i.e. RNA Age Metric) – that is consistent with in vitro B cell class-switching induced in the presence of IL-4, and added to the **Discussion**.



Revised Figure 4. B. *Variation in HAI responses to seasonal influenza strains, comparing day 0 and day 7 post-vaccination in the 2020-2021 and 2021-2022 flu season.*



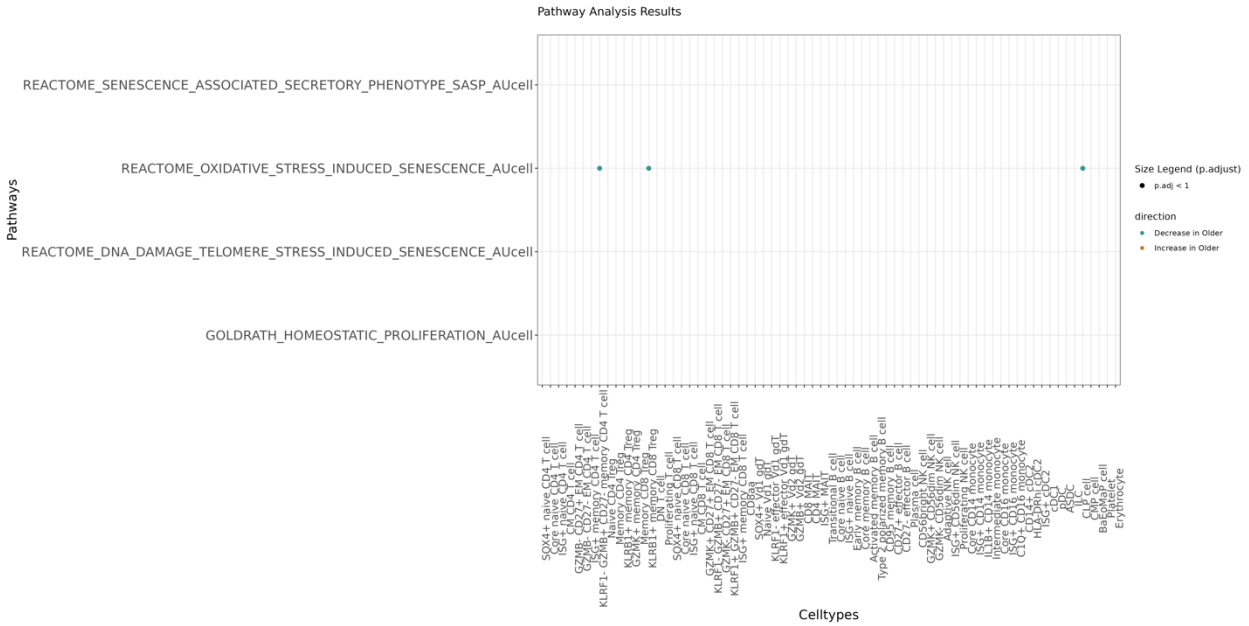
Revised Figure 4. L. *Spearman correlations comparing influenza-specific responses with RNA age metric for CM T cell subsets.*

3) Their data to some extent contradicts previous findings including no increase in inflammatory proteins over time (including IL-11 that was published in Nature recently) and no impact of previous CMV infection on the proteome over time. This raises the questions of ***when exactly inflammation and CMV infection induce their effects on the proteome and on leukocyte subsets and why does this effect not change over time*** *especially as CMV induces lifelong persistent infection? This has not addressed. If not inflammation and/or CMV infection, what* ***maintains these changes over time****? What is the* ***extent of proliferative activity in the different leukocyte populations over time****, especially in the T cell subsets?*

While most immune aging studies examine features of advanced age (>65 yrs of age), here we focus on individuals at the cusp of advanced aging (55-65 years old adults) compared with younger adults (25-35 yrs), to gain insight into the transition period

between functional and dysfunctional immunity. We have added this detail throughout the manuscript for better clarity. Moreover, we further dissected our age-related Olink data and found that although no change in inflammatory cytokines is found before 65 yrs of age - However, in the broader age range of our follow-up cohort, a modest positive correlation of IL-6 (rho=0.35 , padj=2.93e-7) and IL-11 (rho=0.26, padj= 3.27e-4) with advanced chronological age was detected, suggesting that some features of inflammation may contribute to immune dysregulation later in life. These new findings have been added into the **Results** and highlight some of the unique features of more advanced age compared with our relatively "young" older cohort.

In regards to CMV infection, our data, including updated comparisons in **Figure 3**, show that age impacts the composition of the CMV-mediated adaptive NK immune landscape in healthy adults, without significant changes to the T cell compartment prior to transition into advanced aging setting. We further investigated the enrichment of specific transcriptional signatures in immune cell subsets with age, including proliferation and senescence-related pathways. From these analyses, we find no significant pathway differences with age across the 71 immune cell subsets. (***Reviewer only Figure 1***) We have added this finding to the **Results** section.

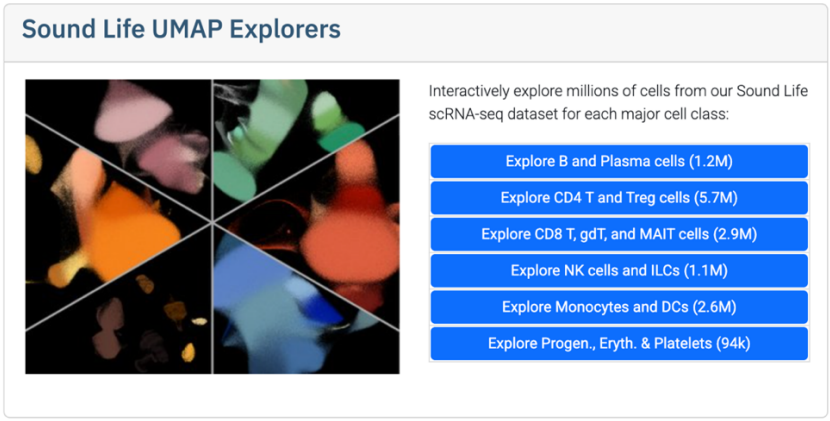


Reviewer only Figure 1 *- showing the results for pathway enrichment by age, across all 71 cell subsets. No significant difference (p<0.05) in proliferation or senescence pathways were observed in any immune cell subset.*

4) It was good to see that vaccination induced antibody production and B cell function overall (but not plasma cells) was reduced during ageing. The extensive datasets that have been generated will be a great resource for further investigations of B cell function before and after vaccination during ageing.

We thank the review for this positive comment on the dataset. To facilitate more detailed data exploration, we have also created new UMAP explorers specific to each

major subset in the Sound Life dataset. (**Reviewer only Figure 2**) The web link to these UMAP explorers has been added into **Methods/Data Visualizations**.



Reviewer only Figure 2: *Sound Life Major Immune Cell Subsets UMAP Explorer. Explore CD4 T, CD8 T, B cell, NK, myeloid and other subsets and their gene expression separately for each major immune cell compartment at higher resolution in the entire Sound Life scRNA-seq data. https://apps.allenimmunology.org/aifi/insights/dynamics-imm-health-age/vis/umap/*

5) In the current clinical climate that is moving to the use of senolytic drugs in the treatment of ageing-related disorders, it would be good to know which leukocyte population acquire senescent characteristics during ageing and whether this affects the outcome of vaccination, especially in the older subjects. It is possible that the close interrogation of their datasets will identify gene signatures associated with cellular senescence in the different populations studied by ScRNAseq to address this point.

We further investigated the enrichment of senescence-related pathways in our immune cell subsets with age. However, we found no significant enrichment in senescence pathways within any immune cell subset with age. (all $p>0.05$) This has been added to the Results section. (see **Reviewer Figure 1** above) This finding is consistent with the age of our older cohort (55-65 yrs) being at the cusp of advanced aging and the lack of evidence for systemic inflammation that is normally associated with increased senescence within our healthy adult cohort.

6) One problem with the interpretation of the results that is present in the current study but also in all previous studies identifying age associated signatures is that it has only been possible to investigate blood leukocytes, for obvious ethical and ethical considerations, that applies to all human work. Is what is being observed reflect immunity globally? For example the data on Tfh/B cell interactions, the blood is not where these interactions take place. It is known that circulating leukocytes generally decrease with age while those in tissues actually increase.

We appreciate the reviewers' comment about broad application of peripheral immune cell studies in humans and agree that more studies into globally diverse populations are needed. In order to help address this, we used two other publically available immune health datasets from cohorts from the East Coast of the US (Terekhova et al. 2023) and

Europe (Yazar et al. 2022), in addition to our follow-up cohort collected on the west coast of the US. (revised **Figure 2F-G** above) As described above, we find increases across age in our RNA Age Metrics in all three cohorts, suggesting this re-programming is a common feature of immune aging. Additionally, a recent study has described elevated Th2 and CD11c+ (effector) B cells in people from rural versus urban environments, potentially linked to higher pathogen exposure in rural communities. (Manurung et al. 2025) This would be consistent with our findings that repetitive, acute stimulation in the context of influenza vaccination may be promoted by Th2-like skewing and altered effector B functionality. A future direction studying the kinetics of immune aging across global populations has been added into the discussion and is an exciting new area of research.

Overall, the current interpretation of the datasets is very superficial given the volume involved, but the value of this work lies in the future when this data becomes available to other researchers. However the inescapable caveat is that as leukocytes age, non-lymphoid cells in every organ also ages. This will lead to altered lymphoid/non-lymphoid cell interactions during immune responses. Ultimately, although extremely difficult, the most valuable insights into understanding age associated changes and strategies for enhancing immunity will come from unravelling these altered interactions.

With the comments collectively provided by the reviewer, we feel we have expanded the novelty of our findings about the aging immune system and its responsiveness, including demonstrating a functional Th2-like skewing in memory T cells with age, and that these findings together with the comprehensive dataset created will help accelerate more human-based immunological insights across the research community. We also agree with the reviewer that future studies into tissue immunity across age will be highly beneficial to understand mechanistic, microenvironmental drivers of these age-related peripheral immune responses. We have added this concept into the **Discussion**. However, developing that body of research is outside the scope of this study.

Referee #2 (Remarks on code availability):

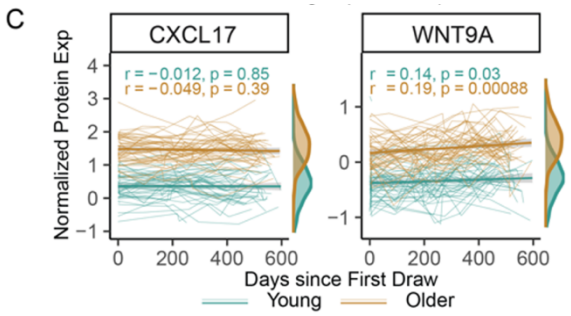
I am not able to comment on the data collation, standardization and statistical analysis as this is beyond my expertise

Referee #3 (Remarks to the Author):

Gong et al. reported a multi-omic profiling study (scRNA-seq, proteomics, flow cytometry) of human blood immune cells, analyzing data from approximately 300 healthy adults and 96 adults followed over two years. Through single-cell RNA sequencing of over 16 million PBMCs, they found that T cells at earlier stages of differentiation accumulate distinct age-related transcriptional changes more than other immune cells, independent of systemic inflammation and chronic perturbation. Additionally, they observed that impaired memory B cell responses to influenza vaccination in older adults are associated with gradual GATA3/Th2 polarization in T cell subsets. The authors have made this extensive data resource available for further data mining and exploration.

1. Given the 60–80 years of human adult life, multiple visits over a longer follow-up period would be ideal for a human longitudinal study. Practically, a minimum of 5 to 10 years would be needed to observe the impact of aging. In this context, a two-year follow-up in relatively younger age groups (>65 years old in the 'old' group) would be more appropriately considered a repeat measurement rather than a longitudinal study. Additionally, the age range of the 'old' group (55–65 years) does not align with the current standard definition of older adults, which is generally >70 years. Therefore, in my opinion, the title may be overstated. A mostly cross-sectional analysis of immune cell aging with over 300 donors is still an impressive amount of work.

Indeed, while most studies examine features of advanced age (>65 yrs of age), here we focus on individuals at the cusp of aging (55-65 years old adults) compared with younger adults (25-35 yrs), to gain insight into the transition period between functional and dysfunctional immunity. We have noted this important distinction throughout the revised manuscript. Although we agree with the reviewer that certain aspects of immunity require longer scales of time to monitor age-related transitions within an individual and have remove “Longitudinal” from our title, we find non-zero slopes over the course of two years that indicate subtle changes in serum proteome, gene expression and cell frequencies in this shorter time period. An example of this is shown in Revised **Figure 1C** - where we find WNT9A levels increase over time in both young and older adults, but CXCL17 had no change. We have added Spearman correlations into all “over time” and “across age” plots to highlight this better - and would argue that even in this short period of time, there are on-going changes in the immune system that occur and can be detected through shorter-term, longitudinal tracking.

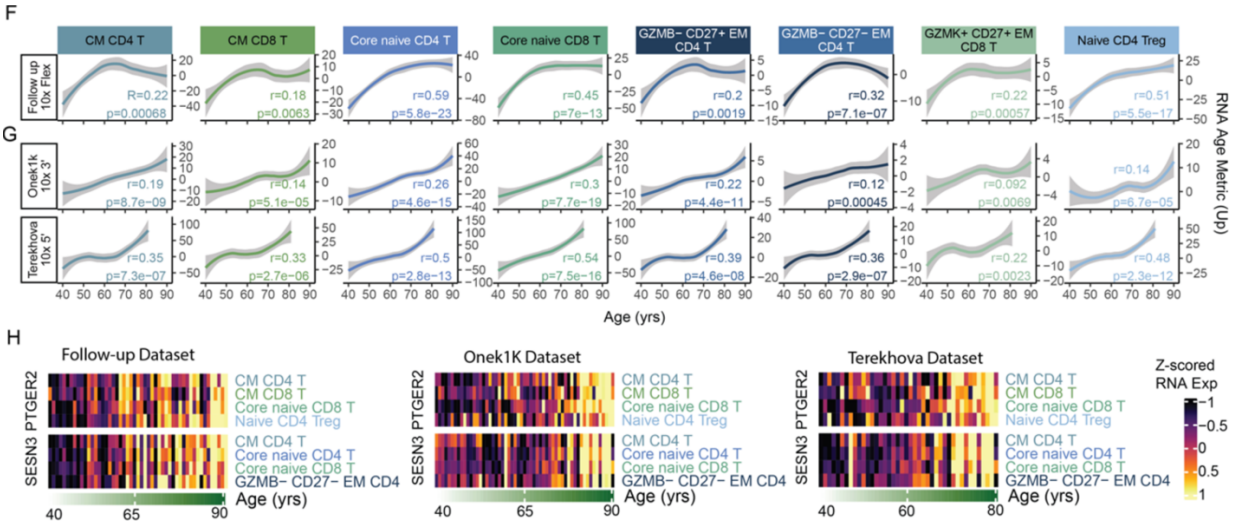


***Revised Figure 1. C**. CXCL17 and WNT9A normalized protein expression (NPX) over time in young (teal) and older (bronze) adult plasma. Each donors’ samples are connected by a line.*

2. The findings on the dynamics of “up- and down-regulated genes within immune cells in adults over 65 years of age, which may differ from those occurring between ages 25 to 65,” are intriguing. It would be valuable if the authors could perform the same analysis on published data, such as Terekhova et al. (Immunity, 2023), to confirm these findings.

To better assess the dynamics of immune cell signatures across age, we performed the same analysis on two additional external datasets. *(Terekhova et al. 2023; Yazar et al. 2022)* From these analyses, we find that trajectories are variable by cohort. While there was no consistent trajectory (or plateau) across these three cohorts, we now demonstrate that our age-related transcriptional signatures hold across T cell subsets in more than 1300 people. (Revised **Figure 2F-G**) This includes the common upregulation of select genes *SESN3* and *PTGER2* with age in multiple T cell subsets. (Figure 2H)

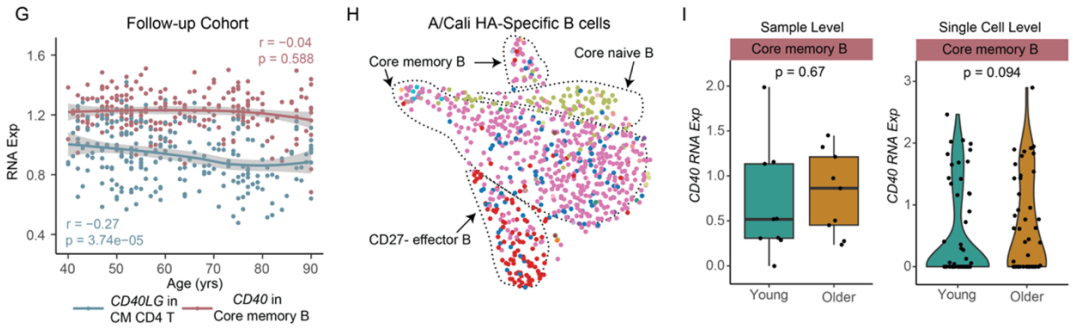
The variance in trajectories across age may be biology or technical, as each cohort was from a different location and utilized different sequencing platforms for scRNA-seq data generation, also highlighted in the Revised **Figure 2F-G**. A large, more cohesive study on immunity across age would be needed to clarify this – and has been added to the **Discussion.**



Revised Figure 2. F-G. *The average RNA Age Metric of upregulated genes for the top age-impacted immune cell subset, shown across age in* ***F.*** *our follow-up cohorts and* ***G.*** *two external immune aging datasets. (Terekhova et al. 2023; Yazar et al. 2022) Regression line shown with 95% confidence intervals in gray.* ***H.*** *Heatmap of mean RNA expression by age for select up-regulated genes identified from initial DEG analysis in the three independent immune aging datasets.*

3. The authors concluded that the reduced transcriptional propensity to support memory B cells with age is based on a general decrease in CD40LG expression in central memory (CM) CD4 T cells, rather than a decrease in CD40 expression in core memory B cells. However, the highly heterogenous nature of these subsets, this finding does not necessarily apply to flu-specific CM CD4 T cells and flu-specific core memory B cells, which represent only a fraction of the total CM CD4 T cells and core memory B cells. If the authors have data on antigen-specific CM CD4 T cells or core memory B cells, it will substantially strengthen this conclusion.

Utilizing an externally available dataset of influenza (A/California) HA-specific B cells (*GSE167823*), we examined CD40 RNA expression on flu-specific core memory B cells between young and older adults and found no significant difference in expression. These data are now shown in **Extended Figure 8H, 8I**. While the HA antigen used here is not B/Phuket-specific, A/California is another highly boosted flu antigen that was present in flu vaccines from 2010-2017, thus is likely reflective of similarly repetitive B/Phuket responses.



Extended Figure 8. ***G.*** *RNA expression of CD40LG in CM CD4 T cells and CD40 in core memory B cells across age in our follow-up cohort (n=234). Regression line shown with 95% confidence intervals in gray with Spearman correlations.* ***H.*** *UMAP of HA-specific B cells from young and older adults (GEO GSE167823), labelled with our Human Immune Health Atlas B cell subsets.* ***I.*** *Comparison of CD40 RNA expression on HA-specific core memory B cells from young (n=9) and older (n=9) adults.*

4. Figure 1: It is essential to include a filter based on gene expression percentage (>60–70%) within the subset, in addition to log2fc and p.adj, for identifying DEGs. The expression percentage-filtered DEGs and related analyses should be presented consistently throughout the results.

The same cut-offs for DEGs were utilized throughout the analyses that included a minimal 10% gene expression cut off on single cell levels by each cell type, p.adj<0.05 and log2fc>0.1. These specific details have now been added to the figure legend for ease of reference.

5. Extended Figure 4: The age-associated transcriptional changes in human T cell subsets, as well as shared transcriptional changes across different T cell subsets, were reported by ***Lu et al. 2022 (DOI: 10.1038/s41467-022-32869-x)*** *and should be cited.*

We apologize for our oversight of this paper nicely characterizing CD8 T cells with age and have added with reference into the **Introduction**.

6. In several places within the Results section, the authors mention previous studies without providing citations. Please add references wherever prior studies are referenced.

We have now added references to areas where we indicate prior study results throughout the manuscript.

7. Based on Figure 6, the authors concluded that there was no age-related alteration in the vaccine-specific expansion of plasma cells. However, since the oldest donors in the 'old' group were 65 years, it remains unclear if such alterations might exist in individuals older than 65. The authors should discuss this limitation and suggest directions for

future studies.

While most studies examine features of advanced age (>65 yrs of age), here we focus on individuals at the cusp of advanced aging (55-65 years old adults) compared with younger adults (25-35 yrs), to gain insight into the transition period between functional and dysfunctional immunity. We have noted this important distinction throughout the revised manuscript, as well as discussed the need for further studies into response features of advanced aging and chronic inflammatory conditions.

Referee #4 (Remarks to the Author):

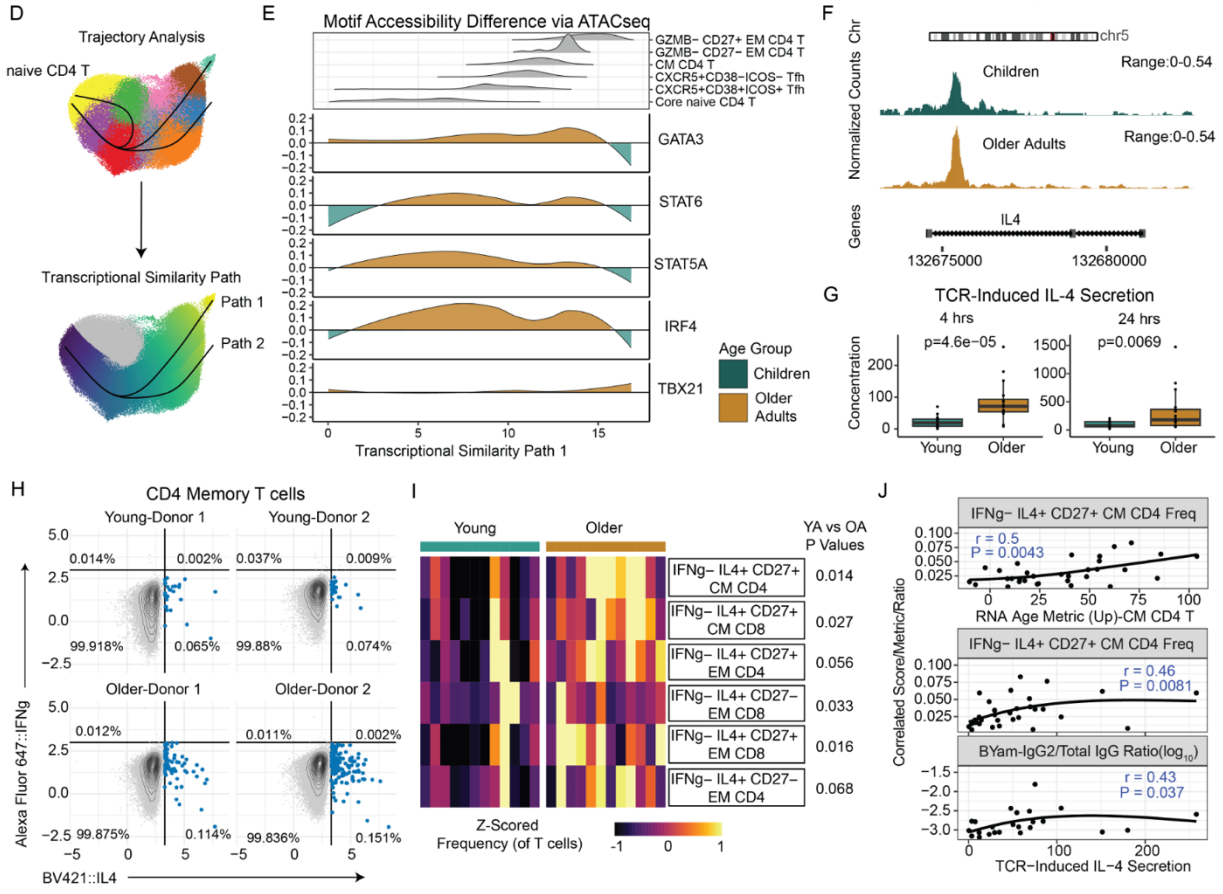
The manuscript provides a detailed analysis of age-related immune dynamics using longitudinal and cross-sectional cohorts, focusing on T and B cell transcriptional and compositional changes. The major findings include identifying age-associated transcriptional changes in naïve and central memory T cells, highlighting a GATA3/Th2-polarized state; impaired memory B cell responses in older adults, marked by reduced class switching and functional antibody production after influenza vaccination; a distinct immune landscape shaped by CMV infection, largely independent of aging; and the development of the Human Immune Health Atlas, a valuable resource for immune research.

While the study presents an impressive dataset that will undoubtedly serve as a significant resource for the scientific community, several findings lack novelty. Nonetheless, the manuscript is of high quality and deserves rapid publication. I would, however, suggest aligning the findings more closely with existing literature and emphasizing their novel insights.

1) The age-related GATA3/Th2 polarization was previously reported by Terekhova et al. (2023). This manuscript acknowledges their findings in the discussion: "Our findings on the age-related increase in a Th2-like state in central memory CD4 T cells align with recent literature finding elevated CCR4+ memory T cells in advanced age (Terekhova et al. 2023), however, we find this GATA3/Th2 polarization is not specific to one T cell type but is found in many T cell subsets, suggesting a mechanism where age-related elevation in GATA3 activity impedes T-bet activation in older adults (Dai et al. 2024; Naradikian et al. 2016)." Terekhova et al. linked GATA3/Th2 polarization to external cytokine signaling (e.g., IL-4/IL-13) and used trajectory analysis to map T cell progression. Here, the authors note no significant increase in circulating Th2-polarizing cytokines (IL-4, IL-5, IL-13). It would be valuable to investigate upstream drivers of GATA3 activity and distinguish between intrinsic and extrinsic mechanisms. Trajectory analysis could model T cell developmental transitions leading to GATA3-mediated Th2 polarization. Additional experiments, such as stimulating naïve T cells from young donors with IL-4 or IL-13 or co-culturing naïve CD4+ T cells with autologous or heterologous APCs, could help disentangle intrinsic T cell changes from APC-driven signals. GATA3 mRNA and protein levels could then be assessed using qPCR and flow cytometry, while downstream cytokines (IL-4, IL-13) could be analyzed with ELISA.

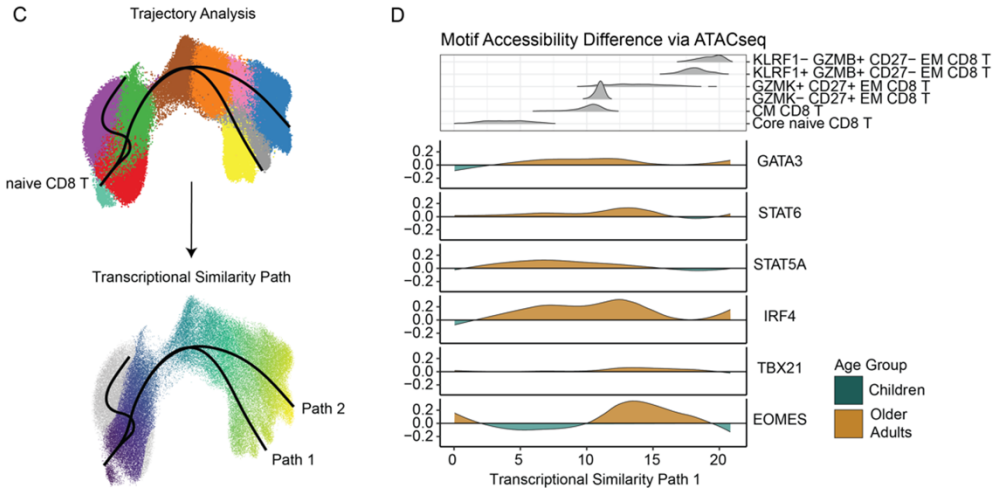
To better demonstrate our GATA3 findings, we utilized trajectory analysis to compare GATA3 and other TH2-related TF activities (based on scATAC-seq Chromvar analyses) across all CD4 and CD8 T cell subsets categorizing them based on transcriptional similarity. (Revised **Figure 5D-E**; **Extended Figure 9C-D**) From these analyses, we see

that the age-related GATA3/TH2-like polarization occurs broadly across the naive and memory T cell compartments but less so in the more terminal GZMB+ subsets. To link these transcriptional and epigenetic indicators of a Th2-like state with functional responses, we then assessed the Th1 (IFNg) and Th2 (IL-4) cytokine profiles of young and older adult T cells. Upon T cell receptor stimulation, we found significantly higher IL-4 secretion from older adult T cells via a multi-plex MSD read-out. (**Figure 5G**) Utilizing intracellular spectral flow cytometry, we also found that several memory CD4 and CD8 T cell subsets, including CM CD4 T cells, displayed higher spontaneous IL-4 production in older adults, aligning with our trajectory analyses of Th2-like state predisposition in memory T cells. (Revised **Figure 5 H-I**) Notably, the Th2-driving TF GATA3 activity/expression can be modulated by features previously linked with age including DNA damage, TCR signaling strength and IL-2 responses (Callender et al. 2021; Al-Aghbar et al. 2024; Yamane, Zhu, and Paul 2005; H. Zhang et al. 2022) – suggesting this age-related dysregulation could likely initiated in an IL-4 independent manner. We further discuss the implications of these findings in the **Discussion** section.



Revised Figure 5. D. *RNA-based trajectory analysis of CD4 T cells from T cell TEA-seq dataset.* ***E.*** *Th1 (TBX21)- and Th2 (GATA3, STAT6, STAT5A, IRF4)-related transcription factor (TF) motif accessibility differences based on Chromvar analysis of scATAC-seq data from TEA-seq across CD4 T cells from children (n=8) and older adults (n=8). Teal indicates lower accessibility in older adults, whereas bronze indicated higher accessibility in older adults compared with children.* ***F****. Chromatin accessibility tracks of the IL4 gene region in CM CD4 T*

cell subsets, showing normalized read coverage. ***G.*** *Concentration of IL-4 (pg/mL) secreted by aCD3/aCD28 T cell receptor (TCR) stimulation of T cells isolated from young (n=16) and older (n=16) adults at 4 and 24hrs.* ***H.*** *Representative IL-4 and IFNg expression by memory CD4 T cells determined by intracellular flow cytometry and* ***I.*** *heatmap of IFNG$^-$IL-4$^+$ memory T cell subset frequencies from young (n=12) and older (n=12) adults rested for 4hrs without TCR stimulation. P-values were calculated using Wilcoxon rank sum test.* ***J.*** *Spearman correlations of IFNG$^-$IL-4$^+$ CM CD4 T cells with RNA Age Metric in CM CD4 T cells and IL-4 secretion post-TCR stimulation with frequencies of IFNG$^-$IL-4$^+$ CM CD4 T cells and normalized B/Phuket-specific IgG2 levels at day 7 post-vaccination.*



Extended Figure 9. C. *RNA-based trajectory analysis of CD8 T cells from T cell TEA-seq dataset.* ***D.*** *Th1 (TBX21)- and Th2 (GATA3, STAT6, STAT5A, IRF4)-related transcription factor (TF) motif accessibility differences based on Chromvar analysis of scATAC-seq data from TEA-seq across CD8 T cells from children (n=8) and older adults (n=8). Teal indicates lower accessibility in older adults, whereas bronze indicated higher accessibility in older adults compared with children.*

2) The manuscript identifies impaired memory B cell responses in older adults, marked by reduced class switching and antibody production after influenza vaccination. While supported by the data, these findings are not particularly novel, as similar results have been reported in several studies on age-related vaccine responses. Relevant references include:

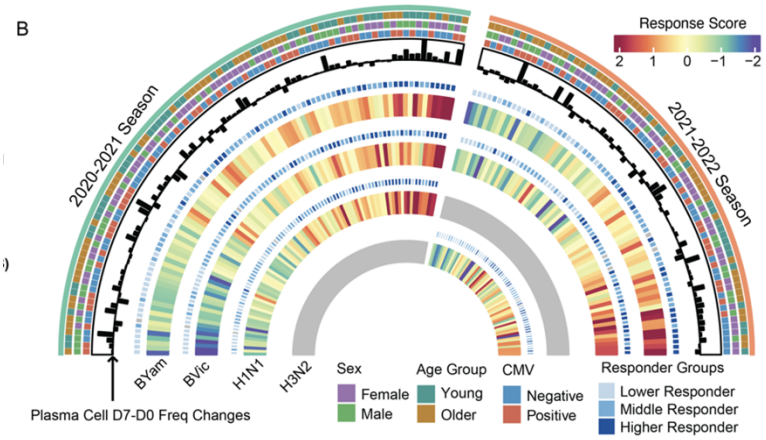
• Nakaya HI et al. Systems Analysis of Immunity to Influenza Vaccination across Multiple Years and in Diverse Populations Reveals Shared Molecular Signatures. Immunity. 2015 doi: 10.1016/j.immuni.2015.11.012

• Furman D et al. Apoptosis and other immune biomarkers predict influenza vaccine responsiveness. Mol Syst Biol. 2013 Apr 16;9:659. doi: 10.1038/msb.2013.15.

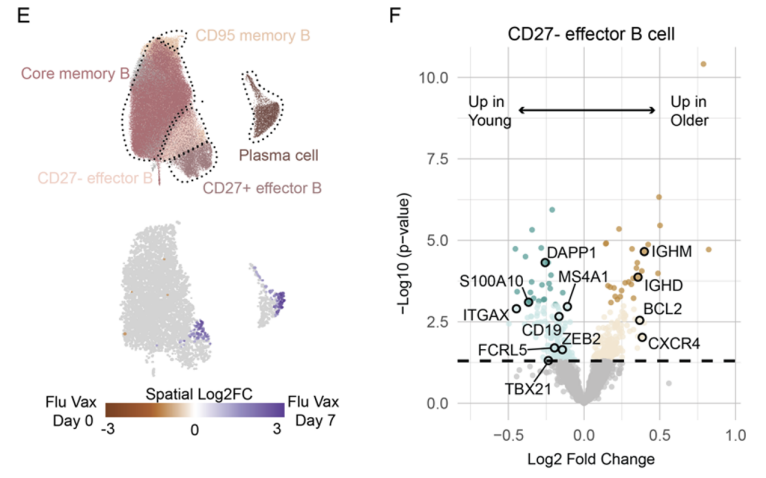
• Frasca D et al. Unique biomarkers for B-cell function predict the serum response to pandemic H1N1 influenza vaccine. Int Immunol. 2012 Mar;24(3):175-82. doi: 10.1093/intimm/dxr123.

• Tsang JS et al. Global analyses of human immune variation reveal baseline predictors of postvaccination responses. Cell. 2014 Apr 10;157(2):499-513. doi: 10.1016/j.cell.2014.03.031.

While we appreciate that a number of studies have looked at B cell responses to vaccination with age, we would like to highlight a few key features of our original and revised manuscript that are unique from these studies and provide deeper insight into immune dysregulation in the context of age. Notably, we use multiple distinct methods to interrogate influenza responses, that include 1) the development of custom multi-plex influenza-specific HAI assays, total IgG expression and IgG isotype levels in collaboration with MSD that provide information on multiple influenza strain responses simultaneously from a single assay (e.g., revised **Figure 4B**); and 2) utilization of scRNA-seq to look at cellular frequencies and transcriptional response states within multiple different immune cell subsets simultaneously, including but not limited to memory B cell subsets pre- and post-vaccination. (e.g., revised **Figure 4E-F**) The combination of these assays provided us the ability to correlate functional B cell responses with transcriptional re-programming within T cell subsets that link to reduced responsiveness in older adults, and we hope that they will provide other novel insights into age-specific vaccine design in the future.



Revised Figure 4. B. *Variation in HAI responses to seasonal influenza strains, comparing day 0 and day 7 post-vaccination in the 2020-2021 and 2021-2022 flu season.*



Revised Figure 4. E. *UMAPs of memory B cell subset and their differential frequencies pre- and day 7 post-vaccination from scRNA-seq data. Bottom plot shows Log_2 fold change of clinical metadata feature of 2020-2021 flu vaccination of day vs day 7 compared using Milo differential abundance testing.* ***F.*** *Volcano plot of DEGs for CD27- Effector B cells between young (n=26) and older (n=42) adults at day 7 post-vaccination. Highlighted genes are those previously shown to define a flu-specific effector B cell subset in a vaccinated adult cohort. (Nellore et al. 2023) Dark teal and bronze dots signify significantly different genes, and light-colored dots indicate nominal significance, while gray dots indicate no significance between age cohorts.*

3) Experiments with aged Cd4cre Gata3f/f KO mice could provide mechanistic insights into the authors' proposed findings regarding antibody production.

While we appreciate the utilization of mouse models to gain more mechanistic insights in many contexts, this work beyond of the scope of this current study. We have focused on understanding how the transcription and epigenetic changes we observe lead to functional differences in T cells with age, which revealed that multiple memory CD4 and CD8 T cell subsets have a higher spontaneous IL-4 production in older adults as well as an association between memory T cell states and influenza-specific IgG class-switching (Revised **Figure 5** shown above). These new data provide a more robust link between Th2-like skewing and age-related vaccine immunity in healthy human adults.

4. The manuscript highlights the decrease of ZBTB38, a marker gene of adaptive NK cells, in CMV+ older adults. The authors could validate this finding using datasets from related studies, such as:
• Aino-Elina Häkkinenet al. Single-Cell Transcriptomics Reveals Adaptive NK Cell and Antigen-Specific T Cell Responses Shaped by CMV Viremia Post-Hematopoietic Stem Cell Transplant. Blood 2023; 142 (Supplement 1): 4786.
doi: https://doi.org/10.1182/blood-2023-179714
• Kar R et al. Single-cell transcriptomic and T cell antigen receptor analysis of human cytomegalovirus (hCMV)-specific memory T cells reveals effectors and pre-effectors of CD8+- and CD4+-cytotoxic T cells. Immunology. 2024 Jul;172(3):420-439. doi: 10.1111/imm.13783.

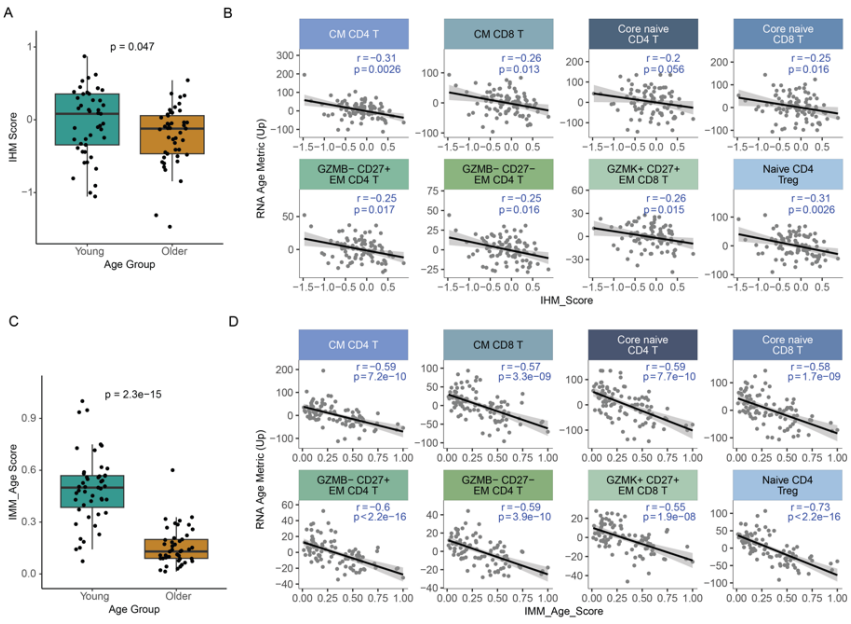
Unfortunately, neither of these datasets has the necessary cohort information to be able to directly assess the question of *ZBTB38* expression in adaptive NK cells within CMV+ adults across age. Moreover, other scRNA-seq immune aging dataset we investigated did not have CMV infection status available on their donors (Terekhova, OneK1), thus we are unable to directly validate these findings. However, it is also important to note that in our more stringent statistical analyses suggested by the reviewers, while we find a positive correlation of *ZBTB38* expression in Adaptive NKs in CMV+ people, it was not statistically different within our follow-up cohort. ($R=0.18$, $p=0.28$) As we are unable to further confirm this finding in other external datasets, we have softened our conclusions about the impact of age on the transcriptome of adaptive NK cells.

5. The manuscript could benefit from refining the classification of participants into age groups by incorporating biological age metrics, which better reflect physiological differences than the current arbitrary cutoff of 55 years (in the first cohort). Leveraging established age-related transcriptomic and their own proteomic markers, a composite biological age score could be developed and applied to the cohorts. This approach would allow grouping participants based on biological age, potentially revealing variability in immune responses and vaccine efficacy.

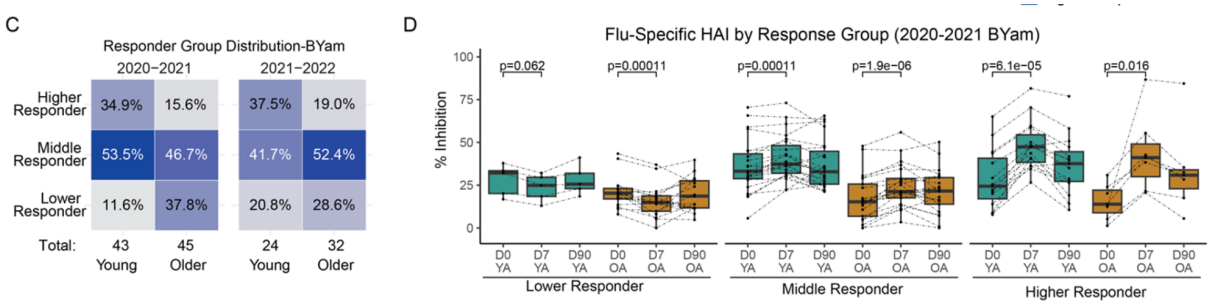
Ultimately, our goal was to better understand the maintenance of transcriptional states within immune cells across age. We thus established a common signature indicative of

the overall transcriptional state of a cell - that could be used to track cellular states more broadly over time and with age (instead of on a gene-by-gene level) – and provide more mechanistic insight into immune dysregulation with age. However, developing metrics of immune health is important when thinking about long-term clinical screening. Thus, we compared our RNA Age Metrics to two Immune Health Signatures, 1) IMM-AGE that is based on cellular frequencies and 2) IHM that is based on whole blood bulk RNA-seq transcriptional signatures. We found that our age-related transcriptional profile metric correlated with both IMM-AGE and IHM. (**Extended Figure 4**) The weaker association with IHM may be due to the fact that whole blood contains other cell types, such as neutrophils, as well as being a mixed signature of both composition and cellular state (i.e. changes in the RNA expression profiles of cells) alterations that cannot be delineated from one another.

We also took a new approach to assess responses to influenza vaccination using linear modeling to account for baseline HAI differences, sex and CMV status - then assessing differences in responder frequencies by age. From these analyses, we found that older adults have fewer high responder compared with young adults, specifically for B/Phuket responses across both vaccination series. (Revised Figure 4,



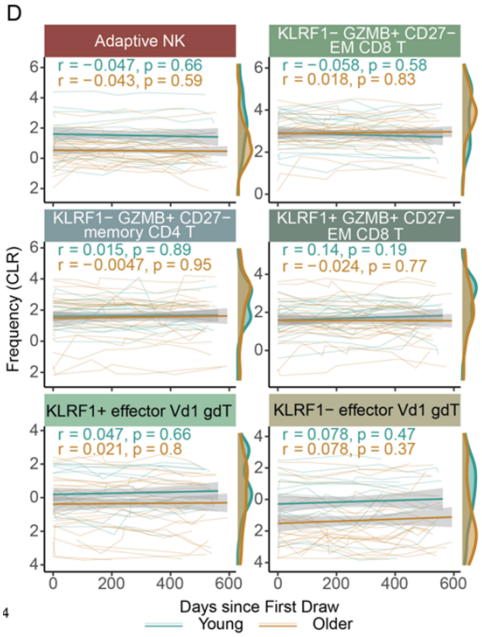
Extended Figure 4. Comparison of RNA Age Metric to IMM-Age and IHM metrics. A. *Comparison of IHM score determined from whole blood bulk RNA-seq for young and older adults from Flu Vax Yr 1 Day 0.* ***B.*** *Spearman correlation between IHM and RNA Age metric (up) in age-susceptible T cell subsets* ***C.*** *Comparison of IMM-AGE score determined from cell frequencies from scRNA-seq data for young and older adults.* ***D.*** *Spearman correlation between IMM-AGE and RNA Age metric (up) in age-susceptible T cell subsets.*



Revised Figure 4. C. *Distribution of response groups across age groups of Byam (B/Phuket) for two separate flu years. 'Higher' represents the top 25%, 'Middle' the middle 50%, and 'Lower' the bottom 25% based on response score cutoffs.* ***D.*** *Mean percent inhibition of flu hemagglutinin (HA) antigen as determined by the HAI assay (**C, E**) for both young and older adults at days 0 7 and 90, separated by responder group. P-values were calculated using Wilcoxon's signed-rank test (paired).*

6. The statistical analysis in the manuscript could benefit from greater clarity and rigor. For instance, the "delta changes" in Figure 1 are not well defined, making it difficult to interpret their biological significance beyond statistical differences. Additionally, some statements, such as the "continuous increase" in the age metric (Figure 2F) and the "trending decrease" in KLRC2 expression, lack statistical evidence or clear descriptions of the methods used to assess these trends. Phrases like "trending decrease" are imprecise and would be strengthened by specific statistical tests and p-values.

We apologize for the oversight of inclusion of statistical correlations for our continuous dataset analyses. We have now included the rho and p-values for Spearman correlations for all relevant figures ("across age" and "over time" plots). (example of "across age" see Revised **Figure 2F-G** above, example of "over time" below)

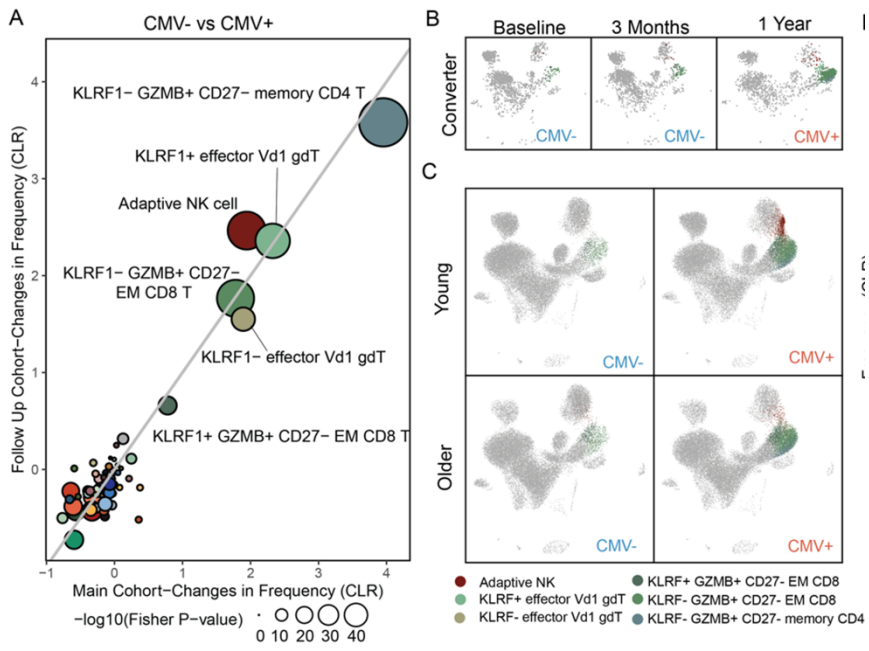


Revised Figure 3. C. *Select subset frequencies in PBMCs shown over time in young (teal) and older (bronze) adults. All regression lines shown with 95% confidence intervals shown in gray with Spearman correlations.*

7. The findings regarding the impact of CMV infection on immune subsets are intriguing, but the robustness of the statistical significance could be further strengthened with an empirical test. To ensure that the observed differences are not simply due to random variability, the authors could perform a permutation analysis. By randomly assigning individuals to CMV+ and CMV- groups multiple times and repeating the differential analysis, they could estimate the likelihood of identifying the same or similar subsets purely by chance. This approach would provide a robust empirical baseline for assessing the true significance of the results. Additionally, combining the confirmation from (EXTENDED) Figure 5A with the findings in Figure 3A could help reinforce the

conclusions by providing a more comprehensive validation of the differential effects observed.

We performed a permutation analysis (with 100,000 iterations per cohort) of our CMV comparisons for cell frequencies. (**Supplemental Table 3**) We then compared our findings from our main cohort with that of our follow-up to robustly identify CMV-responding subsets. (Revised **Figure 3A**) Similar to our previous analyses, we find 6 subsets statically increased in CMV-infected people compared with CMV-negative people. We further identified a single sero-converter in our cohort, that converted from CMV-negative to CMV-positive over the course of this study. To highlight the CMV-specific nature of these 6 populations, we have further included UMAPs of multiple timepoints from this person to demonstrate subset expansion post-infection. (Revised **Figure 3B**)



*Revised **Figure 3. A.** Bubble plot comparison of the change in frequency (using centered log-ratio (CLR) transformation) between CMV$^+$ (n=44) and CMV$^-$ (n=52) adults at 'Flu Vax year 1 day 0' in our main cohort and CMV$^+$ (n=136) and CMV$^-$ (n=98) in our follow-up cohort. Bubble size shows -log10(Fisher P-value) for our main cohort using Wilcoxon rank sum test. **B-C.** UMAPs of T and NK subsets in **B.** one young adult CMV sero-converter over time and **C.** in CMV-infected and uninfected young and older adults, highlighting immune cell subsets that consistently increase in frequencies in CMV infected individuals.*

Response to Reviewers

We again thank the reviewers for their thoughtful comments. With this re-revision, we have addressed all reviewers' comments and feel that we collectively enhanced the novelty and impact of our study by:

1) *Demonstrating the robustness of age-related transcriptional re-programming* in peripheral T cell subsets in more than 1400 people from over 6 different cohorts, translated this finding into T cell subsets within lymph nodes (LNs) across age and revealed accelerated transcriptional aging specifically in central memory CD4 T cells from otherwise healthy people at risk for the development of rheumatoid arthritis - indicating that our RNA Age Metric is reflective of immunological aging, and not just chronological age.

2) *Revealed previously undescribed flu-specific IgG isotype skewing with age*, demonstrating IgG2/3 isotype skewing in flu-specific core memory B cells consistent with that of circulating flu-specific antibody skewing with age.

Thus, from this single, unified study, we have revealed the following new insights::

1) Transcriptional immune aging is a non-linear process,
2) Transcriptional reprogramming leads to functional Th2 bias in memory T cell prior to advanced age,
3) Inflammation is not an underlying driver of healthy immune cell reprogramming,
4) CMV infection significantly alters the immune system but independently from age,
5) Dysregulation of B cell responses to vaccination occurs earlier in aging for more highly-boosted flu antigens and is linked to functional memory T cell reprogramming.

Collectively, we believe that our revised study reveals unique features of immune dysregulation that occur *prior to advanced age* - providing novel targets for age-related immune modulation and potentially prevention of disease onset in more advanced ages.

Our *accompanying suite of Data Apps and visualization tools* will also enable researchers easier, open access to these extensive datasets to promote further immunological discoveries and applications across the broader research community and ultimately help accelerate our fundamental understanding of human immune responses to vaccination, infection and age-specific disease susceptibility.

Referee #1:

Referee #1 (Remarks to the Author):

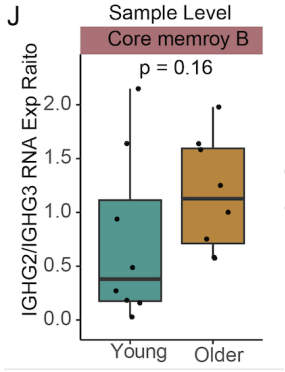
I would like to commend the authors for the extensive and thoughtful revisions they have undertaken in response to the previous review. The additional analyses and data substantially strengthen the manuscript, providing important mechanistic insights and novel findings that enhance the scientific value of the work. The revised version successfully addresses the majority of my prior concerns.

I have only a few remaining points for the authors to consider, which I believe would further clarify and enrich the manuscript:

1. Extended Data Figure 8H and I: The re-analysis of the publicly available dataset to examine CD40 expression in vaccine-specific B cells from young and older adults is appreciated and well executed. However, one of the key conclusions of the manuscript is a skewing toward IgG2

subclass usage in highly boosted responses in older individuals. Given the availability of antigen-specific B cell transcriptomic data in this dataset, I encourage the authors to take this analysis one step further by assessing the expression ratio of IGHG2 to IGHG3 in these cells. This would provide valuable support (or nuance) to the claim of subclass skewing and directly test whether this phenomenon can be detected in the independent dataset.

We agree that this analysis could provide further support for isotype skewing with age. From extended transcriptomic-based analysis, we found elevated IgG2/IgG3 ratios in flu-specific core memory B cells from Cali/A in older adults, albeit not statically significant. (**Extended Figure 8j**) The elevated IgG2/3 ratio in flu-specific core memory B cells aligns with the higher ratios of secreted flu-specific IgG2/3 in circulation of older adults - and suggests that this isotype skewing is a common, age-related feature across highly boosted vaccine antigens.



Revised Extended Figure 8j. Ratio of *IGHG2* and *IGHG3* RNA expression in influenza-specific core memory B cells in young and older adults.

2. Th2 skewing upon repeated exposure: The observation that the reduced antibody responses in older adults are most pronounced for the highly boosted B/Yamagata strain is intriguing. It would benefit the reader if the authors could expand briefly on their interpretation of this result. Specifically, what are the potential immunological mechanisms or contextual cues that might drive a Th2-biased response and the associated antibody response changes following repeated exposure to the same antigen? Speculation on how this might influence the quality and durability of protection in older adults would also be welcome. The authors should also explain how a Th2-skewing affects only the antibody response to one strain. Given that T cells are often cross-reactive and respond to additional antigens within the vaccine, besides HA and NA, one would expect the Th2-related impact to be spread across all strains in the influenza vaccine.

This is an interesting discussion point. GATA3 expression and Th2 skewing are closely associated with reduced TCR signaling strength, including in patients with inborn errors of immunity involving this pathway (Al Aghbar, Biorxiv, 2024; Lyons & Milner, JEM, 2018). Moreover, intrinsic Th2-skewing can also be overcome by strong TCR engagement - driving a more typical Th1 response. The dysregulation in highly repetitive Phuket antigen in older adults suggest that as memory T cells undergo repetitive rounds of activation and quiescence, there may be an overall reduction in intrinsic TCR signal strength in a subset of longer-lived memory T cells that drives a bias towards Th2 responses upon activation. Future exploration into naive and memory T cell activation and functional responses to different flu antigens across age could provide useful insight into these mechanisms. We have added these points to the **Discussion**.

3. Recent supporting literature: A recent study employing human tonsil organoids (PMID: 39986275) highlights the role of Th1 responses in supporting inactivated influenza vaccine (IIV)-induced immunity. This study complements the authors' findings and could usefully be integrated into the discussion to provide additional context and support for the importance of T cell polarization in effective vaccine responses.

Yes, we agree that this study nicely complements our findings and we have added this concept to our **Discussion**.

In summary, the manuscript is much improved and is nearing readiness for publication. The above points are intended to strengthen the clarity and impact of the findings and are not intended as major revisions.

Referee #3 (Remarks to the Author):

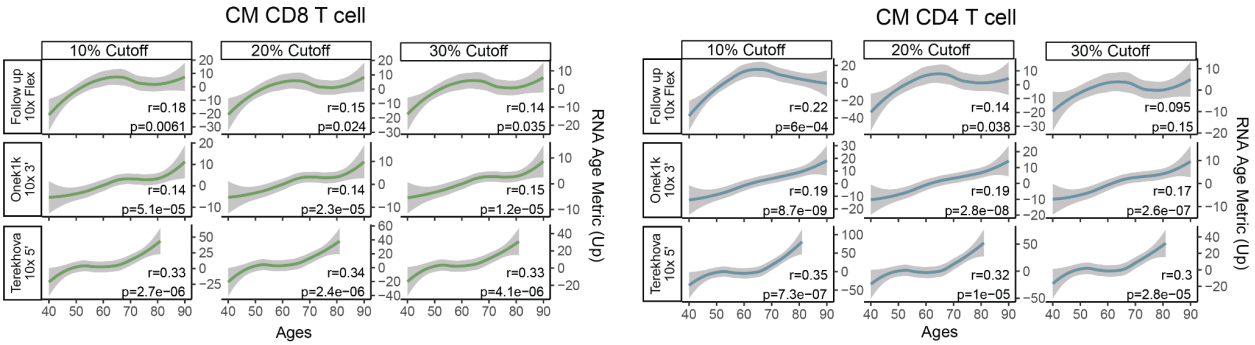
This revision has addressed most of my questions. However, the current differential expression analysis uses a minimal 10% gene expression cutoff, which is quite low. It is difficult to interpret a change observed in only 10% of cells as representative of the entire population. If the cutoff were increased to 50% for DEGs that are increased expression in a cell population, how many of the DEGs would remain valid (e.g. Figure 1F-G and other similar findings)? Would applying a more stringent cutoff improve the consistency of DEG identification across datasets?

During the development of our data analysis approaches, we reviewed the existing literature for guidance on best practices. This included this citation and associated tutorial-based guidance:

- Heumos, L., Schaar, A.C., Lance, C. et al. Best practices for single-cell analysis across modalities. Nat Rev Genet 24, 550–572 (2023).
- "Differential Gene Expression Analysis — Single-Cell Best Practices." Online Tutorial. Accessed 2025. https://www.sc-best-practices.org/conditions/differential_gene_expression.html

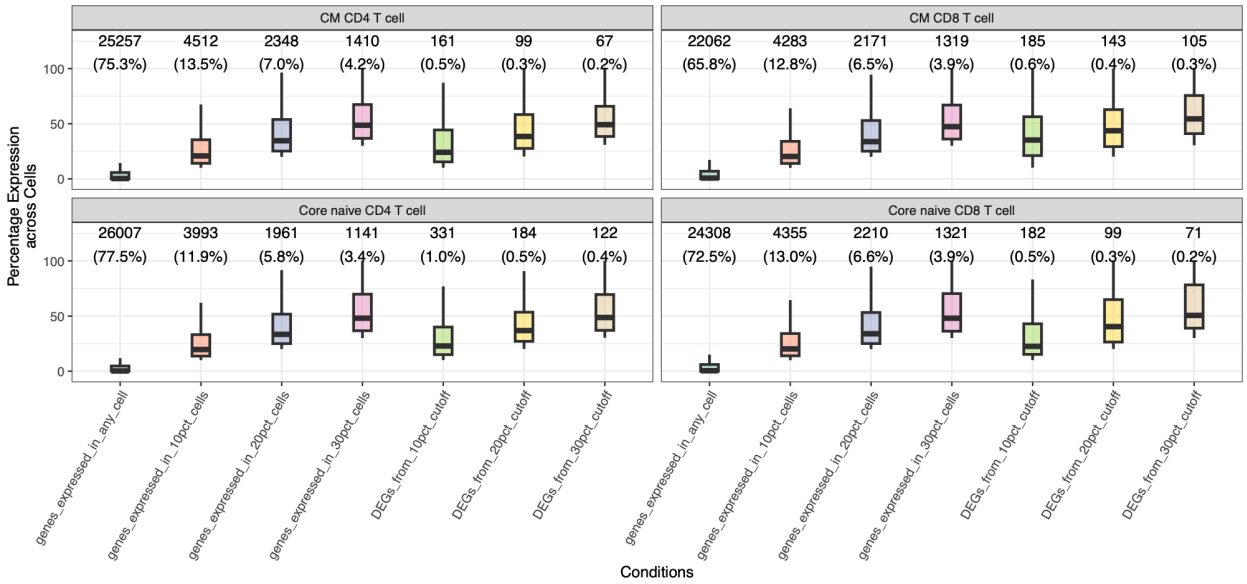
Based on this literature review, a 5-10% gene expression percentage cutoff is commonly used in scRNA-seq studies to identify differentially expressed genes (DEGs)[1,2,3], as it addresses data sparsity, accounts for cellular heterogeneity, and captures lowly-expressed but important functional genes. The 10x Genomics 3' scRNA-seq method (v3.1 chemistry) captures approximately 30-32% of mRNA transcripts per cell[4,5], making data sparsity more pronounced in rare cell populations. In line with other studies, we chose the 10% cutoff to balance discovery of cell-type specific features while excluding random variation.

To further understand and explore the influence of varying cutoffs on identified features, we have included a 20% and 30% cutoff in our DEG analyses of T cell subset age metrics.



Reviewer only Figure 1. RNA Age Metric at Varying % Cut-offs. The RNA Age Metric trajectories calculated using a 10%, 20% and 30% cutoff-based DEGs (with our 3' scRNA-seq data), shown here, *does not alter* the consistency of trajectory r values across the other human aging datasets.

The impact on DEG counts are as follows:



Reviewer only Figure 2. Gene Expression Analysis at Varying % Cut-offs. The percentage of genes expressed across T cell subsets within our 3' scRNA-seq data under different filtering conditions in reference to the total mappable genes in the assay (n=33538). This analysis includes genes expressed by at least a single cell, genes expressed in at least 10%, 20%, or 30% of cells within a subset, and then DEGs between the younger and older age groups based on a 10%, 20%, or 30% cutoff. The number over each box indicates the total number of genes resulting from each filtering condition and the percentage over each box indicates the proportion of genes from each condition relative to total mappable genes in the assay (*i.e*. 'genes from condition' as a percentage of 'all genes').

The 10% cutoff filtering results in approximately 12-13% of mappable genes expressed within the CD4 and CD8 Central Memory (CM) T cell subsets. Additionally, the respective differences in aging-related DEGs represented from the 30% versus 10% cutoff condition, are 94 (0.3%), 80 (0.3%), 209 (0.6%) and 111 (0.3%) when applied to CM CD4, CM CD8, Core Naive CD4 and Core Naive CD8 T cells. These results suggest that the 10% cutoff reduces noise, inconsistently-expressed genes and technical artifacts in the DEG analysis, while balancing potential trade-offs between sensitivity and specificity.

For improved clarity, we have added further details on the cutoff selection for DEG analysis and impacts on our findings to **Methods**.

References:

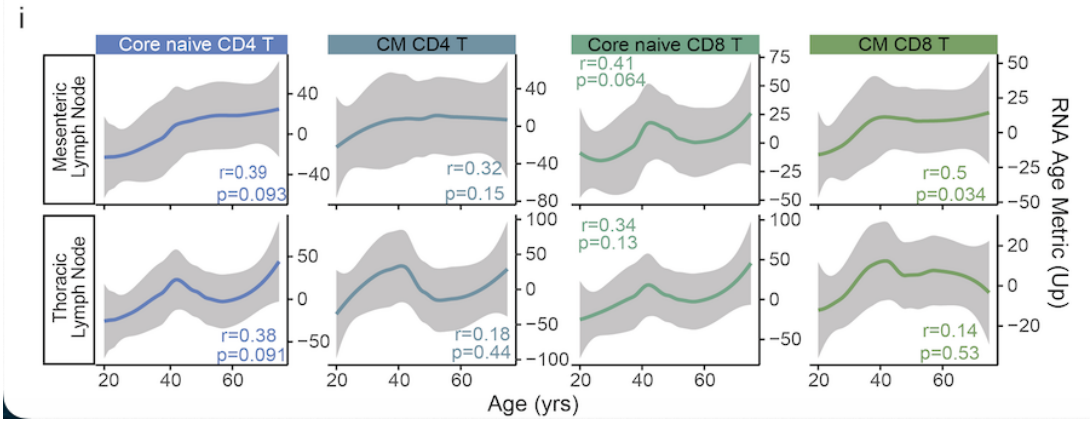
1. Heumos, L., Schaar, A.C., Lance, C. et al. Best practices for single-cell analysis across modalities. Nat Rev Genet 24, 550–572 (2023). https://doi.org/10.1038/s41576-023-00586-w
2. Ravichandran, S., Erra-Diaz, F., Karakaslar, O.E. et al. Distinct baseline immune characteristics associated with responses to conjugated and unconjugated pneumococcal polysaccharide vaccines in older adults. Nat Immunol 25, 316–329 (2024). https://doi.org/10.1038/s41590-023-01717-5
3. Zhu, M., Hsu, CW., Peralta Ogorek, L.L. et al. Single-cell transcriptomics reveal how root tissues adapt to soil stress. Nature 642, 721–729 (2025). https://doi.org/10.1038/s41586-025-08941-z

4. Zheng, G., Terry, J., Belgrader, P. et al. Massively parallel digital transcriptional profiling of single cells. Nat Commun 8, 14049 (2017). https://doi.org/10.1038/ncomms14049 (See **Supplementary Figure 2**)
5. "What Fraction of mRNA Transcripts Are Captured per Cell?" n.d. 10X Genomics. Accessed July 15, 2025. https://kb.10xgenomics.com/hc/en-us/articles/360001539051-What-fraction-of-mRNA-transcripts-are-captured-per-cell

Referee #4 (Remarks to the Author):

While the revisions considerably strengthen the manuscript, particularly through the addition of functional assays and expanded antibody analysis, the work remains largely descriptive and incrementally novel by Nature standards. The resource value is undeniable, and the RNA Age Metric is potentially impactful. However, further mechanistic insights or causal experimentation would be necessary to elevate its significance. I do recommend publication as a resource paper, rather than as a report of conceptual breakthroughs.

We appreciate the reviewer's recognition of the manuscript's strengthened data and potential impact of the RNA Age Metric. To better highlight the translatability of our RNA Age Metric (RAM), we further analyzed this metric in a recently released human dataset on tissue aging in normal LNs (Wells, *et.al.*, Nat Imm, In Press). From these analyses, we show that age-related transcriptional re-programming found in blood is also present in secondary lymphoid tissues. (**Revised Figure 2i**).



Revised Figure 2i. RNA Age Metric in T cell subsets from human mesenteric (n=21) and thoracic (n=21) lymph nodes collected across age. (GEO GSE299043)

Furthermore, to demonstrate the relevance of RAM as a metric of immune functional states more than a direct indicator of age - we analyzed another recently released dataset that profiled the immune system of overtly healthy individuals at risk for the development of rheumatoid arthritis (determined by the presence of anti-citrullinated protein autoantibodies (ACPA)). (He, Glass, *et.al.*, STM, In Press) From these analyses, we find elevated RAM specifically in central memory CD4 T cells (**Revised Figure 2j**) - indicating that immune tolerance dysregulation may stem from altered development or long-term maintenance of memory in these individuals. Moreover, these data highlight that RAM can be used as more than an indicator of age, but as an indicator of immune cell health and functional state. These two new analyses provide additional novel insights into T cell aging in tissues and age as well as demonstrates the broad applicability of our data and analysis as a resource and tool for the field.

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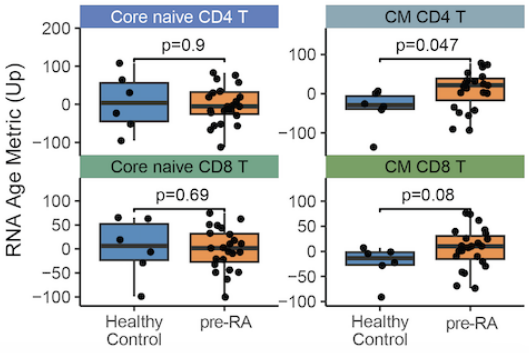

Revised Figure 2j. RNA Age Metric in T cell subsets from young adults (20-40yrs of age) that are healthy (ACPA-) or pre-RA (ACPA+, at risk for the development of rheumatoid arthritis). (GEO GSE274680)

References:

1) Wells, S.B.†, Rainbow, D.B. †, Mark, M. †, Szabo, P.A. †, Ergen, C. †, Caron, D.P. †, Maceiras, A.R., Rahmani, E., Benuck, E., Amiri, V.V.P., Chen, D., Wagner, A., Howlett, S.K., Jarvis, L.B., Ellis, K.L., Kubota, M., Matsumoto, R., Mahbubani, K., Saeb-Parsy, K., Dominguez-Conde, C., Richardson, L., Xu, C., Li, S., Mamanova, L., Bolt, L., Wilk, A., Teichmann, S.A., Farber, D.L.*, Sims, P.A.*,Jones, J.L.*, Yosef, N.* (2025) Multimodal profiling reveals tissue-directed signatures of human immune cells altered with age. *Nat. Immunol*. In Press. (also Biorxiv, 2024)

2) He, Z., Glass, M., Venkatesan, P., et. al,. (2025) Systemic inflammation and lymphocyte activation precede rheumatoid arthritis. Science Trans Med, In Press. (also Biorxiv, 2024)

Response to Reviewers

We thank all of the reviewers for their time, thoughtful input and helpful suggestions that have collectively enhanced the novelty and impact of our study – revealing unique features of immune dysregulation that occur *prior to advanced age*.

Referees' comments:

Referee #1 (Remarks to the Author):

I want to thank the authors for providing additional analyses and thoughts. All my concerns are addressed.

We thank the reviewer for their thoughtful comments and suggestions throughout the revision process.

Referee #3 (Remarks to the Author):

I would like to thank the authors for providing the rationale for using a 10% gene expression cutoff through literature review and comparative data analysis. Establishing an appropriate balance between sensitivity and specificity is always challenging. Given the sparsity of the data (30–40% detection rate) and the heterogeneity of defined cell subpopulations, applying a 10% cutoff is reasonable as an initial step for identifying DEGs. However, it remains difficult to determine how many of the findings represent true versus false positives if the 10% cutoff is used as the basis for biological conclusions. Employing a higher cutoff (20–30%) would likely yield more reproducible results.

We agree that our cut-off analysis provides comparative support for using a 10% cut-off for gene expression in our initial DEGs analyses and, in line with other studies[1-5], balances discovery of cell-type specific features while excluding random variation. Moreover, the 10% cut-off also accounts for cellular heterogeneity and *captures lowly expressed but biological important genes, such as transcription factors and cytokines, that may be lost when using a higher 20-30% cut-off*. We also demonstrated the robustness of our RNA Age Metric with 10, 20 and 30% cut-off analyses, highlighting that the 10% cutoff reduces noise, inconsistently-expressed genes and technical artifacts in downstream DEG analyses, while balancing potential trade-offs between sensitivity and specificity.

References:

1. Heumos, L., Schaar, A.C., Lance, C. et al. Best practices for single-cell analysis across modalities. Nat Rev Genet 24, 550–572 (2023). https://doi.org/10.1038/s41576-023-00586-w
2. Ravichandran, S., Erra-Diaz, F., Karakaslar, O.E. et al. Distinct baseline immune characteristics associated with responses to conjugated and unconjugated pneumococcal polysaccharide vaccines in older adults. Nat Immunol 25, 316–329 (2024). https://doi.org/10.1038/s41590-023-01717-5
3. Zhu, M., Hsu, CW., Peralta Ogorek, L.L. et al. Single-cell transcriptomics reveal how root tissues adapt to soil stress. Nature 642, 721–729 (2025). https://doi.org/10.1038/s41586-025-08941-z
4. Zheng, G., Terry, J., Belgrader, P. et al. Massively parallel digital transcriptional profiling of single cells. Nat Commun 8, 14049 (2017). https://doi.org/10.1038/ncomms14049 (See **Supplementary Figure 2**)
5. "What Fraction of mRNA Transcripts Are Captured per Cell?" n.d. 10X Genomics. Accessed July 15, 2025. https://kb.10xgenomics.com/hc/en-us/articles/360001539051-What-fraction-of-mRNA-transcripts-are-captured-per-cell