Running head: Profiling of aged T-reg at single-cell resolution

Title: Unveiling the immune system ageing in single-cell resolution (Commentary, Clean Version)

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Short Summary:

This writing serves as a commentary on the findings presented in the original manuscript by Yang et al. in 2023, published in the Journal of Leukocyte Biology (JLB). This commentary first summarizes the spatial-temporal dynamics of regulatory T-cells (T-reg) derived from mice (*Tabula Muris Senis*) of different ages (3M, 18M, and 24M) at different anatomical niches like lymph nodes and bone marrow. We also reported possible combinations of receptor-ligand interactions among T follicular regulatory cells (Tfr), T follicular helper cells (Tfr), and Germinal Centre (GC) B-cells, such as Calmodulin/Fas axis and PSGL-1/L-selectin axis. Then, we have elaborated on the significance of understanding aging T-reg, and have offered some possible future research directions for Yang et al., contributing to a critical analysis of their recent study. Building upon these foundations, further investigations and studies can be conducted to delve deeper into the mechanisms by which T-reg influence health upon aging, potentially unveiling novel therapeutic targets to ameliorate age-related pathogenicity.

Conflicts of Interest: None

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Introduction

Regulatory T cells (T-reg) are pivotal players in humoral immunity to serve as a 'brake' to establish immunological homeostasis. This can be achieved by generating cytokines such as TGF-β, IL-35, and IL-10, which inhibit effector T cells (ETCs) [2]. Additionally, T-reg can suppress humoral immunity by triggering ETCs' apoptosis and inducing dendritic cells to produce indoleamine 2,3-dioxygenase, which further suppresses ETCs [3]. Another proposed mechanism involves the generation of adenosine nucleosides, catalyzed by both CD39 and CD73 [3]. T-reg-mediated immunosenescence is believed to be dependent on aging. Palatella et al. have recapitulated several detrimental effects of aging on T-reg [4]. For instance, immunity against non-self-cells is generally weakened in the elderly [4]. Although the mechanisms are intricate, the advancement of single-cell RNA sequencing (sc-RNA seq) has revealed the transcriptional heterogeneity of T-reg, contributing to our understanding of aging immunity. Yang et al. investigated a relevant topic and published their findings in the current issue of the Journal of Leukocyte Biology [1].

In this writing, we aim to summarize some main points covered by Yang et al. and provide comments. Furthermore, we will propose future research directions for studying aging T-reg.

General introduction to sc-RNA seq analysis:

sc-RNA seq analysis by Seurat R package was employed by Yang et al. to study differentially expressed genes (DEG) in mouse (*Tabula Muris Senis*) T-reg [1,8]. The raw data were first obtained from the 'Tabula Muris consortium', a transcriptomic atlas for studying tissue aging in mice [7]. Dead and doublet cells are filtered based on transcript abundance. The dataset was normalized using the *SCTtransform* function to eliminate the effect of sequencing depth on T-reg heterogeneity [1,9]. Principle Component Analysis was conducted for dimensionality reduction of the dataset. Then, cell clusters were identified using *FindClusters* and *FindNeighbours* functions, which are then projected on Uniform Manifold Approximation and Projection [1]. DEG in each cluster are identified by *FindAllMarkers and* quantified based on $\log_2$(fold change) of transcripts [1].

While sc-RNA seq is powerful for understanding T-reg heterogeneity, it is still complicated by its technical limitations [10]. First, sc-RNA seq involves dissociation of viable cells by either enzymatic or heat treatment. Both require rigorous optimization to maintain cell viability [10]. Such dissociation methods can be problematic as they potentially introduce unwanted transcriptional changes, causing errors in downstream analysis. Besides, sc-RNA seq can introduce greater noise than bulk RNA-seq, as each cell is independently considered a biological replicate [10].

The main points covered by Yang et al.

i. Spatial-temporal dynamics of age-dependent T-reg:

Six clusters (C1-C6) of mouse-derived, organ-specific CD4+ T-reg have been identified through sc-RNA seq as described [1]. Most of them come from thymus, as indicated by the expression of thymic origin markers *Ikzf2* and *Nrp1* [1].

Cluster C1 can be further categorized into C1a and C1b, representing marrow-resident and three subgroups of lymphoid-resident T-reg, respectively [1]. C1a has been found to upregulate expression of *Sell, Pi16, Tgfbi,* and *Cpt1a*, while C1b shows upregulation of *Icos*, suggesting that C1a cells are less mature than those in C1b [1]. Generally, *Prkch* and *Il27ra* expressions are absent in C1. In C2, there are likely T-reg cells with compromised oxidative phosphorylation (OXPHOS). C3 consists of $CD4^{+}CD8+$ thymocytes, which may serve as precursors of T-reg, as evidenced by the upregulation *of Cd8a, Cd8b1, Dntt*, and *Rag1* [1]. T-reg cells in C4 and C5 have been identified as T follicular regulatory cells (Tfr), characterized by upregulated expression of Tfr marker genes *Bcl6* and *Cxcr5* [1]. T-reg proliferation in C4 is likely promoted by the upregulation of glycolytic genes [1]. T-reg in C5 highly expresses CD150, suggesting a hematopoietic role in the marrow. Lastly, C6 has been identified as adipocyte-resident T-reg, as evidenced by the upregulation of *Il10, Il1rl1, Pparg*, and *Gata3* [1]. Yang et al. also studied the temporal dynamics of C1-C6 using the propeller R package [1]. The results indicated that the number of cells in C1 and C6 increases from 3M to 24M in mice, while the number of cells in C3 and C5 significantly decreases from 3M to 24M [1]. Additionally, the number of cells in C2 and C4 decreases from 3M to 18M and then increases from 18M to 24M [1].

ii. Interactomes analysis reveals possible therapeutic targets for the aging immune system:

Given the immune-suppressive role of Tfr on germinal center (GC)-derived B cells and T follicular helper cells (Tfh), Yang et al. used the *'iTALK'* R package to investigate whether any age-dependent changes in combinations of receptor-ligand interactions occur between these cells [1]. Interactome analysis revealed that Programmed death-ligand 1 (PD-L1) on GC B cells of aging mice (18 months) can suppress the development of PD-1-expressing Tfh cells and their homing to follicles [1]. The interactome analysis also displayed possible mechanisms of Tfr-mediated regulation of Tfh apoptosis and exhaustion through the Calmodulin/Fas axis and PSGL-1/L-selectin axis, respectively [1]. Furthermore, possible pathways for Tfh activation during aging were discovered. In 18-month-old mice, Lymphotoxin alpha, which is essential for GC formation, was upregulated in Tfh cells [1]. *Jag1* and *Jag2*, both of which encode ligands for *Notch1* receptors, were upregulated in 24-month-old mice [1]. Additionally, significant upregulation of *Notch1* was identified in Tfh cells, indicating ongoing Tfh differentiation mediated by Notch signaling during aging [1]

Significance of the findings:

The aforementioned transcriptomic analysis reveals changes in T-reg subtypes during aging, providing valuable information for diagnostic markers in pathological conditions. For example, T-reg accumulation in adipose tissue is correlated with insulin resistance and an increased risk of type II diabetes [1,5]. Additionally, Safari et al. reported that *STUB1* expression in T-reg inhibits *the FOXP3*-mediated anti-inflammatory program, potentially contributing to the development of rheumatoid arthritis [6]. By studying transcriptional changes or gain/loss of T-reg subsets, we can gather more insights for diagnosing diseases and initiating early treatment.

The interactome analysis identifies receptor-ligand interactions among GC B cells, Tfr, and Tfh during aging [1]. Such understanding enables the identification of potential drug targets, particularly proteins involved in cell signaling associated with aging T-reg [1]. Researchers can then design therapeutic strategies, such as monoclonal antibodies or small molecule inhibitors, to disrupt these interactions. This approach holds the potential to reinvigorate aging immunity and improve immune function.

Comments on Yang et al.'s work and future research directions:

Yang et al. have attempted to identify DEG in clusters of T-reg derived from different lymphoid and non-lymphoid organs through sc-RNA seq [1]. However, such a tremendous load of sc-RNA seq data may be a little bit overwhelming, or even challenging for readers to understand the importance of each piece of data. What are the alternative hypotheses explaining how aging modifies the transcriptional signature of T-reg? What are the central questions to aging T-reg? What knowledge gaps in aging immunity do you want to fill? If these questions are not in mind beforehand, it is like finding a needle in a haystack. Perhaps we can narrow down the scope of the study if we can try to design experiments to test only a limited number of hypotheses.

As mentioned, Yang et al's work has recapitulated many gene signatures of aging T-reg. What is their relative biological significance in contributing to aging immunity? How can experiments be designed to validate the relative significance of certain gene sets in regulating aging immunity? We think gene perturbation assays could be useful for validating the importance of marker genes in driving aging immunity. For instance, the original authors suggested Calmodulin/Fas axis and PSGL-1/L-selectin axis are involved in promoting Tfh apoptosis and exhaustion respectively [1]. Indeed, knocking out (KO) these genes by the Cre-LoxP system in mice, followed by RNA-sequencing of Tfr and Tfh from KO mice of different biological ages, can be conducted. This is useful for studying whether the loss-of-function of these genes can reverse the immuno-suppressive role of Tfr on Tfh, potentially pinpointing targets for rejuvenating T-reg-mediated immunity upon aging. For instance, exhaustion markers (like *PD1, LAG3,*

TIGIT) in mice-derived Tfh can be studied to check if they are downregulated after KO immunosenescence-related genes.

Finally, Yang et.al's mainly attempted to characterize the transcriptional signature of aging T-reg derived from mice. Despite the conservation of gene expression between mice and humans, it is better to study the gene expression of peripheral blood mononuclear cells (PBMC)-derived T-reg collected from donors of different biological ages. Due to discrepancies between human and mouse genomes, resolving their transcriptomic differences provides a more solid foundation for further characterization of aging T-reg in clinical settings. Stratifying cohorts of PBMC donors is necessary such that the transcriptomic heterogeneity of T-reg is minimally confounded by factors like sex, ethnicity, and health conditions of donors.

Figure 1. Possible research directions for Yang et al.
Comments and possible research directions for Yang et al. are recapitulated above. Firstly, knock-out mice model can be generated to study the importance of certain genes driving T-reg aging. Secondly, PBMC-derived T-reg can be studied to better understand transcriptional differences between mouse and human T-reg during aging. Lastly, hypotheses explaining T-reg aging can be set for further testing. Images Created with BioRender.com

Declaration of Interests

The authors declare no financial and personal conflicts of interest.

Reference

1. Yang KY, Liao J, Ma Z, Tse HF, Lu L, Graca L, Lui KO. Single-cell transcriptomics of Treg reveals hallmarks and trajectories of immunological aging. Journal of leukocyte biology. 2023. http://dx.doi.org/10.1093/jleuko/qiad104. doi:10.1093/jleuko/qiad104

2. Kondělková K, Vokurková D, Krejsek J, Borská L, Fiala Z, Ctirad A. Regulatory T cells (TREG) and their roles in the immune system with respect to immunopathological disorders. Acta medica (Hradec Kralove). 2010;53(2):73–77. http://dx.doi.org/10.14712/18059694.2016.63. doi:10.14712/18059694.2016.63

3. Vignali DAA, Collison LW, Workman CJ. How regulatory T cells work. Nature reviews. Immunology. 2008;8(7):523–532. http://dx.doi.org/10.1038/nri2343. doi:10.1038/nri2343

4. Palatella M, Guillaume SM, Linterman MA, Huehn J. The dark side of Tregs during aging. Frontiers in immunology. 2022;13:940705. http://dx.doi.org/10.3389/fimmu.2022.940705. doi:10.3389/fimmu.2022.940705

5. Bapat SP, Myoung Suh J, Fang S, Liu S, Zhang Y, Cheng A, Zhou C, Liang Y, LeBlanc M, Liddle C, et al. Depletion of fat-resident Treg cells prevents age-associated insulin resistance. Nature. 2015;528(7580):137–141. http://dx.doi.org/10.1038/nature16151. doi:10.1038/nature16151

6. Safari F, Farajnia S, Arya M, Zarredar H, Nasrolahi A. CRISPR and personalized Treg therapy: new insights into the treatment of rheumatoid arthritis. Immunopharmacology and immunotoxicology. 2018;40(3):201–211. http://dx.doi.org/10.1080/08923973.2018.1437625. doi:10.1080/08923973.2018.1437625

7. Tabula Muris Consortium. A single-cell transcriptomic atlas characterizes ageing tissues in the mouse. Nature. 2020;583(7817):590–595. http://dx.doi.org/10.1038/s41586-020-2496-1. doi:10.1038/s41586-020-2496-1

8. Hao Y, Hao S, Andersen-Nissen E, Mauck WM III, Zheng S, Butler A, Lee MJ, Wilk AJ, Darby C, Zager M, et al. Integrated analysis of multimodal single-cell data. Cell. 2021;184(13):3573-3587.e29. http://dx.doi.org/10.1016/j.cell.2021.04.048. doi:10.1016/j.cell.2021.04.048

9. Hafemeister C, Satija R. Normalization and variance stabilization of single-cell RNA-seq data using regularized negative binomial regression. Genome biology. 2019;20(1):296. http://dx.doi.org/10.1186/s13059-019-1874-1. doi:10.1186/s13059-019-1874-1

10. Ke M, Elshenawy B, Sheldon H, Arora A, Buffa FM. Single cell RNA-sequencing: A powerful yet still challenging technology to study cellular heterogeneity. BioEssays: news and reviews in molecular, cellular and developmental biology. 2022;44(11). http://dx.doi.org/10.1002/bies.202200084. doi:10.1002/bies.202200084

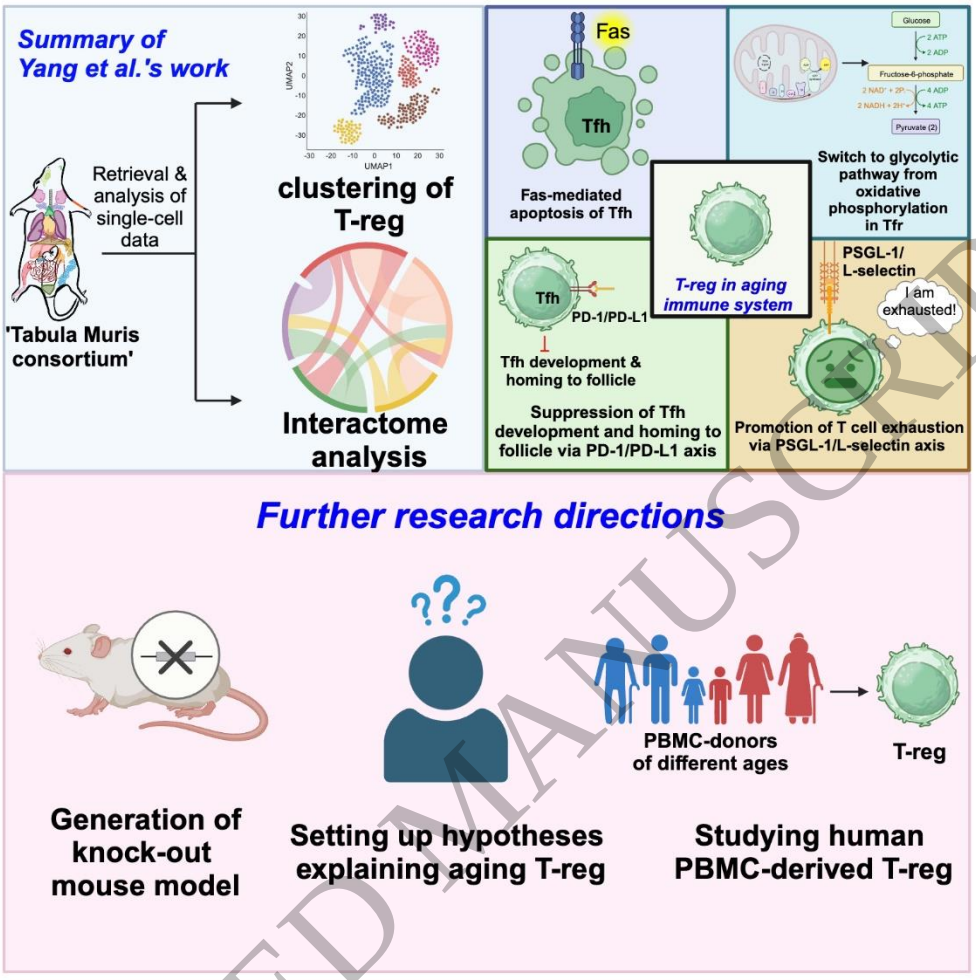

Figure 1
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