הקרן הלאומית למדע
المؤسَّسة الإسرائيلية للعلوم
Israel Science Foundation

ג' באב, תשפ"ד
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לכבוד
פרופ' אלון מונסונגו
מיקרוביולוגיה, אימונולוגיה וגנטיקה
אוניברסיטת בן-גוריון בנגב

לפרופ' מונסונגו שלום רב,

הנדון: בקשתך למענק מחקר בנושא:
<u>השתנות הלימפוציטים עם הגיל והשפעתם על הזדקנות וירידה קוגניטיבית</u>

הצעת המחקר אשר הגשת לקרן הלאומית למדע לא נכללה, לצערנו, בין ההצעות אשר זכו במענקי מחקר השנה.

מצ"ב עיקרי חוות הדעת.

לתשומת לבך, החלטות הנהלת הקרן מתקבלות על סמך סיקור עמיתים ודיונים בוועדות מקצועיות. ראוי להדגיש כי הוועדה המקצועית אשר בחנה את הצעת המחקר התבססה על מכלול ההערות והציונים שהתקבלו מסוקרים חיצוניים ומחברי הוועדה שהם מומחים בתחום, ונתנה משקל, בדיוניה, רק לאותן ביקורות והערכות שהיו מקובלות עליה.

מאחר שכספי ההקצבה השנתית מחולקים עד תום, החלטות הנהלת הקרן הן סופיות ואינן ניתנות לשינוי.

אנו מאחלים לך הצלחה בהמשך דרכך המדעית.

בכבוד רב,

ד"ר תמר יפה-מיטווך
מנכ"ל

העתק: הרשות למחקר ופיתוח, אוניברסיטת בן-גוריון בנגב

מוזמנים לבקר באתר קול המדע https://kolhamada.isf.org.il/ - מיזם של הקרן הלאומית למדע, המגיש מידע מדעי מחזית המדע, לציבור ולקהילה המדעית בישראל.

Reviewer No. 1

- **Originality & innovation**

The most innovative aspect of the application is the hypothesis that the CD4 CTLs that accumulate with age in both rodents and humans may be protective against degeneration (Aim 2). The rest of the application is not as innovative, particularly Aim 1, as there have been several similar multi-modal profiling of peripheral blood of aged vs. young individuals, including both scRNAseq and flow cytometry.

- **Project importance and contribution to scientific knowledge**

Although the overall thematic is of great importance, the significance of the project is dampened by the fact that the most innovative and significant part is underdeveloped. As mentioned above, the most intriguing preliminary data is the fact that CD4 CTLs that accumulate with age in both rodents and humans, driven by senescence (Fig. 5-6), may be protective, as their removal decreased survival and activity, and increased senescence in aged mice (Fig. 3). However, the experiments concerning the mechanisms underlying the accumulation of these cells (Aim 2.3) are only incrementally different from the preliminary data. In particular, the authors don't build on the abundant knowledge on how to manipulate cell senescence in rodents using senolytics to get a better understanding of mechanisms. There is more enthusiasm for Aim 2.1 and 2.2, that will use reporter mice to track the accumulation of these cells in multiple organs and will look in more depth how their removal affects multiple brain cells. However, these studies are mainly descriptive and associative, and do not test hypotheses about these cells' mechanisms of action.
These CD4 CTLs paradoxically appear to be more prevalent in the blood of individuals with mild cognitive impairment (MCI) (Fig. 2), suggesting that these cells develop to try to counter senescence-driven degeneration, making it difficult to interpret its significance in the cross-sectional study proposed in Aim 1. There is more enthusiasm for Aim 3, which is longitudinal, and could thus provide a better understanding of the potential associations between senescence, these cells and MCI progression. However, in absence of a better understanding of the mechanisms of development and action of these cells, which, according to the study timing shown in Scheme 3 will not be available before Aim 3 starts, the very complex and labor-intensive Aim 3 is considered as premature.

- **Adequacy of methods**

The methods are adequate. Preliminary data support feasibility. Power analyses are presented. One important aspect that is missing is the description of how batch effects, inherent to these very large flow cytometry analyses, will be detected and handled.

- **Suitability of investigators' scientific background to the project**

The PI and collaborators have the necessary expertise to conduct this project.

Summary (strengths / weaknesses of the proposal)

The strengths are: (1) the overall thematic importance, (2) the supportive preliminary data for the hypothesis that CD4 CTL accumulation is driven by senescence and is protective against senescence-driven degeneration, (3) the PI's expertise and the preliminary data support feasibility. Despite these strengths, the enthusiasm is diminished by (1) the lack of innovation of Aim 1, (2) the fact that the most innovative and significant part of the proposal is under-developed, and (3) the very complex and labor-intensive Aim 3 is considered as premature.

Reviewer No. 2

The grant application from Prof. Monsonego aims to characterize changes in the immune system associated with aging and utilize them as predictive biomarkers for immune system fitness, aging pace, and cognitive impairment. This proposal is ambitious, combining descriptive research on volunteers with a 4-5 year follow-up, experimental work on transgenic mice, and machine-learning approaches for biomarker identification. The proposal focuses on CD4+ cytotoxic T-cells, a subset recently observed in peripheral blood and associated with aging. The proposal's novelty lies in its exploration of CD4+ cytotoxic T-cells population's development, effector function, and potential association with aging.

The first aim involves screening CD4+ T-cells from peripheral blood in young and old individuals (totaling 36 subjects) using single-cell RNA sequencing. Due to budget constraints, achieving the necessary sequencing depth for biomarker identification may require additional funding or a smaller sample size. Follow-up studies will include FACS and Luminex analysis on a larger cohort. Despite its descriptive nature, this aim can provide valuable insights into the transcriptional profile of CD4+ cytotoxic lymphocytes in humans during aging.

The second aim focuses on elucidating the role of CD4+ cytotoxic lymphocytes using mice bred with CD4creERT crossed with Eomes floxed. These mice will undergo metabolic and behavioral tests, followed by phenotyping using flow cytometry and confocal microscopy. While these experiments provide mechanistic insights, they are largely correlational. Additionally, the proposal lacks evidence of successful CD4creERT-mediated ablation of Eomes, raising concerns about achieving consistent Eomes ablation or potential variability in the ablation of Eomes and cytotoxic CD4+ T cells among individual mice.

Furthermore, the proposal suggests utilizing Eomes creERT x tdTomato mice to create an Eomes reporter strain, enabling precise isolation of CD4+ cytotoxic lymphocytes and their localization across various tissues. However, this approach, primarily descriptive in nature, raises concerns as Eomes creERT targets multiple cell lineages, including NK cells and gdT cells. Differential staining will be necessary to distinguish true CD4+ cytotoxic lymphocytes, making this reporter less than optimal for suggested microscopic studies.

The final part of the second aim explores the interaction between CD4+ cytotoxic lymphocytes and senescent cells, hypothesizing that senescent cells may influence the differentiation or activation of CD4+ cytotoxic lymphocytes. While feasible, the significance of these experiments in the broader context of aging physiology could be elaborated further.

In summary, aim 2 focuses on a more detailed description of CD4+ cytotoxic lymphocyte localization, signal pathways leading to their development, and investigating their

interactions with senescent cells. However, the selected mouse models pose uncertainties in data interpretation, with no alternative strategies proposed or clear evidence provided to support the efficacy of the chosen targeting approaches.

The final aim of the project proposes to employ machine learning techniques on the datasets gathered from volunteers in Aim 1 to identify potential biomarkers associated with aging and immune system perturbations. However, there's a concern that the use of machine learning appears somewhat like a black box or magic wand, lacking a detailed description of the strategy for biomarker identification. Consequently, the approach to rigorously identifying potential biomarkers remains unclear.

In my opinion, the strong side of the submitted proposal is the research question by itself as it aims to experimentally elucidate a crucial phenomenon related to immune system aging, it is highly ambitious and attempts to explore various facets of this phenomenon. However, the weaknesses are that certain sections described above would benefit from a deeper focus and the selection of verified approaches and reagents. This possesses a treat for the successful accomplishment of the project.

Reviewer No. 3

- Originality & innovation

The idea of tracking the role of T-cells in aging using newer single cell tools is not entirely new and, in some ways, Aim1 of the proposal compliments existing data from old (supercentenarian) studies and is not entirely new except for the additional correlation of T cells with immune markers. However, perturbing distinct cell types that accumulate with age and tying this accumulation to physiology, as outlined in Aim2 is highly innovative. One slight concern with the proposal is that it is focused almost entirely on CD4+ CTLs but in their previous work the authors also identified a very large accumulation in exhausted CD4+ cells, but the reasoning to focus on the CTL subset has merits. The use of CD4 cells as a diagnostic tool in Aim3 is interesting and could potentially be an innovative approach for this purpose but for this purpose many patients would need to be sampled with high-dimension data and it is not entirely clear from the proposal what the exact data collected from 60 patients will be and how the model will be used.

- Project importance and contribution to scientific knowledge

Strengths:

Understanding of the aging process is limited and the immune system's role in this process is critical. Therefore, studies that can identify the role of distinct cell populations that arise during aging in a stereotypical way across individuals is very important. Here the authors combine studies in human samples with mechanistic perturbations in an animal model in a way that, if successful, could help us understand the role of specific immune cells in aging.

Weaknesses:

The use of cognitive decline as the central aging correlate in these studies is somewhat limiting. The main reason for this is that the study focuses on circulating immune cells and the Blood Brain Barrier (BBB) limits the contact between these cells and the nervous system itself. This raises several potential issues (here are 3 specific examples): First, the study will be mostly limited to indirect effects of the CD4+ CTLs on the brain. Second, any patients in which leakage or other issues with the barrier arise (mini stroke, for example) may have localized interactions that may not be well correlated with the CD4+ CTL cell load meaning that even individuals with relatively low cell numbers in circulation could have localized maximal cell interactions in cases of barrier dysfunction. Finally, cognitive decline is multi-factorial and it is entirely possible that the link between CD4+ CTLs and decline, even if it exists, will only account for a fraction of the decline, making it hard to account for in any diagnostic meaningful way.

- Adequacy of methods

Strengths:

-The combined use of human and mouse models is a strength of the proposal.
-Exclusion criteria and single cell methods appear robust and thoughtful.

Weaknesses:

- In their previous studies the authors used C57 mice, which are notoriously prone to immune cancer (B-cell lymphomas) in later ages. These lymphomas account for the majority of

mortality in aged mice of this background. It would be advisable to use a different mouse model as much as possible.
- In previous animal work the authors main comparison was of 2 month old mice with aged mice, 2 months is a young age (somewhat analogous to teenage years in humans), in the animal work proposed for aim 2 the authors would ideally make comparisons in more meaningful ages that correspond to young but fully mature animals (6-8 months) or mid-aged mice (approx. 12-16 months) in their comparison with the aged groups.

- Suitability of investigators' scientific background to the project

  Strengths:

- The lead investigator has a strong track record of success and recently discovered an interesting age-related phenomenon in mice
- There are collaborators with clinical sample access and bioinformatic support that will provide guidance and samples.

Weaknesses:

None

- Summary (strengths / weaknesses of the proposal)

This proposal follows up on a very interesting finding out of the Monsonego lab that has potential to yield an important link between aging phenotypes and a distinct subset of T cells that accumulates during aging. The proposal seeks to first identify whether this T-cell population that was identified in mice is also correlated with aging phenotypes in humans. This aim (Aim 1) is important because it ties the original mouse data to human health and seeks to correlate outcome with phenotype, going a step further than other work in this area. The proposal's other strength is in trying to systematically perturb a cell population in aging mice and test the outcome. This is a strength of the proposal and could yield interesting results.

הערות הוועדה המקצועית

While the proposal tackles an important and innovative research question with promising preliminary data, it requires further refinement and development of mechanistic insights, methodological strategies, and clarity in the application of complex techniques to achieve its ambitious goals.
Specifically, the committee would suggest considering improving the following points:

1) Improve mechanistic insights of the proposal: The most significant and innovative parts of the proposal, particularly regarding the mechanisms of CD4+ CTL accumulation and function, are not thoroughly developed. The proposal lacks detailed hypotheses and mechanistic studies beyond descriptive and associative analyses.
2) The longitudinal study proposed in Aim 3 might be premature given the current limited understanding of the underlying mechanisms. The complexity and labor-intensive nature of this study may also present significant challenges. Additionally, the application of machine learning for biomarker identification in Aim 3 appears underdeveloped, with concerns regarding the clarity and rigor of the proposed strategy.
3) Limitations in Cognitive Decline Focus: The focus on cognitive decline as the central aging correlate is considered limiting due to potential issues with the blood-brain barrier and the multifactorial nature of cognitive decline.