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REVIEW

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CD8⁺ T cell heterogeneity in aging: insights from single-cell profiling

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Abstract

Inflammation progressively increases with age, resulting in a broad remodeling of the CD8⁺ T cell compartment. Recent advances in single-cell RNA sequencing (scRNA-seq) have revealed a transcriptional heterogeneity within the CD8⁺ T cell subsets, challenging traditional classifications based on surface markers. This review summarizes current insights from scRNA-seq and multimodal profiling studies, demonstrating a heterogeneity of CD8⁺ T cell states defined by distinct and overlapping transcriptional programs. Notably, GZMK⁺ effector memory T cells expand with age, and recent studies in mouse models suggest that GZMK⁺ T cells can activate the complement system, positioning them as potential mediators of inflammation. In addition, emerging diversity within TEMRA and non-canonical T cell subsets challenges the conventional boundaries between established T cell subsets. Integrative single-cell approaches are essential for deciphering the dynamic interplay between immune aging and chronic disease, and for informing targeted interventions to restore immune resilience in older adults.

Introduction

An adult human is estimated to harbor approximately 4×10^{11} T cells, one-third of which are CD8⁺ T cells [59]. These cells play a crucial role in immune surveillance, detecting and eliminating infected or abnormal cells. With age, the CD8⁺ T cell compartment undergoes significant changes, primarily driven by reduced thymic output and cumulative antigen exposure, resulting in a diminished naïve T cell pool and an accumulation of effector memory cells [39, 66]. CD8⁺ T cells in old individuals exhibit diminished TCR responsiveness, impaired cytotoxic function, and increased production of pro-inflammatory cytokines (Jonsson et al., [32]; Lan et al., [37, 48, 73]. Over the years, particularly in the context of chronic inflammatory diseases, CD8⁺ T cells may become adaptive to the inflammatory environment, but they may also act as active contributors to systemic inflammation, weakening immune defense in older individuals.

A chronic, low-grade inflammation, often referred to as "inflammaging" is considered one of the hallmarks of aging [17, 42, 47]. This persistent inflammatory state develops with age and plays a central role in the development of many age-associated diseases, likely both a consequence and a driver of changes in the T cell compartment [40]. Responses to inflammatory processes in the body may increase susceptibility to infections, cancer, and diminish vaccine efficacy [5, 22, 40].

However, it is challenging to disentangle the relative contributions of aging and chronic inflammation to the remodeling of CD8⁺ T cell subsets, as these processes are highly interdependent. Nevertheless, age and inflammation may also exert partially independent influences

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on CD8⁺ T cell dynamics. For example, a recent study examining circulating proinflammatory cytokine levels in indigenous, non-industrialized populations reported little to no association between inflammatory markers and either age or age-related disease [19]. The authors proposed that inflammaging may be largely a byproduct of industrialized lifestyles, with considerable variation across human populations. It is reasonable to assume that both intrinsic aging and chronic inflammation influence the phenotypes of CD8⁺ T cells across the human lifespan; however, they may affect distinct CD8⁺ T cell subsets. The age-associated decline in naïve CD8⁺ T cell numbers is primarily driven by thymic involution and appears to occur largely independently of systemic inflammation or inflammatory comorbidities. In contrast, the expansion and altered differentiation of effector-memory CD8⁺ T cells are influenced by chronic inflammatory comorbidities and persistent infections. In this review, we use the term inflammation to refer to the chronic proinflammatory state characterized by sustained cytokine and chemokine production that accompanies aging and age-related diseases.

Over the past decade, single-cell technologies have transformed the analysis of CD8⁺ T cells by providing significantly higher resolution and dimensionality than traditional flow cytometry. These advances have enabled detailed insights into cellular heterogeneity, as well as transcriptional, functional, and developmental states. Single-cell analysis has become a standard approach for studying T and B cell dynamics in the context of aging and age-related diseases, playing a key role in identifying changes in cell subsets and discovering novel age-associated populations. Ideally, combining flow cytometry with single-cell approaches would offer the most comprehensive understanding. However, direct comparisons between the two are challenging. Although both methods assess immune cell heterogeneity, they operate at different biological levels (protein vs. transcriptome) and employ distinct measurement principles, defining subsets using different markers and criteria.

Here, we highlight recent findings from scRNA-seq and multimodal profiling studies that reveal a spectrum of CD8⁺ T cell states shaped by unique and shared gene expression patterns and their changes in human aging.

From pre-defined population markers to single-cell omics

Circulating human CD8⁺ T cells have been typically classified by flow cytometry. Flow cytometry excels in phenotyping well-defined cell subsets and has enabled rapid division of CD8⁺ T cells into four main subsets based on their expression of CCR7 and CD45RA cell markers. These are naïve T cells (TN), which have not yet encountered an antigen, (CCR7⁺CD45RA⁺), central memory T cells (TCM), which preferentially traffic to secondary lymphoid organs and have high proliferative potential (CCR7⁺CD45RA⁻), and effector memory T cells (TEM), found predominantly in peripheral tissues and blood with strong cytotoxic potential (CCR7⁻CD45RA⁻). In addition, a distinct differentiated subset, TEMRA cells, re-express CD45RA and are marked as CCR7⁻CD45RA⁺ [24]. Each subset is characterized by distinct phenotypic and functional properties, and additional markers such as CD62L, CD27, CD28, and CD127 have been commonly used for alternative subsetting (Fig. 1).

Using reliably detectable robust markers at the transcript and protein levels, such as CD8A, GZMB, and CCR7, has enabled scRNA-seq analysis to annotate transcriptionally distinct clusters based on shared marker profiles. However, for example, the protein variants of the CD45 receptor, CD45RO and CD45RA, cannot be distinguished by scRNA-seq analysis. Nevertheless, advances in multi-omics have expanded scRNA-seq capacity to combine membrane markers with transcriptomic profiling in the same cell [4]. A key development has been cellular indexing of transcriptomes and epitopes (CITE-seq), which combines surface protein quantification with gene expression profiling in single T cells [62]. CITE-seq has resolved this issue by capturing the expression of two CD45 isoforms that originate from alternative splicing, thereby differentiating between CD45RA⁺ naïve and CD45RO⁺ memory T cells. This has enabled the direct correlation of protein-based flow cytometry markers with gene expression, uncovering rare T cell subsets and differentiation trajectories by profiling millions of individual T cells. Since many critical markers of T cell function are controlled at the protein level rather than through mRNA abundance, CITE-seq enables a more accurate characterization of T cell phenotypes [49]. The approach has enabled the identification of functional states, such as activation or exhaustion, which would be missed by transcriptome data alone, offering a more complete map of T cell responses across health and disease [6, 10, 65].

In parallel, single-cell immune repertoire profiling allows the retrieval of paired T cell receptor (TCR) alpha and beta chain sequences from individual cells, linking antigen specificity to transcriptional state [29]. This integration enables tracking the expansion, differentiation, and function of specific T cell clones within tissues, providing critical insights into immune responses in infection, cancer, and autoimmunity [14, 70, 75]. Additionally, advances in single-cell chromatin accessibility profiling (e.g., scATAC-seq) have made it possible to map the regulatory landscapes of T cells at single-cell resolution [60, 76]. By integrating chromatin accessibility with transcriptomic data from the same cells, the gene regulatory mechanisms can be connected to transcriptional

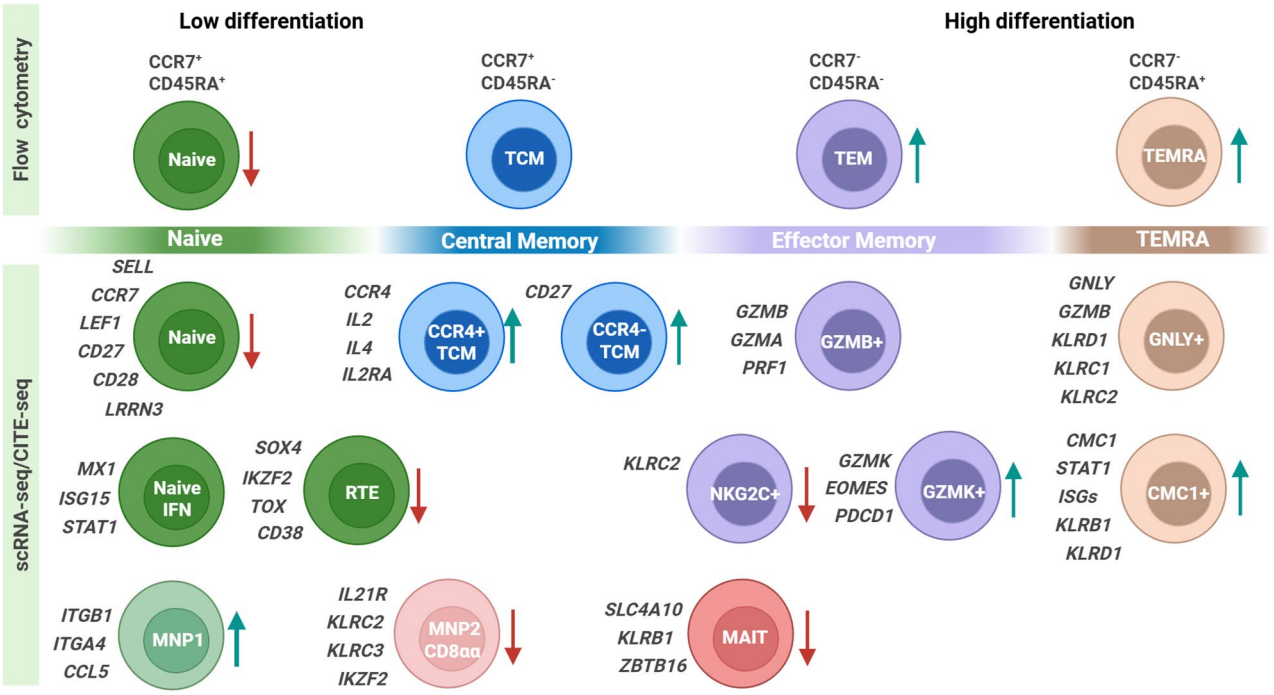

Fig. 1 CD8$^+$ T cell subsets identified by flow cytometry and single-cell transcriptomic analysis. The subsets are colored according to their clustering with naïve (green), central memory (blue), effector memory (violet) or differentiated TEMRA (brown) cells. The genes used in subset annotations are given in italics. The red or green arrows indicate whether the cell subset decreases or increases with age

programs, shedding light on the epigenetic control of T cell development.

Integrating transcriptomic, membrane marker, TCR repertoire and epigenomic data at the single-cell level offers novel insight into CD8$^+$ T cell aging. However, computational challenges remain, including batch correction across diverse aging cohorts, aligning modalities with differing resolutions and data structures, and inferring causality from correlation. Advances in machine learning and multimodal data integration frameworks enable to overcome these obstacles and fully leverage large datasets. Also, single-cell methods use cell suspensions, causing loss of spatial information about cell localization and cell-cell interactions. Spatial approaches such as imaging mass cytometry and spatial transcriptomics are rapidly advancing and help to integrate transcriptional and proteomic data with tissue architecture.

The decline of naïve CD8$^+$ T cell numbers is a hallmark of aging

Studies of T cell dynamics during aging have revealed intermediate states within the CD8$^+$ compartment as well as age-associated changes in the abundance of naïve and memory cells [11, 26, 47, 51]. The decreasing numbers of naïve CD8$^+$ T cells are a hallmark of aging, driven by thymic involution, impaired homeostatic proliferation, and a sustained inflammatory environment [47]. Multiple scRNA-seq studies of human PBMCs have validated the reduction of naïve CD8 + T cell numbers in single or multi-cohort integrative analysis of healthy aging [18, 35].

Adult naïve CD8$^+$ T cells form a relatively uniform cluster in scRNA-seq analysis. Classically characterized by the presence of *CCR7*, *TCF7*, *LEF1*, and *SELL* gene markers, the "true naïve" cells appear to be a relatively homogenous population, constituting 85–95% of all naïve CD8 + T cells (Table 1). However, a single-cell multi-omics profiling, including transcriptomics, surface proteomics, and chromatin accessibility, reported a high-resolution analysis of additional naïve CD8$^+$ T cell subsets and their specificity across childhood and adulthood [67]. In addition to the "true naïve" cells, they characterized T stem cell memory (TSCM) cells and two smaller, memory-like naïve precursor populations (MNP-1 and MNP-2), and the distinct phenotypic features. TSCM cells are circulating memory CD8$^+$ T cells that express CD45RA and CCR7 as well as CD62L, CD27, CD28 and CD127, like naive T cells, but can be distinguished from naive cells by other markers such as CD95, IL2RB, CXCR3, and ITGAL [23]. TSCM cells are similar to TCM cells in functional properties but are considered even more pluripotent with greater longevity [21, 56]. With age, the frequencies of TSCM and MNP-1 subsets increased (from 2.1% in children to 8.8% in adults and from 3% to 7.3%, respectively) while the MNP-2 subset decreased (from 3.3% to 0.8%).

The naïve-like populations maintained the expression of "classical naïve" transcription factors such as *LEF1*,

Table 1 The CD8+ T cell subsets identified by single-cell transcriptomic analyses

Cell subset	Subset Markers	Reference
Memory-like naïve precursor 1 (MNP-1)	Naïve cell markers, ITGB1, ITGA4, CCL5	[67]
Memory-like naïveprecursor 2 (MNP-2)	CD8aa; Naïve cell markers, IL21R,KLRC2, KLRC3, IKZF2; poised to express IFNG	[67]
Naïve-IFN	MX1, STAT1, ISG15 (interferon-stimulated genes)	[64]
Recent thymic emigrants(RTEs)	SOX4, IKZF2, TOX; CD38; open chromatin enriched for SOX4, IKZF2,TOX motifs	[7]
CCR4+ TCM subset	CCR4, IL-2, CD25, IL-4, IL-4R, GATA3	[64]
CCR4- TCM subset	CD27, CD62	[64]
GZMK+ TEM subset	GZMK+, CD28+, CD57-, ITGA4 (CD49d), PD1, EOMES	[48]
GZMB+ TEM subset	PRF1, GZMA, GZMB, GNLY	[2] [16] [37]
KLRC2+ CD8+ cells	KLRC2+ GZMB-	[64]
GNLY+ TEMRA	GNLY	[74]
CMC1+ TEMRA	CMC1, STAT1, ISGs, CD160, KLRB1, KLRD1	[74][68]

BACH2, and *FOXP1*; however, these subsets also exhibited enriched transcription factor motif accessibility linked to enhanced effector function, including motifs for *EOMES* and T-bet (*TBX21*). The MNP-2 population showed a distinct response to IL-21 stimulation compared to naïve T cells, marked by upregulation of *PRF1*, while displaying lower expression of *IFNG* relative to memory T cells. Interestingly, MNP-2 cell abundance declined from childhood to adulthood and had a more moderate reduction in older donors over 60. Terekhova and colleagues also identified a distinct $CD8^+$ Tn population characterized by elevated expression of interferon-stimulated genes such as *MX1* and *ISG15* (termed "Naïve-IFN"). However, unlike canonical naïve $CD8^+$ T cells, the abundance of these cells remained unchanged with age [64].

As a specific subset of naïve cells, recent thymic emigrants (RTEs) constitute developmentally the most immature subset of peripheral T lymphocytes, having only recently egressed from the thymus. They express distinct phenotypic markers that differentiate them from the main naïve T-cell pool, reflecting their transitional state of maturation [13]. Using a multi-omics approach, Bohacova and colleagues identified RTEs by their expression of transcriptional regulators SOX4, IKZF2, and TOX, and elevated levels of the surface protein CD38 [7]. RTEs within the naïve T-cell pool are epigenetically distinct, characterized by open chromatin regions enriched for motifs corresponding to SOX4, IKZF2, and TOX. In line with the thymic involution, the abundance of RTEs, as defined by CD38 and SOX4 expression, declined with age.

The naïve $CD8^+$ T cells undergo a pronounced transcriptional remodeling during aging [7, 71]. These alterations occur in key signaling pathways that underlie the functional decline of the immune system's responsiveness. In older individuals, the naive population shows increased expression of multiple effector and inflammation-associated genes, including *ANXA1*, *PTGER2*, *CLIC1*, *S100A1*, *S100A4*, *PRF1*, *GZMA*, and *CCL5* [7, 18]. Thus, the upregulation of inflammation-associated genes may indicate an enhanced exposure to the pro-inflammatory milieu characteristic of the aging circulatory system.

$CCR4^+$ TCM subset accumulates with age

TCM is a population of long-lived T cells with self-renewal capacity, primarily residing in lymphoid tissues [31]. They play a key role in protecting against recurrent infections and are characterized by high expression of CD27, CCR7, and CD62L, which facilitates homing to secondary lymphoid organs. The overall proportion of TCM cells remains relatively stable with age, although it tends to increase following infections [43]. However, with repeated rounds of proliferation over the lifetime, TCM cells gradually lose their self-renewal potential.

In scRNA-seq analysis, distinguishing TCM from naïve cells has been challenging due to their similar gene expression profiles [44, 77]. Classical naïve T cell gene markers, such as *LEF1*, *TCF7*, *CCR7*, and *SELL*, are expressed in both populations, albeit at lower levels in TCM cells. *LRRN3*, another marker associated with naïve $CD8^+$ cells, is found in only a small subset, while *CD69* is more highly expressed in TCM cells [9, 18]. Due to their similarity to naïve cells, the changes in TCMs with age might have been overlooked in scRNA-seq studies.

Single-cell analysis identified two major subpopulations of $CD8^+$ TCMs, each comprising approximately 10% of the total $CD8^+$ T cell pool, and distinguishable by surface expression of the CCR4 gene [64]. From these, the $CCR4^+$ TCM subset accumulates more rapidly with age than the $CCR4^-$ subset. They express IL-2 and CD25 and are enriched for type 2 immunity-related markers, such as IL-4, IL-4R, and GATA3. This suggests that $CD8^+$ $CCR4^+$ TCMs undergo age-related functional skewing, as their expansion correlates with the age-associated increase in Th2 memory $CD4^+$ T cells [64]. In contrast, the $CCR4^-$ TCM subset exhibits increased TCR clonality with age - a feature also observed in other memory T cell subsets. CCR4 is known to be expressed on Th2 cells and regulatory T cells. Therapeutic targeting of CCR4 with the neutralizing antibody mogamulizumab in advanced solid cancer reduced the number of CCR4-expressing $CD8^+$ TCMs, which are important in antitumor immunity. The cell numbers were ultimately maintained over time in long-term survivors, who had lower CCR4 expression in

their central memory CD8⁺ T cells or NK cells with an exhausted phenotype [45].

A subset of CD8⁺ TEM cells has age-related expression of the GZMK enzyme

scRNA-seq analyses have revealed substantial heterogeneity among CD8⁺ TEM cells, along with a shift toward the expression of inflammation-related genes in aged individuals (Zheng et al., 2020). Age-associated transcriptional changes include altered expression of genes linked to activation and cytotoxicity, increased TCR repertoire clonality, and reduced diversity [43].

A defining characteristic of TEM heterogeneity is based on granzyme expression, dividing the population into GZMK⁺ and GZMB⁺ TEM subsets. Of these, only GZMK⁺ TEM cells increase with age, rising from approximately 9% of CD8⁺ T cells in the youngest individuals to about 20% in the oldest [64]. GZMK⁺ CD8⁺ TEM cells express CD28 but lack CD57, distinguishing them from CD45RA⁺ TEMRA cells. They express high levels of EOMES and PD-1, as well as the tissue-homing marker ITGA4 (CD49d), and show increased expression of inflammatory genes [48]. In chronic rhinosinusitis (CRS), GZMK⁺ CD8⁺ T cells were found to co-express KLRG1⁺, CD27⁺, and ITGAE⁺ [37]. Their TCR repertoire is highly clonal, with clonality increasing alongside their age-related expansion. However, they seem to proliferate primarily in response to innate cytokine signals rather than TCR stimulation [16]. Notably, GZMK⁺ TEMs tend to cluster close to MAIT and iNKT cells—two innate-like cell types known for cytokine production in response to TCR-independent stimulation [32].

In contrast, GZMB⁺ (also GZMB⁺GNLY⁺) TEMs are highly cytotoxic, express high levels of *PRF1*, *GZMA*, and *GZMB*, and their clonal expansion does not appear to be age-dependent. However, in another study in healthy individuals, GZMB-expressing CD8⁺ TEM cells were mostly CD45RA positive, identifying them as the TEMRA subset [2]. In CRS, these subsets show minimal clonal overlap. Still, they are both linked to circulating CD8⁺ T cells, suggesting that the decision to adopt a GZMB⁺ or GZMK⁺ effector pathway may occur early during the immune response [37]. The distinction between GZMB⁺ and GZMK⁺ TEMs may parallel the bifurcation in Th1 versus Th2 lineages. GZMK⁺ CD8⁺ T cells and Th2 CD4⁺ cells tend to co-localize within tertiary lymphoid structures, indicating potential collaboration in promoting CRS pathogenesis.

Another transcriptionally distinct memory T cell subset is KLRC2⁺ (NKG2C⁺ GZMB⁻) CD8⁺ cells, comprising less than 1% of the total CD8⁺ T cell population [64]. *KLRC2*, which encodes the NKG2C receptor, is typically expressed in NKT-like cells and the CD8⁺ TEMRA subpopulation. This subset represents a unique branch in the putative CD8⁺ memory T cell differentiation pathway, distinct from NKT and TEMRA cells. Unlike other memory subsets that remain stable or increase with age, KLRC2⁺ population progressively declines.

CD8⁺ TEMRA cells are heterogeneous and can be divided into GNLY⁺ and CMC1⁺ subsets

Effector memory T cells re-expressing CD45RA (TEMRA cells) represent a distinct subset of CD8⁺ T cells with potent cytotoxic capacity and limited proliferative potential [28]. These cells are characterized by the loss of costimulatory markers CD28 and CD27, the re-expression of CD45RA, and the expression of exhaustion and cytotoxicity-associated markers, such as PD-1, CX3CR1, and CD57 [61]. Flow cytometry studies have shown that CD8⁺ TEMRA cells are a heterogeneous population, with a subset lacking CD57 expression retaining proliferative capacity [58, 69]. Although often characterized as terminally differentiated, a recent paper showed that memory CD8⁺ T cells re-express CD45RA after antigen clearance in vivo as they transition from the effector to memory phase, suggesting that memory CD8⁺ T cells of the TEMRA phenotype should not be generalized as terminally differentiated or dysfunctional [46].

The single-cell resolution has also shown the heterogeneity of CD8⁺ TEMRA or TEMRA-like cells. Pereira and colleagues demonstrated that aging drives the reprogramming of CD27⁻CD28⁻CD8⁺ effector memory T cells through Sestrin 2-mediated signaling, leading to the loss of TCR activity and the acquisition of innate-like cytotoxic function via the NKG2D–DAP12 complex [55]. In their scRNA-seq analysis, they demonstrated that CD8⁺ TEMRA cells uniformly expressed high levels of GNLY and NKG7, whereas subpopulations varied in the expression of other cytotoxic and NK cell–associated genes. Specifically, expression of FCGR3A (CD16), FCRL6, KLRD1 (CD94), KLRG1, TYROBP (DAP12), KLRK1 (NKG2D), KLRC1 (NKG2A), and KLRC2 (NKG2C) was more heterogeneous, suggesting functional diversity within the TEMRA compartment.

Recently, Lu et al. reported the presence of two CD8⁺ TEMRA subpopulations with high expression of *GZMB* and *GZMH*, and which were increased in old individuals [43]. We showed that multiple CD8⁺ TEMRA populations can be identified through single-cell transcriptome profiling of CCR7^{lo}CD45RA^{hi} cells [68]. Specifically, we resolved transcriptionally distinct TEMRA subpopulations based on shared expression of CD3, CD8A, CCL5, KLRD1, and NKG7. These subsets differed in their expression of *CMC1*, granzymes, and *EOMES*, reflecting transcriptional diversity within the TEMRA compartment. CMC1 is a chaperone protein for complex IV of the mitochondrial electron transport chain, ensuring the proper assembly and function of the respiratory

chain [8]. Two *CMC1*-expressing TEMRA clusters were enriched in old and CMV-positive individuals, whereas the third TEMRA cluster, which was *CMC1*-negative but had higher expression of granzymes, was increased in younger and CMV-negative individuals [68]. An integrative analysis of CD8⁺ T cells in cancer and inflammation revealed two distinct CD8⁺ TEMRA populations: GNLY⁺ and CMC1⁺ [74]. Compared to GNLY⁺ TEMRA cells, CMC1⁺ TEMRA cells showed elevated STAT1 and ISG expression, enhanced type 1/2 IFNs, TNF, and IL-12 signaling, and upregulation of innate immunity pathways and markers such as CD160, KLRB1, and KLRD1. Notably, CMC1⁺ CD8⁺ TEMRA cells had lower TCR diversity than GNLY⁺ CD8⁺ TEMRA cells. They also shared clonotypes with GZMK⁺ CD8⁺ T cells with exhausted cell markers LAG3 and TIGIT, and were enriched in immune-related adverse events following checkpoint inhibitor therapy.

CD8⁺ TEMRA cells accumulate in CMV-positive individuals

Substantial evidence indicates that chronic antigenic stimulation contributes to the progressive reshaping of the CD8⁺ T cell compartment during aging. A well-established example is latent CMV infection, which drives the expansion and accumulation of CD8⁺ TEMRA cells [1, 52, 53]. CMV pp65-specific CD8⁺ T cells comprise a heterogeneous population of effector-memory subsets, but are particularly enriched for the TEMRA phenotype [68]. Recent single-cell RNA sequencing analyses further demonstrated that CMV-specific CD8⁺ memory T cells are dominated by a TEMRA cluster characterized by high expression of activation and cytotoxicity-associated genes, including *TNFRSF9*, *XCL1*, *CRTAM*, and *EGR2* [20, 33].

Interestingly, CMV infection represents an exception among chronic viral infections. Virus-specific CD8⁺ T cells in persistent infections such as HIV, HCV, and EBV typically display a CD45RA⁻CCR7⁻ TEM phenotype, with relatively few CD8⁺ TEMRA or TCM cells [1]. In contrast, chronic CMV infection is distinct, as the majority of CMV-specific CD8⁺ T cells exhibit the TEMRA phenotype (CD45RA⁺CCR7⁻). As noted earlier, a recent study by McGuire and colleagues [46] suggested that CD45RA re-expression in CD8⁺ effector memory cells reflects low or absent antigenic stimulation and is dynamically regulated by antigen exposure. CMV-specific CD45RA⁻ effector CD8⁺ T cells begin to re-express CD45RA when cultured ex vivo with PBMCs in the absence of antigen. Conversely, when CD45RA⁺ TEMRA CMV-specific cells are cultured in the presence of CMV antigen, they downregulate CD45RA and upregulate CD45RO expression. In this light, TCR stimulation governs the bidirectional transitions between CD45RA and CD45RO: antigen exposure promotes the CD45RA-to-CD45RO switch, while the absence of antigen favors CD45RO-to-CD45RA conversion in CMV-specific CD8⁺ T cells.

What underlies the prominent accumulation of CMV-specific CD8⁺ TEMRA cells with advancing age? If CD45RA upregulation is related to the presence of its antigen, this expansion should be primarily driven by CMV infection itself. Can the inflammatory environment or intrinsic aging-associated changes in T cell homeostasis also play a role? Because CMV seropositivity is prevalent in older populations, and because subclinical CMV reactivation or intermittent antigenic exposure becomes more frequent in the context of chronic disease and systemic inflammation, it is difficult to disentangle the relative contribution of each factor. By inducing chronic immune stimulation over time, CMV is thought to contribute to inflammation. One plausible scenario is that following transient CMV reactivation, virus-specific CD8⁺ T cells contract into a long-lived CD8⁺ TEMRA pool that remains poised for rapid reactivation upon subsequent antigen encounter. Notably, CMV reactivation has been linked to monocyte differentiation into macrophages and to proinflammatory cytokine production [27] - processes that are themselves enhanced during chronic inflammation and aging. These observations suggest CMV, inflammation, and immune aging to be mechanistically intertwined. Nevertheless, a recent scRNA-seq profiling of CMV-positive and negative individuals suggests distinct impacts of CMV infection and age on CD8⁺ effector memory T cells [25]. The frequencies of CD8⁺ GZMB⁺ CD27⁻ and GZMK⁺ CD27⁻ T cells increased in CMV seropositive individuals regardless of age, and except decrease in adaptive NK cells, age modestly impacted the effector memory cells in healthy adults. Further investigations, particularly longitudinal studies, are warranted to address these questions.

T cell exhaustion in aging

T cell exhaustion is a distinct state of T cell dysfunction that arises during chronic infections and cancer, characterized by the progressive loss of effector functions and sustained expression of multiple inhibitory receptors, including PD-1, CTLA-4, LAG-3, and TIM-3, among others [3], [72] . Chronic antigen stimulation promotes T cell exhaustion, and an increased frequency of exhausted T cells has been observed in chronic infections and cancer, where persistent antigen exposure leads to impaired immune function [3].

Populations of GZMK⁺ T cells and TEMRA cells have been characterized as exhausted due to their expression of *PDCD1*, encoding PD-1. However, several single-cell RNA sequencing studies have identified distinct CD8⁺ T cell subsets that also exhibit features of exhaustion.

Zheng et al. identified a PDCD1$^+$ CD8$^+$ T cell population, different from GZMK$^+$ effector memory cells, that was found to be increased in abundance in aged individuals [77]. More recently, Luo et al. described a population of exhausted T cells characterized by high expression of the long noncoding RNAs NEAT1 and MALAT1 [44]. Interestingly, these cells were found to be increased in abundance in both healthy older adults and frail elderly individuals. Our recent integrative scRNA-seq analysis of MALAT1$^+$ T cells, revealed that they do not universally express the exhaustion marker *PDCD1* and instead comprise a heterogeneous population of naïve-like (CCR7$^+$) and NK/effector-like (NKG7$^+$EOMES$^+$) cells [18]. Notably, we did not observe a consistent increase in the abundance of these cells across aging cohorts.

The functional distinction between GZMK$^+$ TEM cells and exhausted PD-1$^+$ CD8$^+$ T cells remains to be investigated. While both subsets express PD-1 and share transcriptional features, it is unclear whether PD-1 expression on GZMK$^+$ cells denotes early exhaustion or an activated, inflammation-driving phenotype. Further delineation of these subsets using functional assays is needed to clarify their roles in immune aging and disease pathogenesis.

Non-canonical CD8$^+$ T cell subsets in aging

Mucosal-associated invariant T (MAIT) cells are a subset of unconventional T cells defined by their semi-invariant TCRα chain, which includes the *TRAV1-2* gene segment, and by the expression of markers such as *KLRB1*, *ZBTB16*, and *SLC4A10* [57]. These cells recognize small microbial metabolite ligands presented by the monomorphic MHC class I-related protein MR1 and typically express either CD8αα or CD8αβ coreceptors, with CD8 interacting directly with MR1 to promote optimal MAIT cell activation and cytokine production. CD8$^+$ MAIT cells become enriched during adolescence; however, multiple studies have shown a specific, age-dependent decline in their frequency after the age of 20–30 years [18, 41, 71]. Despite reduced numbers and clonal expansions with age, MAIT cells generally retain their function, though chronic inflammation may impair their effectiveness [36, 41]. Increase in MAIT cells in adulthood can be associated with their antimicrobial effector function as the antimicrobial activities of MAIT cells from young adults (aged 18 years) are higher than those from middle-aged counterparts (aged 50 years) [71].

MAIT cells have been identified within the naïve CD8$^+$ T cell pool, but with no age-related decline observed in this subpopulation [67]. In the same study, Thomson et al. also described a distinct, non-canonical CD8αα population within the naïve compartment, which was present in children but nearly absent in adults. These cells exhibited features of both naïve (*LEF1*) and effector cells (*EOMES* and *TBX21*), and were characterized by elevated *IL21R* and poised *IFNG* expression.

The majority of γδ T cells are negative for CD4 and CD8 markers, although some express the CD8 marker in chronic infection and inflammation in humans (Chowdhury et al., 2023). Similar to MAIT cells, γδ T cells show an increase from childhood to adolescence and subsequent decline in older adulthood [71]. The age-dependent decrease of γδ T cells is seen among the innate-like Vγ9$^+$Vδ2$^+$ population, while Vδ1$^+$ T cells are maintained upon aging [36]. A recent study clustered γδ T cells into three subsets: GZMB$^+$, GZMK$^+$, and naïve SOX4$^+$ γδ T cells, which all follow the pattern of increase and decline with age [50].

Natural killer T (NKT) cells are another innate-like T cell subset that recognizes lipid antigens presented by CD1d, contributing to immune regulation and host defense through rapid cytokine production and cytotoxic activity [54]. Terekhova et al. identified a GZMB-expressing cluster marked by high surface levels of CD8 and CD56, along with strong transcriptional expression of *TYROBP*, and classified these cells as NKT-like based on this distinct marker combination [64]. These NKT-like cells did not change in abundance with age; however, they showed extensive TCR clonotype overlap—sharing 70–90% of clones—with effector memory, HLA-DR$^+$, and proliferative CD8$^+$ T cell subsets.

Clonal decline and antigen experience shape aging CD8$^+$ T cells

A study analyzing paired αβ TCRs from 30 human donors estimated the lower bound of TCR richness at 3 × 10^8 and 8 × 10^7 for CD4$^+$ and CD8$^+$ T cells per individual, respectively. However, these calculations likely underestimate the true diversity [63]. Although highly individual, the overall richness of the TCR repertoire is approximately 3–5 times lower in CD8$^+$ T cells than in CD4$^+$ T cells [38].

While both CD4$^+$ and CD8$^+$ T cells show age-related declines in diversity, the reduction is more prominent in CD8$^+$ T cells. As expected, clonal richness is greatest in the naïve CD8$^+$ TCRβ repertoire [38]. In naïve CD8$^+$ cells, TCRα richness decreases by 2.2% per year and TCRβ by 3.5%, compared to declines of 0.7% and 2.3% per year in naïve CD4$^+$ cells, respectively [63]. In contrast, memory CD8$^+$ TCRβ richness remains relatively stable with age. This disproportionate erosion of diversity in naïve CD8$^+$ T cells likely contributes to impaired cytotoxic responses in the aging population. Another key difference is the greater age-related increase in TCR sequence overlap between naïve and memory compartments in CD8$^+$ versus CD4$^+$ T cells. In donors over 70, shared TCR sequences reached 42% (TCRα) and 45% (TCRβ) in CD8$^+$ T cells, compared to just 8% and 10% in CD4$^+$ cells [63],

suggesting stronger lineage convergence in the CD8⁺ pool with aging.

Age results in a significant clonal expansion of TCRβ repertoires in CD8⁺ memory cells [63]. Recent integrated single-cell RNA and TCR sequencing analyses have demonstrated that effector memory subsets, including CD4⁺ and CD8⁺ GNLY⁺ cells, as well as CD8⁺ CMC1⁺ cells, account for the predominant proportion of clonally expanded cells in aging [71]. Many TCR clonotypes span multiple subsets, particularly among CD8⁺ TCM, TEM, and exhausted cells, but rarely overlap with naïve T cells. Nevertheless, in aged donors, the sharing of identical TCR sequences between naïve and memory cells is 4–5 times greater in CD8⁺ T cells than in CD4 + cells [63]. These public or shared clonotypes appear more frequently in adults and elderly individuals, indicating a gradual decline in repertoire complexity over time [44, 63]. Expectedly, the public clonotypes often recognize common and recurrent viruses such as CMV, EBV, and IAV. Luo et al. demonstrated that CMV-specific αβ TCR clones were abundant and exclusively detected in older adults, reflecting in vivo clonal expansion over time. CMV-specific CD8⁺ T cells are memory cells, consistent with prior exposure to the antigen [44, 68]. While cumulative antigen stimulation appears to shape TCR persistence, the influence of shared MHC-independent factors in promoting public TCRs remains unresolved.

Accumulation of transcriptome heterogeneity and loss of effector cell identity

The single-cell transcriptome analysis can reveal patterns not detectable at the bulk cell level. For example, the transcriptomes from 120,000 cells across CD8⁺ T-cell subpopulations showed that more genes exhibit increased rather than decreased expression with age [43]. Lu et al. identified three mechanisms underlying age-associated changes in CD8⁺ T cells: changes in the proportion of cells expressing a gene, changes in the expression level per cell, or both. Among genes that increase with age, 46% changed in percentage, 12% in expression level, and 14% in both, whereas for genes that decrease with age, these proportions were 3%, 24%, and 20%, respectively. The increased-percentage genes were linked to exocytosis and oxidative phosphorylation, suggesting heightened cytotoxic and metabolic activity in aged T cells, while increased-expression genes related to antigen processing and presentation. Decreased-expression genes were associated with DNA replication, indicating diminished proliferative capacity. These findings underscore the cell-state-dependent gene regulatory changes that occur during immune aging.

The concept of "exdifferentiation" suggests that cells progressively lose their specialized identity with age. The bulk transcriptomic studies have suggested that cellular identity declines in aging tissues. scRNA-seq can provide a more detailed analysis of whether individual cells deviate from their transcriptional programs over time. First studies in mice examining cell-to-cell gene expression variability identified age-related effects in CD8⁺ T cells [34]. The direction of this variability, whether increased or decreased, depended on the tissue of origin. For example, kidney-resident CD8⁺ T cells exhibited reduced transcriptional heterogeneity with age, while CD8⁺ T cells from the lung and spleen showed increased variability. In humans, Luo and colleagues assessed transcriptional heterogeneity among CD8⁺ T cells from umbilical cord blood of young, old, and frail individuals [44]. Measuring heterogeneity using the average transcriptional distance from each cell to the group centroid, they observed differences between frail individuals and the other groups. More recently, analyzing gene expression across two large cohorts, comprising four million immune cells, Connolly and colleagues showed a pronounced decline in the number of differentially expressed genes in effector CD8⁺ T cells with age [12].

While cellular identity tends to decline with age, highly differentiated T cell subsets increasingly emerge [61]. A decline in effector cell plasticity, which may impair the ability to respond efficiently to immune challenges, is one of the hallmarks of T cell aging [47]. This loss of plasticity is primarily associated with CD4⁺ T cell lineages; however, oligoclonal CD8⁺ T cells expressing GZMK, along with TEMRA cells, also represent differentiated states. An extreme polarization likely reflects tight regulation by specific transcription factors.

The interaction between the decline of cellular plasticity and the increased transcriptional heterogeneity observed in scRNA-seq studies remains an open question. The accumulation of transcriptomic variation with age may depend on the number of cell divisions undergone by CD8⁺ T cells. Single-cell analyses of aged individuals show that naïve CD8⁺ T cells remain a relatively homogeneous population, whereas most diversification occurs within the memory subsets [30] (Fig. 2). However, the extent of transcriptomic variation differs due to differences in proliferation dynamics. It is conceivable that in naïve CD8⁺ T cells, slow homeostatic turnover, combined with efficient DNA repair mechanisms, helps to prevent the accumulation of genetic and transcriptomic variation. In contrast, memory cells undergo rapid and extensive clonal expansion, which accelerates the buildup of variation. Over time, the accumulation of genetic damage in memory cells disrupts cellular homeostasis, ultimately leading to cell death and the loss of intermediate subsets within the memory compartment. For example, Lu et al. used single-cell transcriptome data from CD8⁺ T cells to construct a linear model that predicts biological age, which they then correlated with the mutational

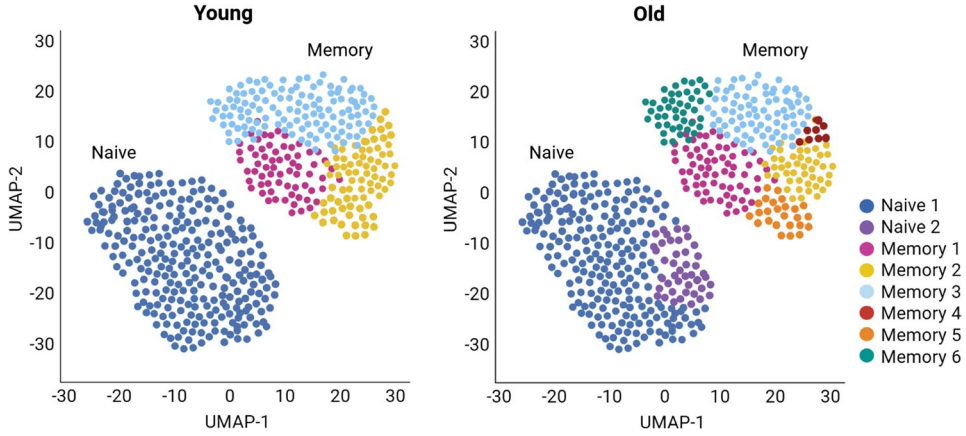

Fig. 2 In aged individuals, naïve CD8⁺ T cells remain relatively homogeneous, while most transcriptional and cellular diversification occurs within memory subsets. This difference reflects distinct proliferation dynamics: naïve CD8⁺ T cells undergo slow homeostatic turnover with minimal genetic and transcriptomic variation, whereas memory cells experience rapid clonal expansion that accelerates variation. Over time, accumulated genetic damage in memory cells amplifies transcriptomic diversity, potentially disrupting homeostasis, and reshaping the memory compartment

burden across naive, EM, CM and TEMRA subsets. Among these, the TEMRA population showed the greatest age-associated increase in mutational burden [43].

GZMK⁺ cells May have a key role in triggering complement activation

GZMK is a conserved serine protease, typically expressed at higher levels by GZMK⁺ cells than by GZMB⁺. In contrast to classical cytotoxic GZMB⁺ effector memory cells, GZMK⁺ cells show low or no perforin expression, but secrete high concentrations of GZMK and can produce cytokines through both TCR-dependent and -independent mechanisms (Fig. 3). They are enriched in peripheral tissues such as the liver and adipose tissue, where GZMK expression is observed in 20–60% of CD8⁺ T cells [15]. GZMK⁺ CD8⁺ T cells are also enriched in several inflammatory conditions, including inflamed synovial fluid from rheumatoid arthritis patients, chronic rhinosinusitis, lupus nephritis, ulcerative colitis, Crohn's disease, COVID-19, and tumors. These cells have been shown to promote airway inflammation.

While GZMB is the most cytotoxic of all granzymes, GZMK acts primarily as an extracellular protease, binding to plasma membranes via interactions with heparan sulfate glycosaminoglycans. GZMK⁺ CD8⁺ T cells are found in high numbers in aged humans and mice [48], and their accumulation has been linked to inflammation in the absence of infection. Inflammatory disease models using Gzmk-deficient mice have shown reduced disease severity and diminished complement activation, indicating that GZMK contributes to the inflammatory pathology of these conditions [15]. In addition to CD8⁺ T cells, human GZMK is highly expressed in innate lymphocytes, such as invariant NKT (iNKT) cells, γδ T cells, MAIT cells, CD56⁺ NK cells, and a subset of non-MAIT CD8⁺ T cells. Some of these innate T cell subsets express GZMK exclusively, without perforin or GZMB, further supporting the notion that GZMK is functionally distinct from GZMB.

Recent studies in mice demonstrated that GZMK acts as an initiator protease capable of cleaving complement components C2, C3, C4, and C5, collectively triggering complement activation [15, 37]. The complement system is an ancient immune defense mechanism composed of over 30 proteins, activated through the classical, lectin, or alternative pathways. These pathways converge at the formation of C3 and C5 convertases, which drive pathogen elimination through the membrane attack complex, opsonization, and inflammation. Although critical for host defense, excessive complement activation can damage tissues and exacerbate autoimmune diseases such as RA and lupus, with the initiating triggers in these diseases largely unknown. Thus, the investigations in mouse models suggest that GZMK-mediated complement activation represents a fourth pathway and may directly promote inflammation. Its ability to bind heparan sulfate glycosaminoglycans and cleave C2 and C4 enables GZMK to facilitate the assembly of membrane-bound C3 and C5 convertases, thus initiating the full complement cascade [15].

These findings uncover a novel mechanistic link between adaptive and innate immunity where a T cell–derived protease directly activates an ancient innate immune system. However, the antigenic or inflammatory signals that drive GZMK⁺ T-cell clones into tissues and trigger GZMK secretion are still unknown. Also, the potential role of GZMK as a complement activator in humans remains to be clarified and needs further investigation. Nevertheless, targeting GZMK, rather than employing systemic complement inhibitors, could selectively disrupt pathogenic feedback loops in inflamed

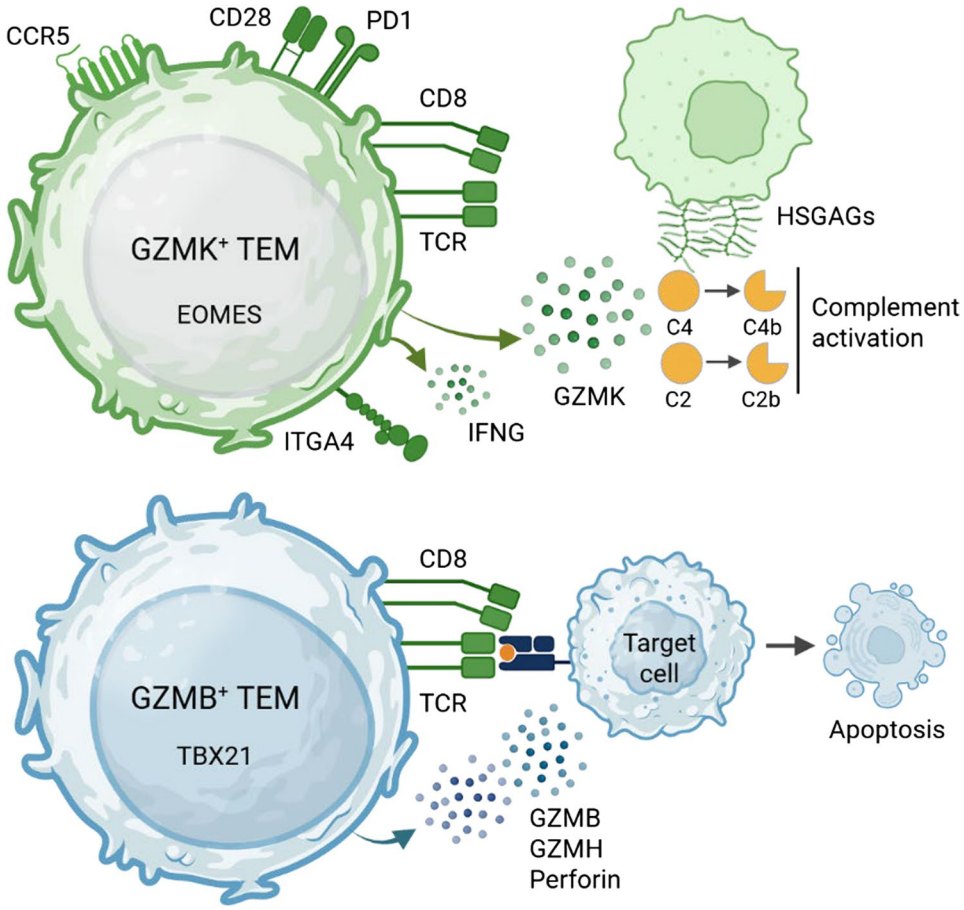

Fig. 3 $CD8^+$ $GZMK^+$ and $GZMB^+$ effector memory T cells. $GZMK^+$ and $GZMB^+$ $CD8^+$ T cells display distinct profiles of surface markers, cytotoxic enzymes, and transcription factors. $GZMK^+$ cells produce high levels of IFNγ and TNFα. They cleave complement components such as C2 and C4, promoting the formation of a C5 convertase that subsequently cleaves C5. In contrast, $GZMB^+$ cells represent the classical cytotoxic $CD8^+$ T cell subset, characterized by high levels of GZMB, GZMH, and perforin, which engage target cells through TCR and induce apoptosis

tissues while preserving the antimicrobial functions of the complement system.

Conclusion

Advancements in single-cell technologies have substantially deepened our understanding of $CD8^+$ T cell heterogeneity and their changes in human aging and chronic inflammatory diseases. Over the years, the $CD8^+$ T cell compartment changes through complex shifts in subset composition, clonal dynamics, and transcriptional states, which is reflected by the decrease in naïve cells, expansion of pro-inflammatory $GZMK^+$ effector memory cells and the accumulation of functionally diverse TEMRA subsets. While the phenotypic and transcriptional heterogeneity of $CD8^+$ T cells in the context of aging and age-related inflammation is well-documented, further studies on the functional consequences are needed. For instance, the expansion of $GZMK^+$ TEM cells likely contributes not only to inflammation but may also impair effective antiviral and vaccine responses in older adults by skewing cytokine profiles. Similarly, increased clonal expansions and reduced TCR diversity in aged TEMRA and exhausted $CD8^+$ T cells can compromise immune surveillance against infections and malignancies, highlighting translational opportunities for immune rejuvenation therapies. Integrative multi-omics and longitudinal studies will be important for elucidating the regulatory networks that drive $CD8^+$ T cell aging and for identifying potential drug targets for immunomodulatory therapies. Harnessing the plasticity within the aged $CD8^+$ T cell pool offers a promising strategy for restoring immune resilience and reducing inflammation-driven pathology in older adults.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
The authors give consent for the publication of the manuscript.

Competing interests
The authors declare no competing interests.

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