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Investigating age-related changes in the immune system and
how they impact cognitive function

by
Juliana Sucharov Costa

THESIS

Submitted in partial satisfaction of the requirements for degree of
DOCTOR OF PHILOSOPHY

in

Biomedical Sciences

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

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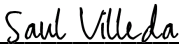


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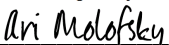
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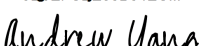
Saul Villeda

DocuSigned by:



Ari Molofsky

DocuSigned by:



Andrew Yang

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Committee Members

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Acknowledgements

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undergraduate, post-baccalaureate, and graduate students; serving as a teaching assistant for his course, Critical Thinking and Scientific Communication, and advising students through the SRTP program at UCSF. These opportunities helped me discover how deeply I value mentorship and teaching, and they will remain among my favorite parts of graduate school.

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Contributions

Chapter 1

J.S. and S.A.V. developed the concept and designed experiments. J.S. performed in vivo and molecular studies. J.S. wrote this chapter. S.A.V. supervised all aspects of the project.

Chapter 2

J.S. and S.A.V. developed the concept and designed experiments. J.S. collected and analyzed data. J.S. performed molecular, biochemical, and in vivo studies and behavioral analyses. G.B., K.J.B.P., T.A., A.P., R.L., R.C., C.H., and A.S. assisted with molecular, biochemical, and in vivo studies. J.C. assisted with RNAseq analysis. J.S. and G.B. generated schematics. J.S. and S.A.V. wrote the manuscript. S.A.V. supervised all aspects of this project. All authors had the opportunity to discuss results and comment on the manuscript.

Investigating age-related changes in the immune system and how they impact cognitive function

Juliana Sucharov

ABSTRACT

Changes in peripheral CD8⁺ T cells are a hallmark of immune aging. However, the role of aged non-infiltrating CD8⁺ T cells in brain aging remains to be fully defined. Here, we show that aged circulating CD8⁺ T cells and their secreted factors drive hippocampal-dependent cognitive decline. Using heterochronic parabiosis and transcriptomics analysis we observed that peripheral CD8⁺ T cells maintain properties intrinsic to their age. Systemic exposure of young mice to aged CD8⁺ T cells elicited synaptic-related hippocampal changes and impaired cognition, while inhibiting activation, rather than infiltration, mitigated their pro-aging effects. Conversely, targeting aged circulating CD8⁺ T cells restored youthful signatures and rescued cognition. Mechanistically, we identified Granzyme K (GZMK) as a secreted pro-aging CD8⁺ T cell-derived factor in plasma, inhibition of which rescues cognition in aged animals. Together, our data identify activated aged CD8⁺ T cell-derived circulating factors as potential therapeutic targets to rescue cognition in old age.

Table of Contents

Chapter 1. Defining shifts in immune cell states in aging and models of cognitive rescue and decline	1
Materials and Methods	22
References	31
Chapter 2. Peripheral aged CD8+ T cells drive cognitive decline through secreted factors	35
Materials and Methods	66
References	80

List of Figures

Chapter 1

Figure 1.1. Hippocampal-dependent learning and memory declines with age.....	7
Figure 1.2. The peripheral immune system shows major population shifts that coincide with age-related cognitive decline.....	9
Figure 1.3. CD8+ T cell infiltration into the vascular and perivascular spaces of the hippocampus increases with age	11
Figure 1.4. CITE-seq analysis identifies splenic immune cells across parabiosis conditions.....	13
Figure 1.5. Peripheral T cell populations are largely refractory to a young or aged systemic milieu.....	15
Figure 1.6. Peripheral CD8+ T cells maintain transcriptomic profiles regardless of environment	17
Figure 1.7. CD8+ T cells retain age-specific pseudotime profiles regardless of environment.....	19

Chapter 2

Figure 2.1. Aged circulating CD8+ T cells elicit aging transcriptional signature in the young hippocampus and promote cognitive impairments	40
Figure 2.2. Aged circulating CD8+ T cells elicit changes in neurons and barrier cells without driving neuroinflammation.....	43
Figure 2.3. Pro-aging hippocampal effects of aged CD8+ T cells are dependent on GPCR signaling.....	47

Figure 2.4. Activation of aged CD8+ T cells, rather than infiltration into tissue, is necessary for pro-aging cognitive effects	50
Figure 2.5. Depletion of circulating aged CD8+ T cells restores hippocampal transcriptional profiles and rescues cognition.....	54
Figure 2.6. Aged CD8+ T cell-derived systemic GZMK promotes cognitive decline	60

Chapter 1

Defining shifts in immune cell states in aging and models of cognitive rescue and decline

ABSTRACT

Aging is associated with both cognitive decline and prominent changes in the immune system, driven in part by the systemic environment. However, the link between the peripheral immune system and cognitive aging is not fully defined. Additionally, while age-related shifts in immune cell states are reported in heterochronic parabiosis, a model of cognitive rescue and decline, lack of separation from donor and recipient has left the role of the systemic environment on the immune system unknown. Here, we confirm age-related decline in hippocampal dependent cognitive function. Within young and cognitively impaired aged mice, we report shifts in populations across a variety of immune cells in the periphery, however, only identify an increase of CD8⁺ T cells within the hippocampus. Utilizing heterochronic parabiosis, we find that T cells exhibit age-intrinsic transcriptional programs that are resistant to rejuvenation by a young systemic environment. These findings highlight the critical role of immune aging in cognitive decline and establish parabiosis as a valuable model for understanding how individual immune populations respond to an aging or rejuvenating environment, as well as identify T cells as uniquely refractory to their systemic milieu.

Introduction

Healthy aging is associated with significant impairments in cognitive function and given the rate at which the human population is aging, it has become critical to identify key drivers of age-related cognitive decline. The hippocampus, or the region of the brain primarily responsible for learning and memory, is particularly susceptible to age-related cognitive decline and is one of the first regions to show hallmarks of brain aging. We and others have demonstrated that pro-aging systemic interventions, such as heterochronic parabiosis, aged plasma transfer, and transplantation of an aged hematopoietic system into young mice, promote cognitive dysfunction in young animals, yet the systemic drivers of these impairments remain incompletely understood. Interestingly, there are known age-related changes in the peripheral immune system, with an emerging role as a driver of brain dysfunction. Indeed, the peripheral immune system is associated with hippocampal function, with B cells, myeloid cells, natural killer cells, neutrophils, and T cells implicated in models of brain aging or neurodegeneration.

Transcriptional and flow cytometry analyses of young and aged peripheral immune cells identify a multitude of changes in the immune system. With age, in a process dubbed “inflammaging”, immune cells become less functional, yet more inflammatory with an increased release of cytotoxic molecules. Specifically, there is a shift in the myeloid-lymphoid output from hematopoietic stem cells, with an increased production of myeloid cells in age, and a shift in the CD8⁺:CD4⁺ T cell ratio, resulting in a higher proportion of CD8⁺ T cells. Within B and T cells, there is a loss of naïve, antigen inexperienced cells, and an increase in memory populations as well as in regulatory T cells. With chronic inflammation, CD8⁺ T cells develop into an exhausted state, where

they can no longer sufficiently respond to immune challenges and have increased resistance to apoptosis and a loss of regenerative capacity, but are maintained in a highly inflammatory state. CD8⁺ T cells in particular are identified as a prominent hallmark of immune aging, with the emergence of an age-associated population characterized by expression of the serine protease Granzyme K (GZMK) as the most highly increased population with age across a variety of mouse tissues. This population is also identified in humans with age, as a pre-exhausted population. While CD8⁺ T cells are a strong hallmark of peripheral immune aging, only modest CD8⁺ T cell infiltration was reported in the aged hippocampus in the absence of neurodegenerative disease pathology in mouse models, while analysis of post-mortem human hippocampi found no age-related changes in infiltration.

Heterochronic parabiosis, a model of cognitive and systemic decline where the circulatory systems of a young and aged animal are joined, identified age-related shifts in the immune system of the young animal and rejuvenation of the immune system in the aged animal. However, as donor and recipient cells have not been studied individually in previous studies, the question remains as to whether these changes are driven by migration of aged cells to the young heterochronic parabiont, and vice versa, or if the immune cells are responding to the young or aged environment. As the hippocampus, and subsequently cognitive function, are affected by the systemic environment, it is unknown whether immune cell changes are also driven by the systemic environment, or whether there may be cells that are refractory and therefore potential drivers of aging.

RESULTS

Assessing age-related changes in hippocampal-dependent learning and memory

To identify a baseline for age-related changes in cognition, we performed several hippocampal-dependent behavioral tasks on young (4 months) and aged (22 months) male mice (Figure 1.1A). We first assessed cognition with the novel object recognition (NOR) task, where mice are introduced to two identical objects for 5 minutes. 24 hours later, on day 2, one of the objects is swapped for a novel object and the amount of time the mouse spends with each object is recorded. Cognition is measured as percent time spent with the novel object, and learning is measured as significance of time spent above a baseline of 50%, or equal time with both objects. Young mice were able to differentiate between the novel and trained object, spending significantly more than 50% of the time with the novel object. Aged mice, however, were unable to differentiate between the novel and trained object and spent on average 50% of the time with each object (Figure 1.1B). To assess spatial learning and memory, we utilized the radial arm water maze (RAWM) task, where mice are placed in a random start arm within an 8-arm water maze and must find their exit platform. Cognition is measured as the number of errors made, or entries into a wrong arm, prior to reaching the goal arm or end time of 60 seconds. Aged mice performed significantly worse than young mice during the training phase, as well as when testing short-term and long-term memory, or day 1 block 5 and day 2, respectively, demonstrating age-related deficits in spatial learning and memory (Figure 1.1C). Lastly, we tested working memory with the Y-maze paradigm. Within this task, mice are trained on two arms of a Y-shaped maze. After 30 minutes, the mice are returned to the maze with all three arms open, and time within each arm is tracked. Young mice spent

significantly more time in the novel arm, both compared to the trained arm and above baseline, or 33% of the time. In contrast, aged mice spent equal time across arms, indicating an inability to distinguish the novel from the trained arm (Figure 1.1D). Z-score analysis of the behavioral results and hierarchical clustering largely separates the young and aged animals, showing a consistent pattern of decline across behavioral tasks, with individual animal to animal variation across tasks (Figure 1.1E). Overall, mice display a decreased cognitive capacity in several aspects of hippocampal-dependent learning and memory.

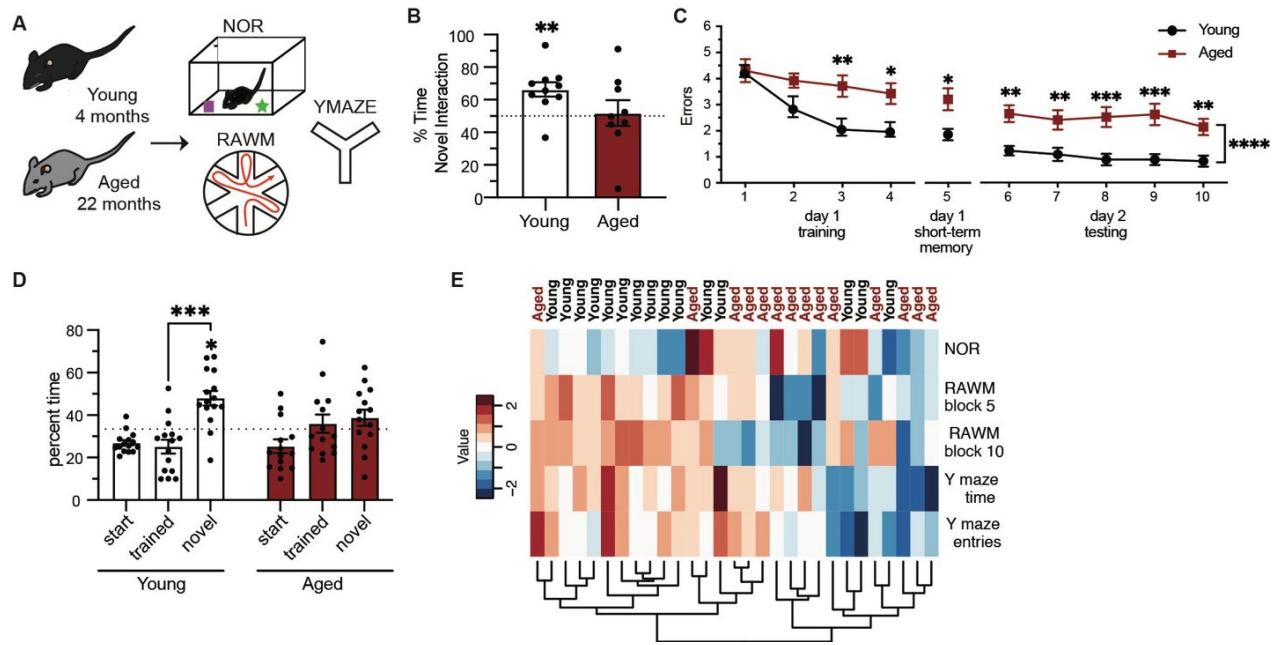


Figure 1.1. Hippocampal-dependent learning and memory declines with age.

A, Schematic illustrating the behavior paradigms for young (4 months) or aged (22 months) mice (n=9-10/group).

B, Object recognition memory as assessed by Novel Object Recognition (NOR) and quantified as percent time spent exploring the novel object (n=15/group). Data is compared against 50%, or equal time with each object.

C, Hippocampal-dependent learning and memory as evaluated by radial arm water maze (RAWM). Changes in cognition were quantified as number of errors while attempting to find the goal arm (n=12-15/group).

D, Short-term memory as assessed by Y Maze and quantified as percent entries into each arm (n=14-15/group)

E, Z-scores of behavioral results, ordered by hierarchical clustering.

Data shown as mean $\pm$ SEM; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Statistical analysis was performed using (B) one-sample t-test vs 50%; (C,D) and two-way ANOVA with Tukey's post hoc.

Immune cell populations show widespread changes in aging

To next characterize shifts in the peripheral immune system with age, spleens from young (4 months) and aged (22 months) mice were dissociated, and the immune cell populations were profiled by flow cytometry analysis. While we observed no change in lymphoid populations B cells, NKT cells, and overall CD8⁺ T cells, there was an age-related loss of NK cells and CD4⁺ T cells within the spleen (Figure 1.2A). Within the CD4⁺ and CD8⁺ T cells, there was loss of naïve and an increase of memory/effector populations, as well as an increase of Tregs in age. Notably, we observed an increase in the TOX⁺ portion of effector memory (EM) CD8⁺ T cells, which denote the age-associated population seen to increase across a variety of tissues (Figure 1.2A). Within the myeloid lineage of immune cells, only macrophages and neutrophils changed in frequency, with a loss of macrophages in the spleen and an increase of neutrophils ($p=0.0628$) (Figure 1.2B). Data suggest shifts in various immune populations in age that co-occur with decline in cognitive ability.

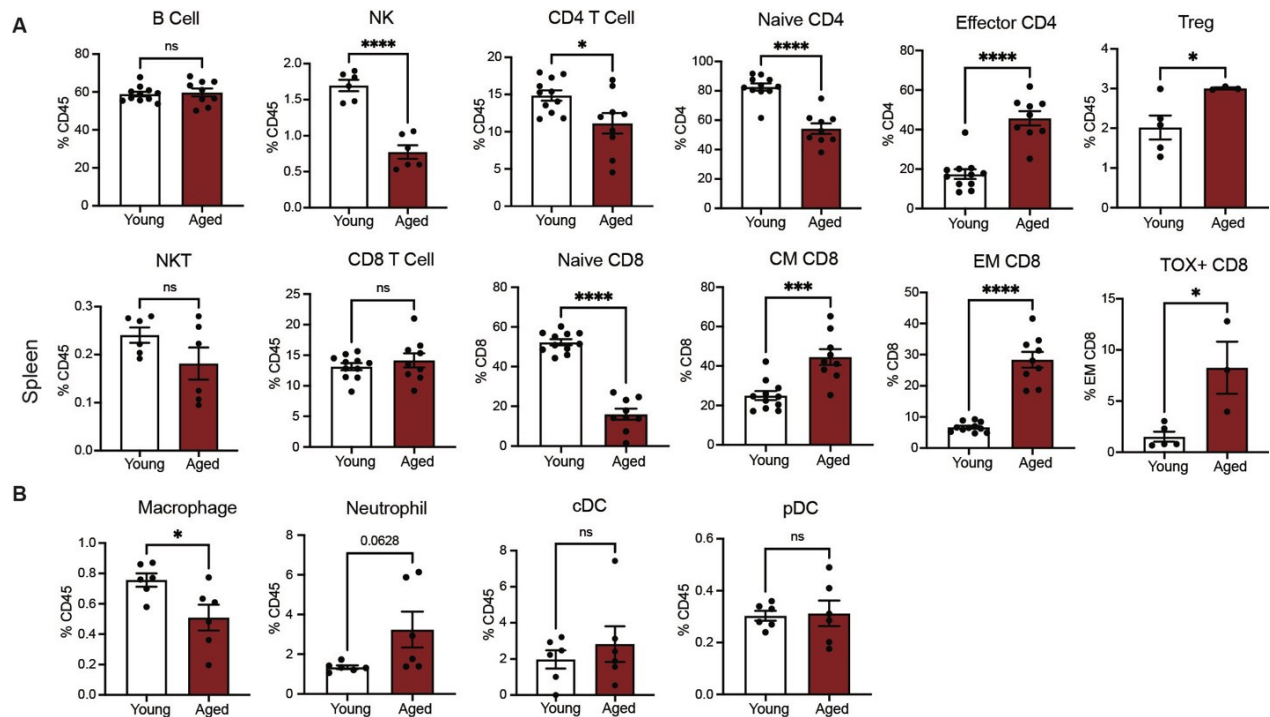


Figure 1.2. The peripheral immune system shows major population shifts that coincide with age-related cognitive decline.

A, Schematic illustrating the behavior paradigms for young (4 months) or aged (22 months) mice (n=9-10/group).

B, Object recognition memory as assessed by Novel Object Recognition (NOR) and quantified as percent time spent exploring the novel object (n=15/group). Data is compared against 50%, or equal time with each object.

Data shown as mean $\pm$ SEM; *p<0.05, ***p<0.001, ****p<0.0001. Statistical analysis was performed using (A,B) two-tail t-test.

Profiling immune cells in the hippocampus identifies increased T cell infiltration

While we observed broad age-related shifts in immune populations in the spleen, analysis of peripheral immune cells located in the hippocampus identified shifts in T cells and macrophages, with T cells being the only population to demonstrate increased localization to the hippocampus (Fig 1.3A). To further identify T cells localized in the hippocampus versus the surrounding vasculature, we injected the mice *i.v.* with a PE tagged CD45 antibody 2 minutes prior to hippocampal isolation to tag cells localized within the vasculature (Fig 1.3B). We identified the increase in T cells as specific to the CD8⁺ T cell compartment, with the increase primarily identified in infiltrating (*i.v.-*) cells (Fig 1.3C). To further classify the regions where infiltrating CD8⁺ T cells were located, we assessed CD8⁺ T cell infiltration with immunohistochemistry (IHC) in the young and aged hippocampus and surrounding regions and observed increased localization of CD8⁺ T cells in the vascular and perivascular spaces of the aged hippocampus, and in the vascular, perivascular, and parenchymal regions surrounding the aged hippocampus (Fig 1.3D-F). Overall, while we identified a variety of immune cells affected by aging, only the CD8⁺ T cells showed significant changes in population proportion and localization within the periphery and the hippocampus, with the age-related increase of CD8⁺ T cells within the hippocampus largely localized to the vascular and perivascular spaces.

CITE-sequencing to identify immune cell populations after heterochronic parabiosis

Considering the pro-aging effects observed in the hippocampus following heterochronic parabiosis and the widespread age-related changes in the peripheral immune system, we first assessed changes in peripheral immune cell trafficking and transcriptional responses to a young and aging systemic milieu using cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq) analysis. Young (4 months) and aged (20 months) mice were surgically joined into isochronic (young-young and old-old) and heterochronic (young-old) parabiotic pairings for 28 days (Fig 1.4A). To distinguish young versus aged cells in heterochronic parabionts, young CD45.1 B6/BoyJ and aged CD45.2 B6 mice were joined (Fig 1.4A). We performed CITE-seq analysis on splenocytes of young isochronic, aged isochronic, young heterochronic and aged heterochronic parabionts (Fig 1.4B,C). Signatures for each cluster were generated by differential gene expression analyses and cell types were established based on transcriptomic signature and canonical surface-markers (Fig 1.4D,E). All suspected immune populations were identified from each animal of identification and origin.

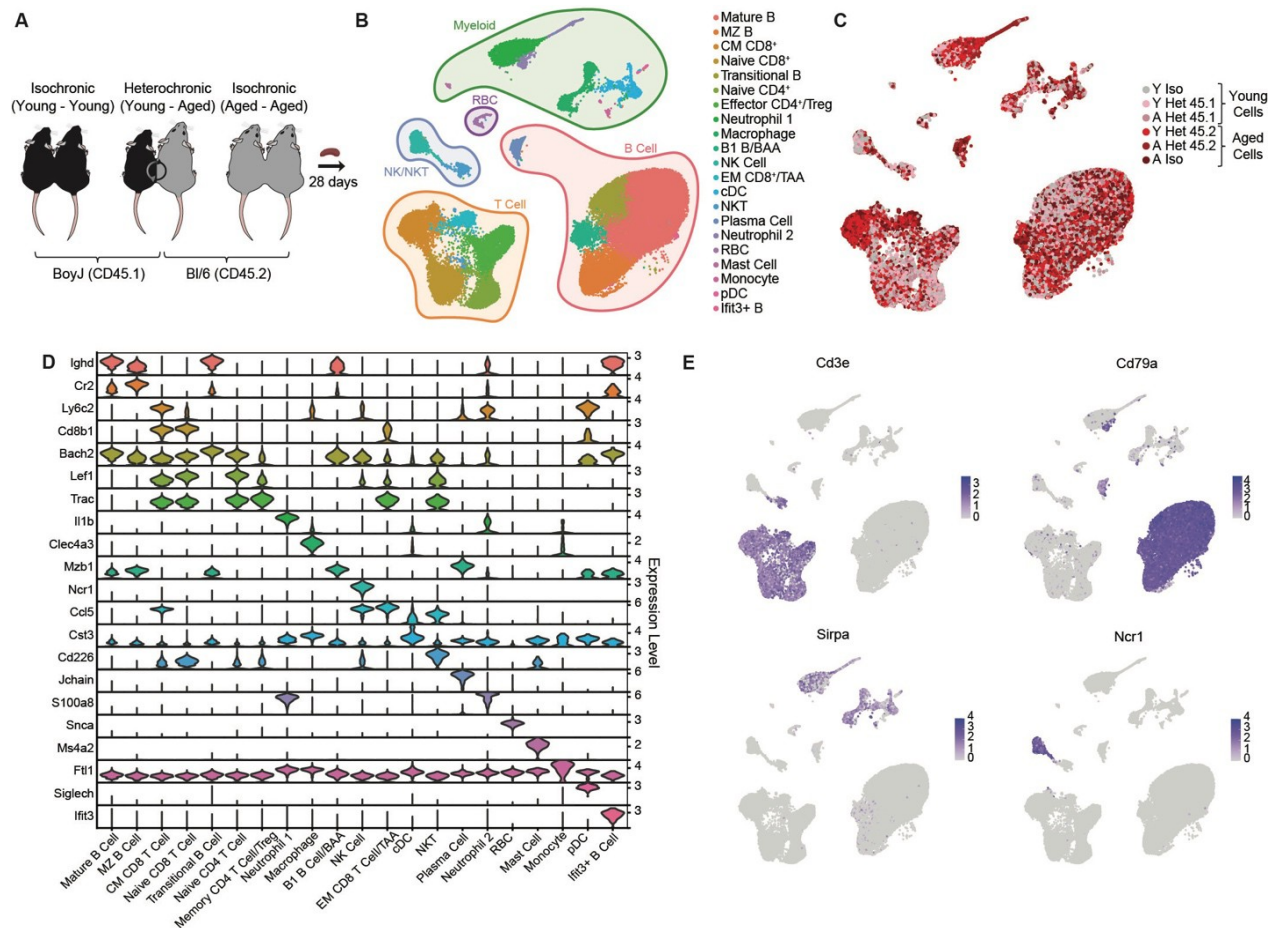


Figure 1.4. CITE-seq analysis identifies splenic immune cells across parabiosis conditions.

A, Schematic illustrating parabiosis pairs, followed by single cell RNA sequencing of splenocytes, sorted for CD45.1+ (4 months, BoyJ) or CD45.2+ (20 months BI/6), (n=4/group, combined after sorting).

B,C, UMAP projection of identified cell types (B) and of mouse identity of cell origin (C).

D, Representative genes for each cell type identified via single cell RNA sequencing of splenocytes (n=1/group).

E, Expression of cell type markers for T cells (Cd3e), B cells (Cd79a), Myeloid cells (Sirpa), or NK cells (Ncr1), overlain on UMAP projection of splenocytes (n=1/group). Statistical analysis was performed using (D,E) DESeq2.

Age-related immune population changes vary in response to environment, as measured by heterochronic parabiosis

We first confirmed bi-directional trafficking of heterochronic parabionts with flow cytometry (Fig 1.5A). Consistent with age-related changes, and as previously reported in heterochronic parabiosis, we observed a decrease in naïve CD4⁺ and CD8⁺ T cells, an increase in effector and memory CD4⁺ and CD8⁺ T cells, and the emergence of age-associated effector memory CD8⁺ T cells in aged isochronic compared to young isochronic parabionts (Fig 1.5B,C). In heterochronic parabiosis, we observed bidirectional trafficking of CD4⁺ and CD8⁺ T cells with the percentage of T cell populations reaching near equilibrium between young and aged parabionts (Fig 1.5B,C). Subsequently, we examined whether exposure to a young or aged systemic milieu altered the distribution of T cell populations intrinsic to their cellular age. We characterized T cells derived from young CD45.1 versus aged CD45.2 animals across isochronic and heterochronic parabiotic conditions and observed that age-related differences in the distribution of CD4⁺ and CD8⁺ T cell populations persisted irrespective of the age of the systemic milieu (Fig 1.5D), with separation of the biological replicates with CITE-seq antibodies CD45 and CD8a identifying statistical significance across young or aged groups compared to the aged or young control, respectively. Flow cytometry of effector memory (EM) CD8⁺ T cells confirmed sequencing results, as we observed significant increases in EM cells by age, not parabiotic condition. In contrast, other immune cell populations were not refractory to their environment (Fig 1.5G-I).

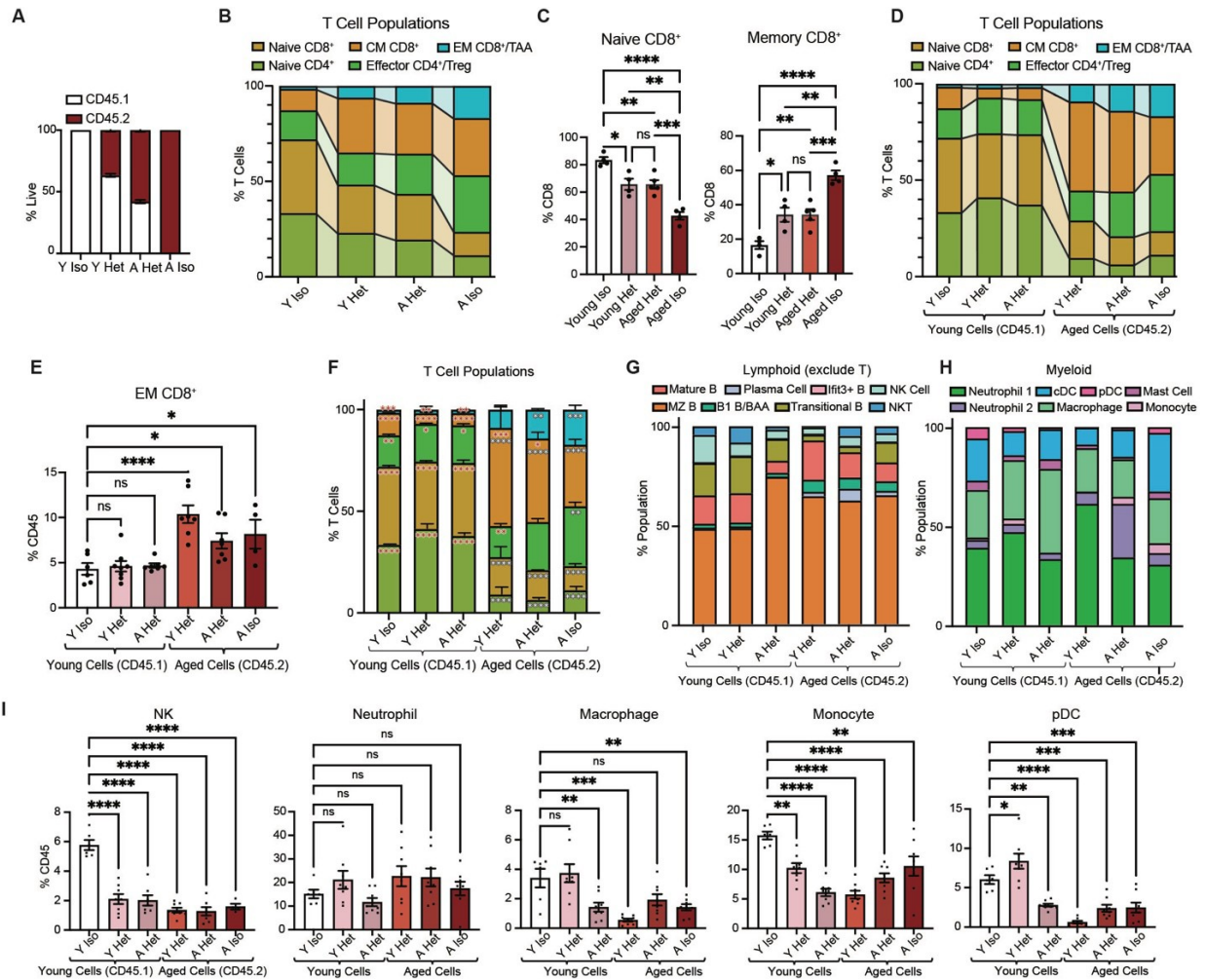


Figure 1.5. Peripheral T cell populations are largely refractory to a young or aged systemic milieu.

A, Ratio of CD45.1+ (young) or CD45.2+ (aged) cells in the spleen of parabionts by flow cytometry, showing chimerism in heterochronic pairs.

B, Percentages of splenocyte T cell populations by scRNAseq, split by mouse of origin.

C, Flow cytometry confirmation of changes in Naïve (CD44-) and Memory (CD44+) CD8⁺ T cell populations (n=4/group).

D, Percentages of splenocyte T cell populations by scRNAseq, split by origin of cells.

E, Flow cytometry confirmation of changes in Effector Memory (CD62L-) CD8⁺ T cell populations (n=4-7/group).

F, Percentages of splenocyte T cell populations by scRNAseq, split by origin of cells after CD45 +/- cell separation. White stars indicate significant population changes from the aged isochronic control and black stars indicate significant population changes from the young isochronic control.

G,H, Percentages of splenocyte lymphocyte (G) and myeloid (H) cell populations by scRNAseq.

I, Populations of NK cells, Neutrophils, Macrophages, Monocytes, and pDCs as measured by flow cytometry (n=4-7/group).

Statistical analysis was performed using one-way ANOVA with Tukey's post hoc (C,E,F,I).

The transcriptome of CD8⁺ T cells is refractory to the environment, as measured by heterochronic parabiosis

Considering that the populations of CD8⁺ T cells were refractory to their environment, we next assessed whether exposure to a young or aged systemic milieu altered the transcriptome of CD8⁺ T cells. While exposure to a young or aged systemic milieu elicited prominent transcriptional changes in myeloid and B cells, the number of differentially expressed genes (DEGs) remained similar within young or aged CD8⁺ T cells, regardless of environment (Figure 1.6A,B). Prominent age-dependent transcriptional changes were detected in CD8⁺ T cells from young compared to aged isochronic parabionts (Figure 1.6C), however those changes persisted in young and aged cells across heterochronic conditions (Figure 1.6D-G). Moreover, in aged CD45.2 CD8⁺ T cells, increased expression of age-associated effector memory T cell markers (e.g., *Gzmk*) and decreased expression of naïve T cell markers (e.g., *Lef1* and *Sell*) were observed regardless of the age of the systemic milieu and few significant changes in expression occurred compared to the age-matched control (Figure 1.6H).

Pseudotime analysis to assess T cell states after young or aged environment

Additionally, we examined the transcriptional aging progression of T cells following parabiosis by pseudotime analysis. Pseudotime analysis identified 6 pseudotime states along the T cell populations, which represent different T cell populations (Figure 1.7A-C). We observed that pseudotime trajectories from naïve to aging-associated effector memory states persisted in aged CD45.2 CD8⁺ T cells in both isochronic and heterochronic parabionts (Figure 1.7D). In particular, the pseudotime module representing the naïve cell states was increased in all young T cells, regardless of environment, and decreased in all aged T cells (Figure 1.7E). Conversely, the central memory CD8⁺ module showed the opposite pattern, with consistent expression across the same age of cells regardless of young or aged environment (Figure 1.7F).

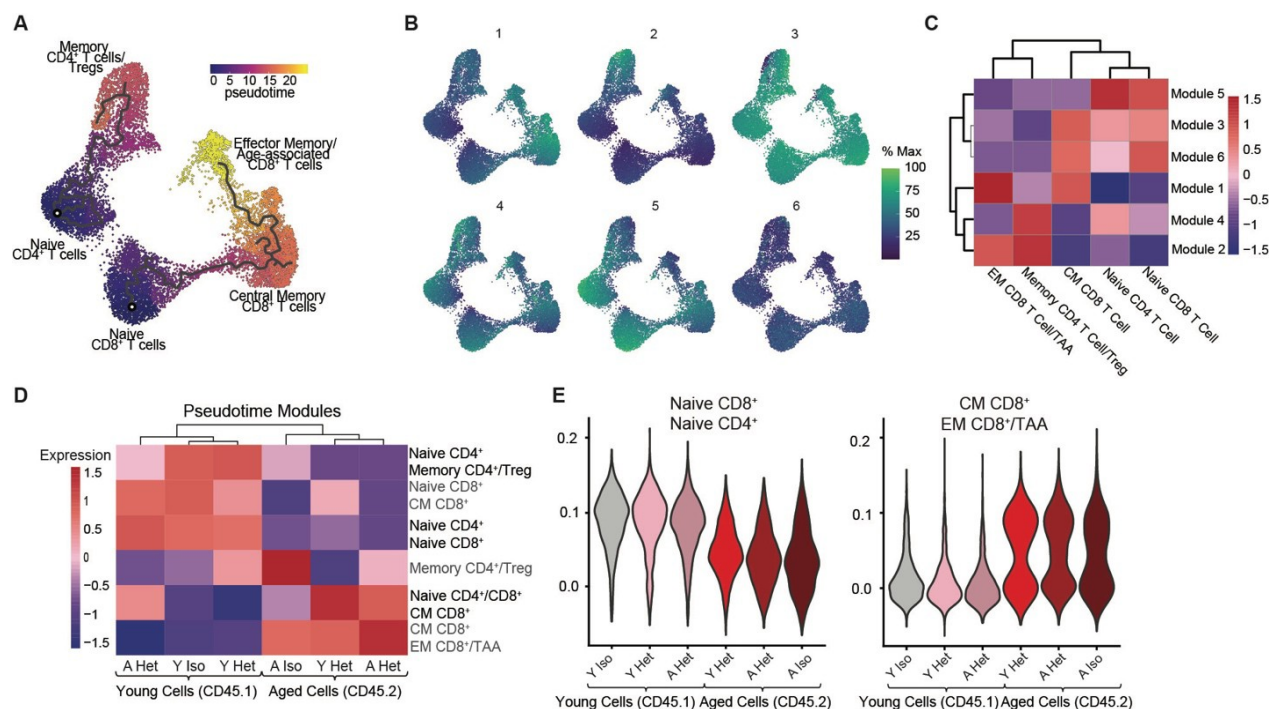


Figure 1.7. CD8⁺ T cells retain age-specific pseudotime profiles regardless of environment.

- A**, Pseudotime analysis of T cell populations overlain on UMAP projection of T cells.
B, Expression of pseudotime modules overlain on UMAP projection of T cells.
C, Heatmap of expression of pseudotime module genes within each T cell cluster.
D, Heatmap of pseudotime modules showing average expression of each module split by origin of cells and hierarchical clustering of cell origin.
E,F, Violin plot of pseudotime modules representing Naive (E) or Memory CD8⁺ T cells (F), split by origin of cells.
 Statistical analysis was performed using DESeq2 and Pseudotime analysis (A-F).

Discussion

This work identifies age-associated CD8⁺ T cells as a stable feature of immune aging that is refractory to exposure to a youthful systemic environment. While aging is accompanied by widespread cognitive decline and remodeling of the peripheral immune system, CD8⁺ T cells uniquely exhibited altered localization, persistent transcriptional identity, and resistance to environmental rejuvenation. Together, these findings suggest that intrinsic immune aging, rather than changes in circulating factors alone, may contribute to hippocampal dysfunction during aging.

Consistent with previous studies, aged mice displayed impairments across multiple hippocampal-dependent behavioral tasks, including object recognition memory, spatial learning, and working memory. The consistent deficits observed across independent assays indicate a broad decline in hippocampal function rather than impairments specific to a single behavioral paradigm. These behavioral changes occurred alongside hallmarks of immune aging, including reduced naïve T cells, expansion of memory and effector T-cell populations, increased regulatory T cells, and alterations within the myeloid compartment. Although these findings are consistent with previous descriptions of immune aging, they also provide a framework for identifying immune populations that may contribute to age-related cognitive decline.

Among the immune populations examined, CD8⁺ T cells emerged as the most striking age-associated population. Intravenous labeling and immunohistochemical analyses further revealed that these cells were predominantly located within vascular and perivascular compartments within the hippocampus. This localization is particularly intriguing given the growing recognition that immune cells residing at the brain borders

can influence neuronal function without extensive infiltration into the parenchyma. Rather than representing widespread immune invasion, the accumulation of CD8⁺ T cells at these interfaces may alter communication between the peripheral immune system and the aging brain.

These observations have important implications for therapeutic approaches targeting aging. While systemic rejuvenation strategies have shown considerable promise, our data suggest that not all immune populations are equally responsive to environmental manipulation. Instead, age-associated CD8⁺ T cells appear to retain stable molecular programs that may limit the effectiveness of interventions based solely on altering circulating factors. This resistance to rejuvenation may help explain why some aspects of immune aging persist despite improvements in systemic physiology.

Overall, these findings support a model in which cognitive aging occurs alongside widespread immune remodeling, yet CD8⁺ T cells emerge as a uniquely stable immune population whose localization and transcriptional identity are largely resistant to changes in the systemic milieu. Targeting the intrinsic aging programs of CD8⁺ T cells may therefore represent an important strategy for overcoming limitations of current rejuvenation therapies and preserving cognitive function during aging. Collectively, these data suggest that CD8⁺ T cells maintain properties intrinsic to their cellular age, being in large part refractory to the effects of a young or aged systemic milieu over the timeframe of parabiosis.

Materials and Methods

Mouse strains. The following mouse lines were used: C57BL/6J mice (Jackson Laboratory line 000664), C57BL/6 aged mice (National Institutes of Aging), C57BL/6-Tg(UBC-GFP)30Scha/J mice (Jackson Laboratory line 004353), and B6.SJL-Ptprca Pepcb/BoyJ mice (Jackson Laboratory line 002014). All studies were done in young (4-5 month) or aged (22-24 month) male mice. The numbers of mice used to result in statistically significant differences were calculated using standard power calculations with $\alpha = 0.05$ and a power of 0.8. We used an online tool to calculate power (<https://www.stat.uiowa.edu/~rlenth/Power/index.html>) and sample size based on experience with the respective tests, variability of the assays and inter-individual differences within groups. All other studies were performed with male mice. Mice were housed under specific pathogen-free conditions under a 12h light-dark cycle, and all animal handling and use was in accordance with institutional guidelines approved by the University of California San Francisco Institutional Animal Care and Use Committee (IACUC) and the Stanford University Administrative Panel on Laboratory Animal Care (APLAC).

Tissue collection. Mice were anesthetized with 87.5 mg per kg ketamine and 12.5 mg per kg xylazine and transcardially perfused with ice-cold phosphate-buffered saline. To process the brains, the whole brain was sectioned in half along the sagittal plane. The hippocampus from one hemisphere was subdissected and snap-frozen and the other was fixed in phosphate-buffered 4% paraformaldehyde, pH 7.4 at 4 °C for 48 h before cryoprotection with 30% sucrose.

Lymphocyte isolation. For flow cytometry analysis, the hippocampus was subdissected and dissociated with the Neural Tissue Dissociation Kit (P) (Miltenyi Biotech) according to manufacturer instructions, then filtered through a 70 µm cell strainer and washed with 10 mL of MACS buffer (PBS with 2% FBS). For collection of splenocytes, spleens were removed, mechanically dissociated with a syringe plunger over a 70 µm cell strainer and washed with 10 ml of MACS. Cells were centrifuged and RBC lysis was performed (155 mM NH₄Cl, 1 mM KHCO₃ and 0.1 mM EDTA) with 2 ml for 5 minutes room temperature (RT). Subsequently, cells were washed, spun 500 RCF for 5 minutes, and resuspended in MACS. For lymph node isolation, cervical, axillary, brachial, and inguinal lymph nodes were removed and mechanically dissociated with a syringe plunger over a 70 µm cell strainer and washed with 10 mL of MACS. PBMCs were isolated from blood through RBC lysis (2 mL, 5 minutes RT) followed by washing with MACS and repeated lysis until sample was mainly colorless or with a 2:1 Ficoll gradient.

Parabiosis. Parabiosis surgery followed previously described procedures^{54,55}. Pairs of C57BL/6J (22 months, aged) or B6.SJL-Ptprca Pepcb/BoyJ (3 months, young) mice were anaesthetized and prepared for surgery. Mirror-image incisions at the left and right flanks were made through the skin, and shorter incisions were made through the abdominal wall. The peritoneal openings of the adjacent parabionts were sutured together. Elbow and knee joints from each parabiont were sutured together, and the skin of each mouse was stapled (9-mm Autoclip, Clay Adams) to the skin of the adjacent parabiont. Each mouse was injected subcutaneously with enrofloxacin (Bayer) antibiotic, buprenorphine (Butler Schein), and carprofen (Rimadyl) as directed for pain and monitored during recovery. For overall health and maintenance behavior, recovery characteristics of paired weights and

grooming were analyzed at various times after surgery. Parabionts were sacrificed 4 weeks after surgery.

CITE-seq. CITE-seq analysis was performed on splenocytes isolated from four mice per group. Splenocytes were isolated as described above, blocked (2.4G2), then labeled with CD45.1 (A20, 1:200) and CD45.2 (104, 1:200) antibodies in MACS for 30 minutes on ice. Samples were washed, then stained with a live/dead marker (Hoechst 33342, 1:10,000) before filtering through a 35 µm FACS tube filter sorting on a BD FACSAria Fusion with a 70 µm nozzle and with a flow rate of 1–2.5. Cells were first gated by forward and side scatter, then gated for doublets with height and width. 40,000 cells per sample that were Hoechst- and CD45.1+ or CD45.2+ were collected for sequencing analysis. Collected cells from each group were labeled with CITE-seq antibodies, as previously described⁵⁶. Sorted cells were first blocked with TruStain fcX (BioLegend) for 10 min on ice, then stained with either CD8a or CD45 antibodies for sample separation during CITE-seq analysis. After 30 min of labelling, samples were washed three times then pooled into 6 groups (CD45.1+ young isochronic, CD45.1+ young heterochronic, CD45.2+ young heterochronic, CD45.1 + aged heterochronic, CD45.2+ aged heterochronic, and CD45.2+ aged isochronic). After combining grouped samples, the samples were incubated on ice with Total-seqB antibodies purchased from BioLegend. After 30 min of labelling, samples were washed three times before delivering the prepared samples to the UCSF-IHG Genomics Core for analysis with the 10x Genomics Chromium Single Cell Expression Solution 3' kit with Feature Barcode Technology (v.3.1). The Genomics Core prepared cells for 10x Genomics Chromium single-cell capture. 25,000 cells were loaded per sample. cDNA libraries were prepared according to the standard 10x Genomics protocols.

The final library pool was sequenced to a depth of 30,000 cDNA reads per cell and 3,000 ADT reads per cell on the NovaSeq X 10B system at the UCSF CAT Core. The raw base sequence calls were demultiplexed into sample-specific cDNA and ADT files with `bcl2fastq/mkfastq` sample sheet using Cell Ranger 7.1 (10x Genomics).

Single cell and single nucleus RNA sequencing analysis. Raw FASTQ files were processed and aligned to mm10 using the Cell Ranger software package (10x Genomics) for the RNA expression matrix, including introns in analysis for single nucleus analysis. Outputs from parabiosis CITE-seq and adoptive transfer snRNAseq were integrated with Cell Ranger Aggr (10x Genomics) to normalize for sequencing depth. For parabiosis sequencing, a total of 59,846 cells were sequenced at a depth of 30,977 reads per cell and approximately a 68% sequencing saturation. Further QC and analysis were performed on R 4.2.2. Ambient RNA was removed with the SoupX package and data from the four samples was integrated using Seurat (2000 variable features, 1:20 dimensions). For all experiments, downstream analysis and data visualization was performed using Seurat, as well as packages DropletUtils, ggplot2, knitr, WriteXLS, RColorBrewer, data.table, stringr, ggplot2, forcats, dplyr, Nebulosa, and htmlwidgets. Data were processed to remove doublets and unwanted sources of variation by removing cells with more than 3,500 and fewer than 300 genes per cell or nuclei with more than 5,000 and fewer than 300 genes per cell and regressing on number of UMIs. Genes expressed in fewer than three cells or nuclei were filtered out. Cells with a percentage of mitochondrial genes of higher than 10% or nuclei with a percentage higher than 0.1% were removed. Final cell counts of 8341 (Young Iso), 8418 (Young Het 45.1), 9981 (Young Het 45.2), 5694 (Aged Het 45.1), 8480 (Aged Het 45.2), and 7364 (Aged Iso) were used for gene

expression analysis. The matrices of data were log-normalized in a sparse data matrix and PCA was applied to reduce dimensionality. The first 20 (parabiosis, CD8 depletion) or 30 (adoptive transfer) PCA components were used to cluster cells by Louvain clustering implemented in Seurat while UMAP plots were independently generated to aid in 2D representation of multidimensional data independent of the clustering. Cell types were identified using known markers, and clusters containing more than one cell type specific marker were excluded. Differential gene expression was performed with a minimum of 10% of cells or 5% nuclei expressed, log fold change threshold of 0.15, and a pseudocount of 0.1. Log-normalized gene expression data were used for visualizations with violin plots and UMAP feature plots. Average expression matrices were used for heatmap visualization. Volcano plots were created using the EnhancedVolcano package and UpSet plots with the ComplexHeatmap package. Pseudotime analysis was performed with monocle3. Predicted interactions were identified with CellChat.

Flow Cytometry. Single cell suspensions of splenocytes, PBMCs, lymph nodes, and hippocampal cells were isolated as stated above, then approximately 1×10^6 cells per sample were stained on a round-bottom 96-well plate with a live/dead marker (GhostDye UV 450, Tonbo, 13-0868) 1:100 in PBS for 20 minutes RT. Cells were subsequently washed in FACS buffer (PBS with 2% FBS, 2 mM EDTA, 2mM NaN₃) and spun for 2 minutes at 300 RCF. Samples were blocked with 2.4G2 (1:100 in FACS) for 10 minutes on ice. Cells were washed with FACS, then resuspended in an antibody cocktail in FACS buffer with Brilliant Stain Buffer (BD, 56379) at a 1:1 ratio for cell surface staining and incubated on ice for 30-60 minutes. After washing, cells were either fixed with 4% paraformaldehyde, washed and resuspended in FACS buffer for storage until analysis or

followed with intracellular staining with the Foxp3 / Transcription Factor Staining Buffer Set (eBioscience, 00-5523-00) according to manufacturer instructions. Cells were analyzed using the BD LSR II Flow Cytometer at the UCSF Parnassus Flow Cytometry CoLab and Flow Jo software (TreeStar). Fluorescence minus one (FMO) controls were used for the initial antibody tests, then thereafter for certain stains. Immune cell populations after parabiosis were analyzed according to published protocols, with the same antibodies and concentrations used. For T cell populations, antibodies used were as follows: CD45-BV711 (1:200, BD Biosciences, 563709), CD45-PE (1:200, ThermoFisher Scientific, 5010370), CD45.2-FITC (1:200, eBioscience, 11-0454-82), CD45.1-PE (1:200, eBioscience, 12-0453-83), CD3-APC (1:200, Tonbo Biosciences, 20-0031-U025), CD8a-PB (1:200, ThermoFisher Scientific, MCD0828), CD4-PE-Cyanine7 (1:200, ThermoFisher Scientific, 25-0041-82), CD44-APC-eFluor 780 (1:100, ThermoFisher Scientific, MCD0828), CD69-AF700 (1:100, ThermoFisher Scientific, 56-0691-82), CD62L-PerCP-Cyanine5.5 (1:100, ThermoFisher Scientific, 45-0621-82), GZMK-FITC (1:100, LSBio, LS-C692079-200), or CCL5-PE-Cyanine7 (1:100, BioLegend, 2308-03). For CD45-PE labeling injections, mice were injected i.v. with 200 μ L of CD45-PE (1 μ L/100 μ L) in saline 3 minutes before sacrifice.

Immunohistochemistry. Tissue processing and immunohistochemistry was performed on free-floating sections according to standard published techniques. Cryoprotected brains were sectioned coronally at 40 μ m with a cryomicrotome (Leica Camera). Free-floating coronal sections (40 μ m) were permeabilized with pre-treatment buffer (0.2% TritonX-100 in TBST) for 30 minutes RT, then washed 3x with TBST and blocked with TBST + 5% NDS. Sections were then incubated overnight at 4 degrees C with anti-Iba1

(1:1,000, Wako, 01919741), anti-Iba1 (1:1000, Synaptic Systems, 234-004), anti-CD68 (1:250, FA-11, Bio-Rad, MCA1957T), anti-cFos (1:100, 96F, Cell Signaling, 2250), anti-CD31 (1:200, R&D, AF3628), anti-CD8 (1:250, 4SM15, eBioscience, 14-0808-82), anti-Collagen1 (1:500, Abcam, ab21286), or anti-Acta2 (1:500, ThermoFisher Scientific, 14-9760-82), or anti-Par1 (1:200, Invitrogen, PA5-116040 in TBST + 5% NDS. Labelling was revealed using secondary antibodies at 1:500 in TBST + 3% NDS for 1 hour room temperature. Sections were imaged using confocal microscopy (Zeiss LSM800 or Zeiss LSM900) or bright-field microscopy (Keyence). Individual cell numbers and intensity in the dentate gyrus was quantified using ImageJ.

NOR. The NOR task was performed as previously described. On day one (the habituation phase), mice performed open field testing by exploring an empty arena for 10 min. Infrared photobeam breaks were recorded and movement metrics were analyzed using the MotorMonitor software (Kinder Scientific). On day two (the training phase), two identical objects were placed into the habituated arena, and the mice were allowed to explore for 5 min. On day three (the testing phase), one object was replaced with a novel object, and the mice were allowed to explore for 5 min. The time spent exploring each object was quantified using the Smart Video Tracking Software (Panlab; Harvard Apparatus). Two different sets of objects were used. To control for any inherent object preference, half of the mice were exposed to object A as their novel object and half to object B. To control for any potential object-independent location preference, the location of the novel object relative to the trained object was also counterbalanced. To determine the percentage of time with the novel object, we calculate (time with novel object)/ (time

with trained object + time with novel object) $\times$ 100. Mice that did not explore both objects during the training phase were excluded from the analysis.

RAWM. Spatial learning and memory were assessed using the RAWM paradigm, according to an established protocol. In this task, the mouse was trained to the location of a constant goal arm throughout the training and testing phase. The start arm changed each trial. Entry into an incorrect arm was scored as an error, and errors were averaged over training blocks (three consecutive trials). During training (day 1), the mice were trained for 12 trials (blocks 1–4), with trials alternating between a visible and hidden platform. After an hour break, learning was tested for 3 trials (block 5) using only a hidden platform. During testing (day 2), the mice were tested for 15 trials (blocks 6–10) with a hidden platform. When scoring, investigators were blinded to treatment. All blocks were analyzed for significance via two-way ANOVA with Šídák's post-hoc, and only significant data points were annotated on figures. Brackets represent significance of area under the curve.

Y maze. The Y Maze task was conducted as previously described. During the training phase, the mice were placed into the start arm facing the wall and were allowed to explore the start and trained arm for 5 min, while the entry to the 3rd arm (novel arm) was blocked. The maze was cleaned between each mouse to remove odor cues, and the trained arm was alternated between mice. After training, the mouse was returned to its home cage. After 45 min, the mouse was returned to the start arm and was allowed to explore all three arms for 5 min. The number of entries in each arm was quantified using the Smart Video Tracking Software (Panlab; Harvard Apparatus). The percentage of entries in each arm was defined as the number of entries in each arm divided by the total number of entries

in all arms during the first minute of the task. Mice that did not perform three entries during the first minute of testing were excluded.

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Chapter 2

Peripheral aged CD8⁺ T cells drive cognitive decline through secreted factors

ABSTRACT

Aging is associated with both cognitive decline and prominent changes in the immune system. Peripheral CD8⁺ T cells undergo significant age-related changes, but their functional impact on the brain remains poorly defined. Here, we demonstrate that aged circulating CD8⁺ T cells directly contribute to hippocampal dysfunction and cognitive decline. Using adoptive transfer models, we show that exposure of young mice to aged CD8⁺ T cells is sufficient to induce impairments in hippocampal-dependent learning and memory, along with synaptic- and brain-barrier cell-related transcriptional alterations. Systemic exposure of young mice to aged CD8⁺ T cells elicited synaptic-related hippocampal changes and impaired cognition, while inhibiting activation, rather than infiltration, mitigated their pro-aging effects. Conversely, targeting aged circulating CD8⁺ T cells restored youthful signatures and rescued cognition. Mechanistically, we identified Granzyme K (GZMK) as a secreted pro-aging CD8⁺ T cell-derived factor in plasma, inhibition of which rescues cognition in aged animals. Together, our data identify activated aged CD8⁺ T cell-derived circulating factors as potential therapeutic targets to rescue cognition in old age.

Introduction

The hippocampus, or the region of the brain primarily responsible for learning and memory, is particularly susceptible to age-related cognitive decline and is one of the first regions to show hallmarks of brain aging. Within the brain, decreased synaptic plasticity, loss of dendritic spine density, loss of regenerative capacity, increased microglial activation, and dysfunction of the blood brain barrier all underly these cognitive changes. While the brain is largely an immune privileged organ, mounting evidence demonstrates that immune function is tightly coupled to cognition. Immune cell populations undergo profound changes with age and disease and have been shown to drive changes within the central nervous system, yet the mechanisms linking age-related immune dysfunction to impaired brain function remain poorly understood. Among these populations, CD8⁺ T cells have emerged as important regulators of brain function in aging. In the brain, infiltrating CD8⁺ T cells in aged mice decrease adult neurogenesis in the subventricular zone of the lateral ventricles promote axon degeneration in the optic nerve and induce neuroinflammation in the white matter. By contrast, only modest CD8⁺ T cell infiltration was reported in the aged hippocampus in the absence of neurodegenerative disease pathology in mouse models while analysis of post-mortem human hippocampi found no age-related changes in infiltration. Notwithstanding, despite the vast majority of CD8⁺ T cells residing in the periphery, the role of aged non-infiltrating circulating CD8⁺ T cells or their secreted factors in hippocampal-dependent cognitive decline has yet to be fully defined. We therefore sought to investigate the effect of peripheral aged CD8⁺ T cells on hippocampus and ascertain the rejuvenating potential of selectively targeting this immune cell population in circulation at old age.

RESULTS

Circulating aged CD8⁺ T cells alter hippocampal transcriptional profiles and impair cognition

Having determined the refractory nature of aged CD8⁺ T cells to their systemic environment, we investigated the pro-aging potential of aged circulating CD8⁺ T cells on the young hippocampus. For this, we performed an adoptive transfer of aged (22 month) or young (4 month) CD8⁺ T cells into young (4 months) animals (Figure 2.1A).

To investigate molecular changes elicited in the young hippocampus by aged circulating CD8⁺ T cells, we performed bulk RNA-sequencing analysis and detected differential expression of 2080 genes relative to control animals. Overlaying significantly expressed genes (DEGs) from our bulk RNA-sequencing data over a single nucleus RNA sequencing (snRNAseq) hippocampal dataset identified that differential genes were significantly enriched in neuron and brain barrier cell populations (Figure 2.1B). Gene ontology (GO) analysis of up- and down-regulated genes identified synaptic plasticity-related biological processes (Figure 2.1C,D). Additionally, we detected decreased hippocampal expression of synaptic-plasticity related genes *Synapsin1*, *Dlg4*, *CamkII*, and *Homer1* by quantitative PCR (qPCR) as well as a decrease in the number of neurons expressing the immediate early gene c-Fos, in young mice exposed to aged circulating CD8⁺ T cells (Figure 2.1E,F). In brain-barrier populations, young mice that received aged CD8⁺ T cells had decreased coverage of CD31 and Collagen1 and increased coverage of Acta2 by immunohistochemistry (IHC) (Figure 2.1G,H).

At a functional level, hippocampal-dependent learning and memory was assessed using novel object recognition (NOR) and radial arm water maze (RAWM). During NOR

testing, young control mice exposed to young CD8⁺ T cells were biased towards a novel object relative to a familiar condition whereas young mice exposed to aged CD8⁺ T cells showed no preference (Figure 2.1I). In the training phase of the RAWM paradigm, all mice showed similar spatial learning ability regardless of adoptive transfer condition (Figure 2.1J). However, young mice exposed to aged CD8⁺ T cells demonstrated impaired learning and memory for the platform location during the testing phase of the task compared with young control mice exposed to young CD8⁺ T cells (Figure 2.1J). These behavioral data indicate that aged circulating CD8⁺ T cells are negative regulators of hippocampal-dependent learning and memory.

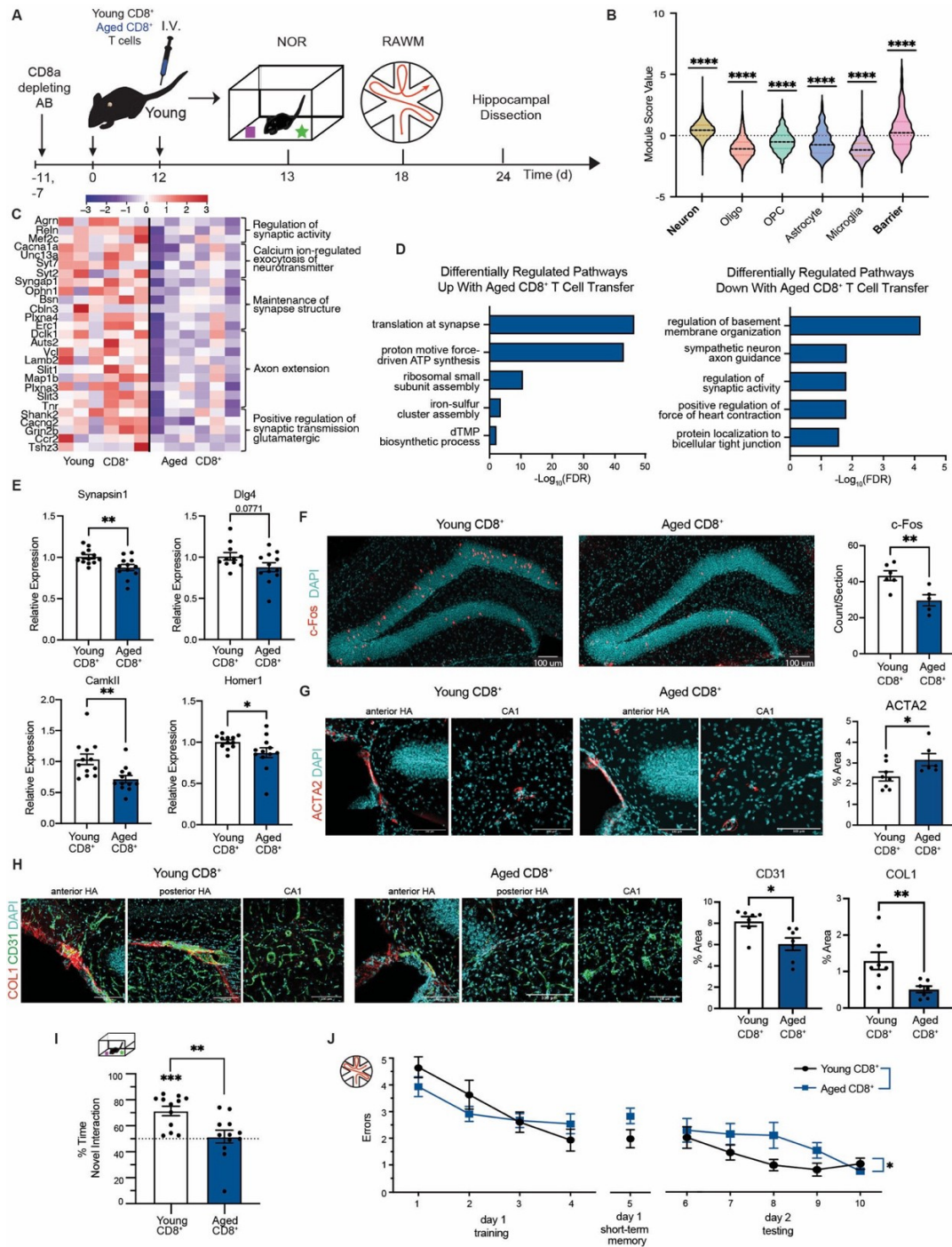


Figure 2.1. Aged circulating CD8⁺ T cells elicit aging transcriptional signature in the young hippocampus and promote cognitive impairments.
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A, Schematic illustrating the timeline of adoptive transfer of 3×10^5 - 3×10^6 young (4 months) or aged (22 months) CD8⁺ T cells to young mice (4 months, BoyJ) after treatment with 200ng CD8a depletion antibody, i.p., followed by cognitive testing.

B, Module score of differentially expressed genes ($p < 0.05$) in aged versus young CD8⁺ transfer overlain onto single nucleus RNA sequencing to identify over-represented cell types ($n=6$ /group).

C, Heatmap of representative differentially expressed genes in Gene Ontology (GO) pathways related to synapses, along with the identified differentially regulated pathways ($FDR > 0.05$).

D, Top GO terms of biological processes associated with differentially expressed up or downregulated genes.

E, Synapse-related gene expression as measured by RT-qPCR ($n=12$ /group).

F, Representative image and quantification of c-Fos (red) expression in hippocampi of mice receiving young or aged CD8⁺ T cell as measured by immunohistochemistry ($n=5-6$ /group).

G, Representative image in key areas of expression and quantification of Acta2 (red) in the full hippocampi as measured by immunohistochemistry ($n=6-8$ /group).

H, Representative image in key areas of expression and quantification of Collagen1 (red) and CD31 (green) in the full hippocampi as measured by immunohistochemistry ($n=7$ /group).

I, Object recognition memory as assessed by Novel Object Recognition (NOR) and quantified as percent time spent exploring the novel object.

J, Hippocampal-dependent learning and memory as evaluated by radial arm water maze (RAWM). Changes in cognition were quantified as number of errors while attempting to find the goal arm ($n=12$ /group).

Statistical analysis was performed using one-sample t-test vs 0 (B) or vs 50% (I), Fisher's exact-tests (D), two-tailed t-test (E-I), and two-way ANOVA with Tukey's post hoc (J).

Circulating aged CD8⁺ T cells affect neurons and barrier cells without driving neuroinflammation

Having determined aged CD8⁺ T cells drive neuronal and cognitive decline, we further assessed other known age-related changes. First, we assessed if the aged CD8⁺ T cells were infiltrating into the hippocampus and observed that transferred young and aged CD8⁺ T cells were observed in the spleen at equal levels (Figure 2.2A). Minimal infiltration into the young hippocampus was detected following adoptive transfer (Figure 2.2B).

To investigate other aging hallmarks elicited in the young hippocampus by aged circulating CD8⁺ T cells, we detected decreased levels of PSD-95 and the NR2B:NR2A ratio by Western blot analysis (Figure 2.2C,D). In brain-barrier populations, we detected decreased levels of ZO-1, a tight junction protein, by Western blot analysis (Figure 2.2C). No changes in microglial reactivity or inflammatory markers were observed by IHC or qPCR, respectively (Figure 2.2E-L). These data indicate that aged circulating CD8⁺ T cells drive changes primarily in neurons and barrier populations.

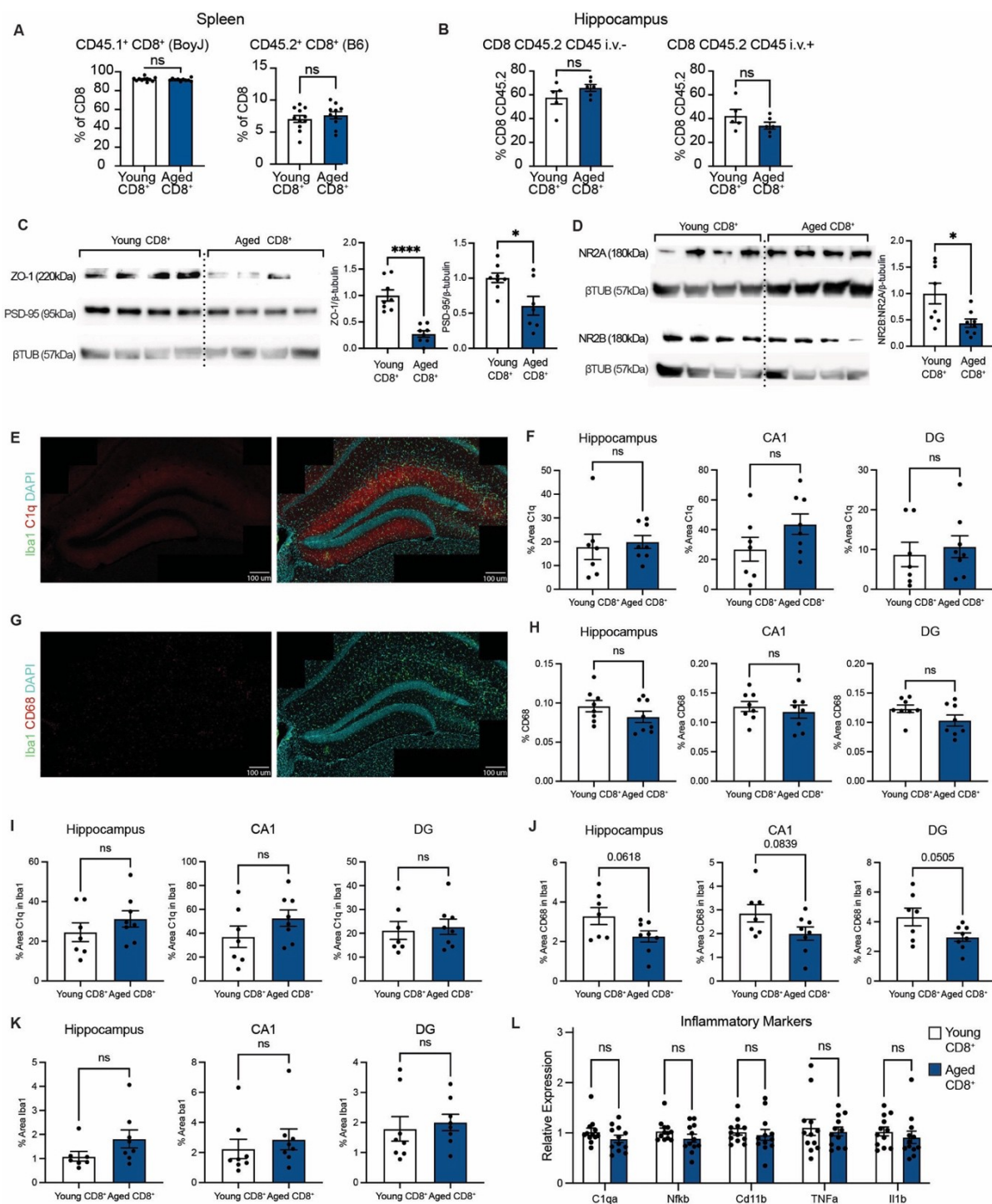


Figure 2.2. Aged circulating CD8⁺ T cells elicit changes in neurons and barrier cells without driving neuroinflammation.

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A, Percent of host (CD45.1) or donor (CD45.2) CD8⁺ T cells located in the spleen 24 days after adoptive transfer, as measured by flow cytometry (n=10-11/group).

B, Percent of i.v.- or i.v.+ donor (CD45.2) CD8⁺ T cells located in the hippocampus 24 days after adoptive transfer, as measured by flow cytometry (n = 5-6/group).

C, D, Representative Western Blot plots and quantification of ZO-1 and PSD-95 I and NR2A and NR2B (**D**) normalized to beta-tubulin (n=7-8/group).

E,G, Representative images in the full hippocampus of C1q (red) and Iba1 (green) I or CD68 (red) and Iba1 (green) (**G**).

F,H,I,J,K, Quantification of percent area of C1q (**F**), CD68 (**H**), C1q in area Iba1 (**I**), CD68 in area Iba1 (**J**), or Iba1 (**K**) in the full hippocampus, CA1 region, and DG region of mice receiving young or aged CD8⁺ T cell as measured by immunohistochemistry (n=7-8/group).

L, Inflammatory-related gene expression as measured by RT-qPCR (n=12/group).

Statistical analysis was performed using a two-tail t-test (A-D,F,H-L).

Inhibiting GPCR signaling of aged circulating CD8⁺ T cells abrogates pro-aging hippocampal effects

As shifts from naïve to age-associated cytotoxic CD8⁺ T cell populations persisted in aged cells irrespective of the age of their systemic environment, we investigated functionally how these aged CD8⁺ T exerted their pro-aging effects on the young hippocampus. A variety of CD8⁺ T cell functions are dependent on Gai/o-coupled G protein-coupled receptor (GPCR)-mediated signaling, such as migration to lymph nodes were naïve CD8⁺ T cells become activated upon encountering their cognate antigen, migration along chemokine gradients to sites of secondary activation, and strong physical interactions with and migration across tissue barriers. Correspondingly, we pre-treated aged CD8⁺ T cells with pertussis toxin (PTx), a known inhibitor of Gi/o protein signaling, to block these functions following adoptive transfer. Specifically, we performed an adoptive transfer of PTx or vehicle treated old (26 months) CD8⁺ T cells into young (5 months) mice, and vehicle treated young CD8⁺ T cells into age-matched young animals as a baseline control for age-related changes (Figure 2.3A).

PTx pre-treatment has been shown to prevent T cell migration to lymph nodes without altering migration to open circulation tissues such as spleen, bone marrow, or liver. Moreover, the timeframe used for our studies was selected in line with previous reports showing minimal entry into the brain parenchyma with no impact on migration to the brain by PTx treatment. Concordantly, we observed decreased levels of infiltrating CD8⁺ T cells into lymph nodes and increased levels in blood circulation in the PTx compared to vehicle treated aged cells but detected no differences in migration to the spleen or brain between treatment groups (Figure 2.3B-D). While age-related shifts from

naïve to effector memory CD8⁺ T cells were observed between vehicle treated young and aged CD8⁺ T cells following adoptive transfer to young animals, these shifts were abrogated in the spleen of young animals following adoptive transfer of PTx-treated aged CD8⁺ T cells (Figure 2.3E).

To investigate the impact on the young hippocampus, we performed snRNAseq analysis. Nuclei were isolated from hippocampi of all treatment groups (Figure 2.3A). A total of 20 cell clusters were identified, and populations were compared across treatment groups (Figure 2.3F,G). Signatures for each cluster were generated by differential gene expression analyses and cell types were established based on transcriptomic signatures and canonical markers. We detected prominent transcriptional changes in the hippocampus of young mice following adoptive transfer with vehicle treated aged compared to young CD8⁺ T cells, which were in large part restored towards young control conditions in the PTx treated group (Figure 2.3H,J). We found that transcriptional responses in the young hippocampus following adoptive transfer with vehicle treated young or aged CD8⁺ T cell or PTx treated aged CD8⁺ T cells are encoded in different cell types of the brain, with changes prominently observed across excitatory neuronal populations (DG, CA1, CA2/CA3) (Figure 2.3I,J). DEGs were largely unique to cell type (Figure 2.3K,L). GO analysis of overlapping DEGs in separate excitatory neuronal populations between young animals exposed to vehicle treated young and aged CD8⁺ T cells and between aged vehicle and PTx treatment groups identified synaptic- and metabolic-related biological processes (Figure 2.3M), in which aged CD8⁺ T cell driven transcriptional signatures elicited in excitatory neurons were restored to more youthful levels by blocking T cell GPCR signaling.

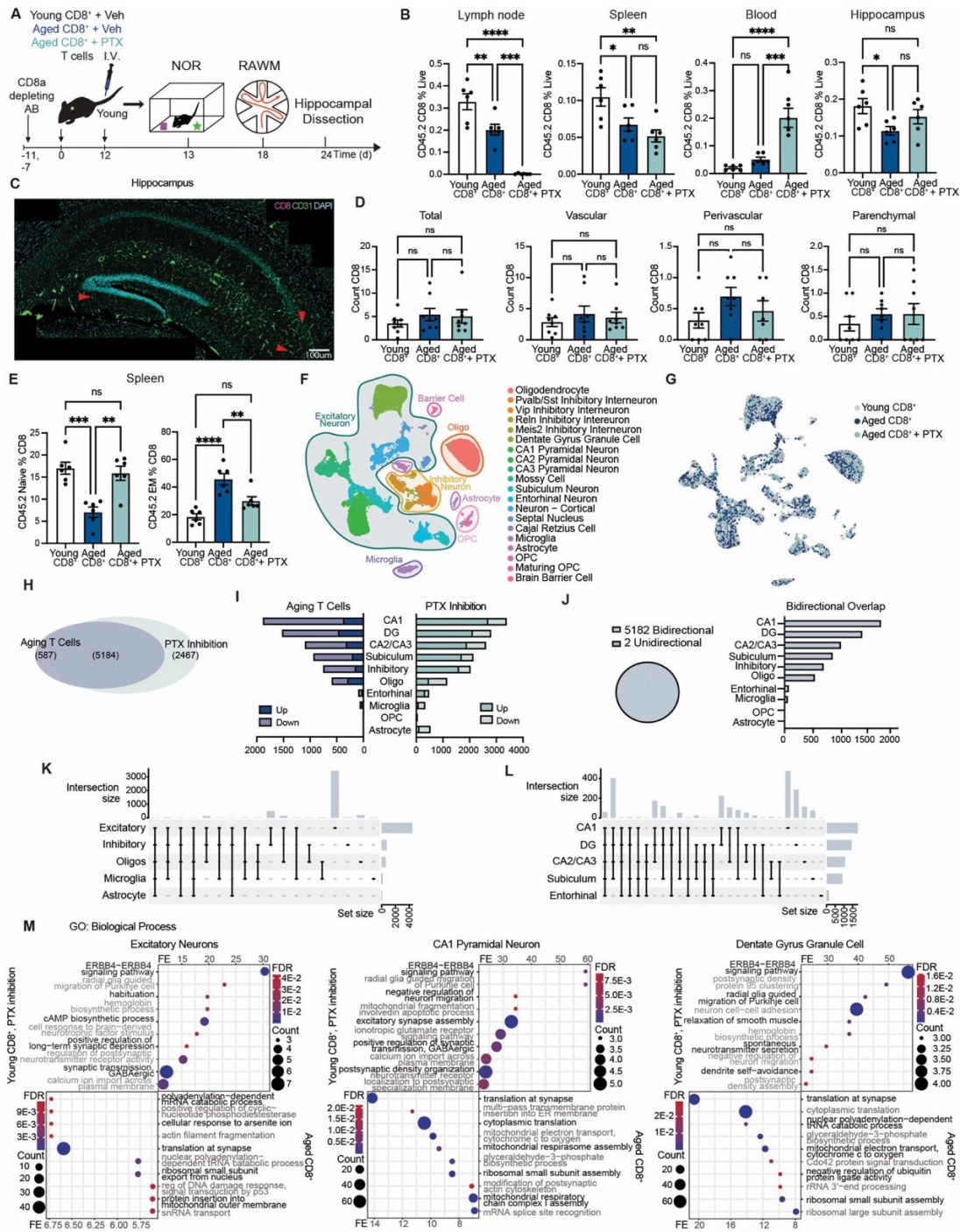


Figure 2.3. Pro-aging hippocampal effects of aged CD8⁺ T cells are dependent on GPCR signaling.

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A, Schematic illustrating the timeline of adoptive transfer of 3×10^5 - 3×10^6 young (5 months) vehicle treated, aged (24-28 months) vehicle treated, or aged PTx treated CD8⁺ T cells to young mice (5 months, BoyJ) after treatment with 200ng CD8a depletion antibody, i.p., followed by cognitive testing.

B, Percentages of donor cells (CD45.2+CD3+CD8+) identified in lymph nodes, spleen, blood, and hippocampus 24 days after adoptive transfer.

C, D, Representative image (**C**) and quantification (**D**) of CD8+ cells (red) in the hippocampus localized in the total area or the vascular, perivascular, or parenchymal regions identified by localization with CD31 (green) as measured by immunohistochemistry (n=8/group).

E, Percentage of splenocyte populations of Naïve (CD44-CD62l+) or Effector Memory (EM; CD44-CD62l-) donor CD8⁺ T cells identified by flow cytometry.

F,G, UMAP projection of identified cell types (**F**) and of mouse identity of cell origin (**G**) of snRNAseq of hippocampi from young mice receiving young vehicle treated, aged vehicle treated, or aged PTx treated CD8⁺ T cells (n=3/group, combined).

H, Overlap between total DEGs in mice receiving young versus aged vehicle treated CD8⁺ T cells and mice receiving vehicle treated aged versus PTx treated aged CD8⁺ T cells (FDR<0.05).

I, Number of genes per cell type driven by aged CD8⁺ T cells or PTx treatment.

J, Directionality and number of overlapped DEGs in excitatory neurons driven by aged CD8⁺ T cells or PTx treatment.

K, L, UpSet plots displaying shared and unique DEGs per cell type (**K**) and excitatory neuron subtype (**L**).

M, Top GO terms of biological processes associated with overlapping DEGs.

Statistical analysis was performed using one-way ANOVA with Tukey's post hoc (B,D,E), DESeq2 (H-L), Fisher's exact-tests (M).

Blocking activation, and not entry into tissue, mitigated pro-aging cognitive effects of aged circulating CD8⁺ T cells

We next examined whether blocking CD8⁺ T cell GPCR signaling could mitigate pro-aging cognitive effects of aged CD8⁺ T cells (Figure 2.4A). Hippocampal-dependent learning and memory was assessed using RAWM. Young control mice exposed to vehicle treated young CD8⁺ T cells committed fewer errors locating the hidden platform compared to young mice exposed to vehicle treated aged CD8⁺ T cells (Figure 2.4B). Congruent with snRNAseq analysis, young mice exposed to PTx treated aged CD8⁺ T cells did not exhibit cognitive impairments in hippocampal-dependent spatial memory (Figure 2.4B).

To delineate the role of activation in mediating the pro-aging cognitive effect of aged circulating CD8⁺ T cells, we treated aged CD8⁺ T cells *ex-vivo* with Tofacitinib, a JAK-STAT inhibitor, to block secondary CD8⁺ T cell activation and cytokine release, or with an anti-VLA-4 antibody to block strong CD8⁺ T cell-barrier interaction and subsequent entry into tissue (Figure 2.4C,D). Treatment with either drug did not alter T cell phenotypes compared to the young control, however treatment with Tofacitinib resulted in shifts towards the young phenotype compared to the aged control (Figure 2.4E,F). Young mice exposed to vehicle treated aged CD8⁺ T cells made significantly more errors before identifying the hidden platform (Figure 2.4G,H). Inhibiting activation and cytokine release by Tofacitinib treatment, and not entry into tissue by anti-VLA-4 antibody treatment, mitigated aged CD8⁺ T cell driven cognitive deficits (Figure 2.4G,H). Together, these behavioral data indicate that T cell migration and subsequent activation are necessary, at least in part, for the pro-aging effects of aged circulating CD8⁺ T cells on the hippocampus.

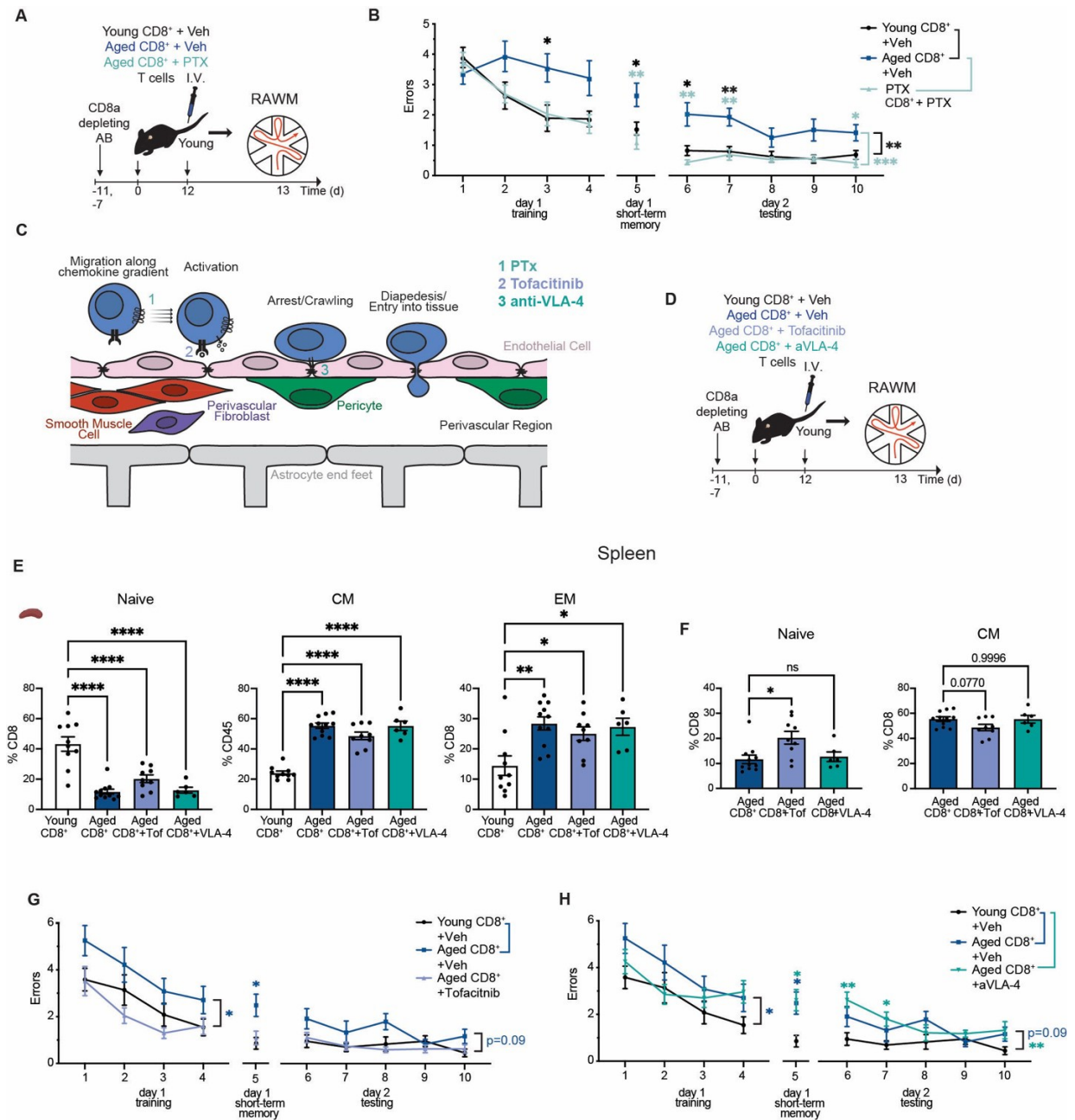


Figure 2.4. Activation of aged CD8⁺ T cells, rather than infiltration into tissue, is necessary for pro-aging cognitive effects.

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A, Schematic illustrating the timeline of adoptive transfer of 3×10^5 - 3×10^6 young (5 months) vehicle treated, aged (24-28 months) vehicle treated, or aged PTx treated CD8⁺ T cells to young mice (5 months, BoyJ) after treatment with 200ng CD8a depletion antibody, i.p., followed by cognitive testing.

B, Hippocampal-dependent learning and memory as evaluated by RAWM (n=11-12/group).

C, Schematic representing CD8⁺ T cell functions inhibited by PTx (1), Tofacitinib (2), or anti-VLA-4(3).

D, Schematic illustrating the timeline of adoptive transfer of 3×10^5 - 3×10^6 young (4 months) vehicle treated, aged (24 months) vehicle treated, aged Tofacitinib treated CD8⁺ T cells, or aged anti-VLA-4 treated CD8⁺ T cells to young mice (4 months, BoyJ) after treatment with 200ng CD8a depletion antibody, i.p., followed by cognitive testing.

E, Percentages of CD8, CD45.2 (donor) and donor Naïve, Central Memory, and Effector Memory young vehicle treated, aged vehicle treated, aged Tofacitinib treated, or anti-VLA-4 treated CD8⁺ T cells located in the spleen 24 days after adoptive transfer, as measured by flow cytometry (n=6-10/group) and compared to the young vehicle treated control.

F, Percentages of donor Naïve and Central Memory aged vehicle treated, aged Tofacitinib treated, or anti-VLA-4 treated CD8⁺ T cells located in the spleen 24 days after adoptive transfer, as measured by flow cytometry (n=6-10/group) and compared to the aged vehicle treated control.

G, H, Hippocampal-dependent learning and memory as evaluated by RAWM after treatment with Tofacitinib (**G**) or anti-VLA-4 (**H**) (n=9/group).

Statistical analysis was performed using two-way ANOVA with Šídák's post-hoc (B,G,H) and with Tukey's post-hoc (E,F)

Targeting aged circulating CD8⁺ T cells restores hippocampal transcriptional profiles and improves cognition at old age

Having observed pro-aging effects of aged circulating CD8⁺ T cells on hippocampal-dependent cognitive function, we explored the possibility that targeting CD8⁺ T cells can rescue cognition in aged mice. We employed a genetic model in which absence of transporter associated with antigen processing 1 (TAP1) results in reduced levels of class 1 surface molecules and a loss of CD8⁺ T cell. We assessed hippocampal-dependent learning and memory in young (3 months) and aged (20 months) *Tap1*-knockout (*Tap1*^{-/-}) and wild type (WT) littermate control mice using RAWM. In young animals, we observed no difference in spatial learning and memory between *Tap1*^{-/-} and WT controls during RAWM training or testing (Figure 2.5A). However, aged *Tap1*^{-/-} mice committed significantly fewer errors locating a hidden platform during RAWM testing compared to WT controls (Figure 2.5B), indicating enhanced cognition in an age-dependent manner.

To selectively target aged peripheral CD8⁺ T cells late in life, aged (22 months) mice were given intraperitoneal injections of a CD8a depleting antibody or IgG2b isotype control (Figure 2.5C). While we observed a decrease in peripheral CD8⁺ T cells, no differences in the number of CD8⁺ T cells in the hippocampus were observed (Figure 2.5D). To investigate molecular changes elicited in the aged hippocampus, we performed bulk RNA-sequencing analysis and detected differential expression of 1488 genes in aged mice receiving a CD8a depleting antibody relative to the IgG2b control. Overlaying significantly expressed genes from our bulk RNA-sequencing data over a snRNAseq hippocampal dataset identified that differential genes were significantly enriched in

neuron populations (Figure 2.5E). GO analysis of up- and down-regulated genes identified synaptic- and vascular-related biological processes (Figure 2.5F). Concordantly, we detected increased expression of synaptic-plasticity related genes *Synapsin1*, *Dlg4*, and *CamkII* by qPCR (Figure 2.5G).

To gain further cell type specific resolution, we performed snRNAseq analysis of hippocampi from aged mice treated with CD8a depleting antibody or isotype control (Figure 2.5H,I). We detected prominent transcriptional changes in excitatory neuronal populations and oligodendrocytes in the hippocampus of aged mice treated with CD8a depleting antibody compared to isotype control (Figure 2.5J). Of the excitatory neuronal populations, we observed predominant transcriptional changes in DG and CA1 neurons (Figure 2.5K). We found that transcriptional responses following CD8⁺ T cell depletion are encoded in different cell types with genes that are largely unique to cell type (Figure 2.5L,M). GO analysis of DEGs in excitatory neuronal populations identified synaptic- and metabolic-related biological processes (Figure 2.5N,O).

To complement transcriptomics analysis, we assessed hippocampal-dependent learning and memory using NOR and RAWM. While both treatment groups showed preference for the novel object during NOR testing, aged mice with depleted CD8⁺ T cells spent significantly more time with the novel object compared to isotype control treated mice (Figure 2.5P). Aged mice with depleted CD8⁺ T cells demonstrated improved memory for the platform location during the testing phase of the task compared with aged control mice (Figure 2.5Q). Together, these data indicate that targeting aged peripheral CD8⁺ T cells restores synaptic-related transcriptional profiles and cognitive function in the aged hippocampus.

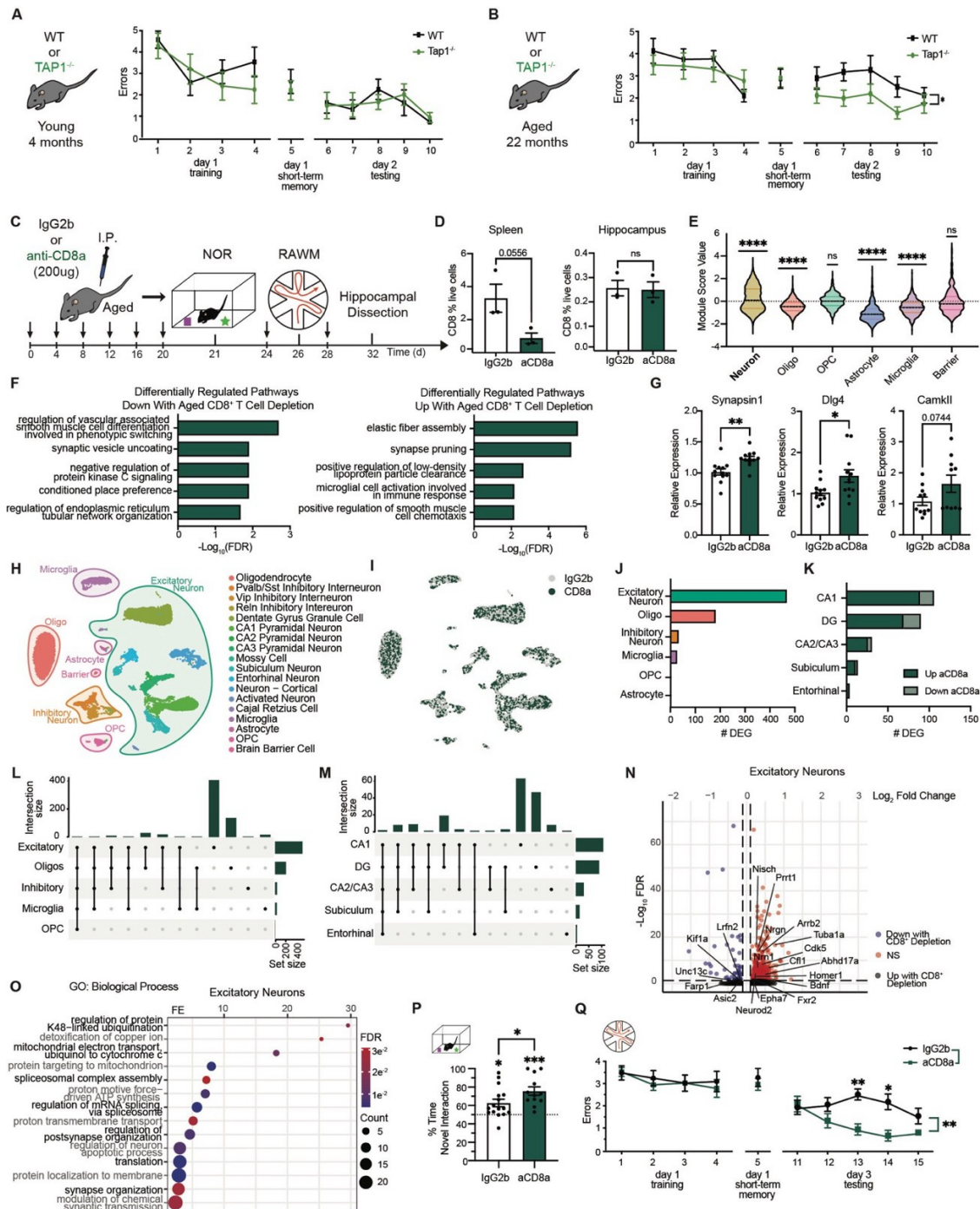


Figure 2.5. Depletion of circulating aged CD8⁺ T cells restores hippocampal transcriptional profiles and rescues cognition.
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A, Representative schematic and hippocampal-dependent learning and memory of young (4 months) WT and TAP1^{-/-} mice as evaluated by RAWM (n=11/group).

B, Representative schematic and hippocampal-dependent learning and memory of aged (22 months) WT and TAP1^{-/-} mice as evaluated by RAWM (n=11/group).

C, Schematic illustrating timeline of treatment of aged mice (22 months) with 200ng CD8a depletion antibody or IgG2b isotype control, i.p., followed by cognitive testing.

D, Numbers of CD8⁺ T cells in the spleen and hippocampus, as a percent of all live cells, measured by flow cytometry.

E, Module score of differentially expressed genes (DEGs) ($p < 0.05$) in CD8a depletion hippocampi versus isotype control, overlain onto single nucleus RNA sequencing to identify over-represented cell types.

F, Top GO terms of biological processes associated with differentially expressed up or downregulated genes ($FDR < 0.05$).

G, Synapse-related or inflammatory gene expression as measured by RT-qPCR.

H,I, UMAP projection of identified cell types (**H**) and of mouse identity of cell origin (**I**) of single nucleus RNA sequencing of hippocampi from aged mice receiving CD8a depleting antibody or isotype control (n=3/group, combined after sorting).

J,K, Number of DEGs per cell type (**J**) and number of up or downregulated genes per excitatory neuron subtype ($FDR < 0.05$) (**K**).

L,M, UpSet plots displaying shared and unique DEGs per cell type (**L**) and excitatory neuron subtype (**M**).

N, Volcano plot of DEGs in excitatory neurons, with genes identified in synapse-related GO terms labeled.

O, Top GO terms of biological processes associated with DEGs in excitatory neurons.

P, Object recognition memory as assessed by NOR.

Q, Hippocampal-dependent learning and memory as evaluated by RAWM (n=11-12/group).

Statistical analysis was performed using two-way ANOVA with Šídák's post-hoc (A,B,Q), t-test (D,G,P), one-sample t-test vs 0 (E) or 50% (P), Fisher's exact-tests (F,O), and DESeq2 (J-N).

Targeting circulating GZMK ameliorates aging-associated hippocampal-dependent cognitive decline

Given pro-aging effects of activated aged circulating CD8⁺ T cells, the possibility arises that systemic factors in circulation secreted by aged peripheral CD8⁺ T cells upon activation may promote cognitive decline. Age-associated effector memory CD8⁺ T cells are the primary source of secreted GZMK in the aging systemic milieu, and extracellular GZMK has been implicated in promoting inflammation and senescence in the periphery. Therefore, we elected to investigate the functional consequence of mimicking an age-related increase in systemic GZMK.

To begin, we confirmed that the age-related emergence of age-associated effector memory CD8⁺ T cell populations persisted in aged cells irrespective of the age of the systemic milieu (Figure 2.6A,B). We corroborated increased GZMK expression exclusively in age-associated effector memory CD8⁺ T cells from isochronic parabionts (Figure 2.6C). We confirmed these changes by flow cytometry of cultured young and aged CD8⁺ T cells and observed increased levels of activated CD69⁺ CD8⁺ T cells and activated cells expressing GZMK (Figure 2.6D). To investigate the effect of CD8⁺ T cells expressing GZMK, we overexpressed GZMK or GFP control in young CD8⁺ T cells prior to adoptive transfer using a retroviral-mediated approach followed by cognitive testing by RAWM (Figure 2.6E,F). Young animals exposed to young CD8⁺ T cells expressing GZMK exhibited cognitive deficits in hippocampal-dependent spatial memory compared to GFP control (Figure 2.6G).

Additionally, we observed increased levels of circulating GZMK in plasma derived from aged compared to young mice, as well as in plasma derived from young mice following

adoptive transfer of aged CD8⁺ T cells, while this increase was no longer observed following adoptive transfer of PTx-treated aged CD8⁺ T cells (Figure 2.6H). To investigate the potential pro-aging activity of circulating GZMK on cognitive function, we used a hydrodynamic tail vein injection (HDTVl)-mediated *in vivo* overexpression approach to increase levels of circulating GZMK. To confirm increased expression in circulation, we generated expression constructs encoding a HiBiT-tagged version of GZMK. HiBiT is a small peptide that forms a complex with LgBiT to produce a luminescent signal, allowing for sensitive detection of tagged proteins. Young (4 months) mice were given HDTVl with expression constructs encoding GZMK-HiBiT or GFP. Increases in luminescent signal were confirmed in blood plasma and liver of GZMK treated young animals (Figure 2.6I). Hippocampal-dependent learning and memory was assessed by RAWM in a larger cohort of young mice administered expression constructs encoding GZMK or GFP by HDTVl (Figure 2.6K). Increased systemic GZMK resulted in more errors committed locating a hidden platform during RAWM testing compared to young control mice (Figure 2.6L). Together, these data indicate that selectively increasing circulating levels of the age-associated CD8⁺ T cell-secreted factor GZMK impairs cognitive function in the young hippocampus.

To ascertain mechanisms of action, we measured luminescence signal for GZMK-HiBiT in fat, cortex, and hippocampus. While tagged GZMK was identified in peripheral tissues, there was no detection in the hippocampus or cortex, suggesting circulating GZMK does not readily cross the blood-brain-barrier (Figure 2.6J). To identify potential cellular targets of circulating GZMK upstream of cognitive impairments, we overlaid previously reported putative GZMK substrates²⁶ over a snRNAseq hippocampal

and vascular dataset. GZMK substrates were over-represented in many populations of barrier cells (e.g., vascular smooth muscle cells, endothelial cells, choroid plexus epithelial cells, pericytes, and perivascular fibroblasts) and under-represented in glial and neuronal populations (Figure 2.6M). Comparison of targets expressed within the hippocampus and DEGs in the hippocampus after systemic exposure to aged CD8⁺ T cells identified 46 overlapping substrates and GO analysis of these overlapping genes identified a downregulation of various brain-border pathways (Figure 2.6N-P). To further investigate, we utilized the Cell Chat package to assess potential signaling pathway changes between hippocampal cells and young or aged CD8⁺ T cells. While we identified changes in reported pathways, such as increases in CCL and IL1 and decreases in SELL signaling, we also observed predicted changes in T cell to brain-barrier signaling pathways, such as Collagen, FN1, and Laminin (Figure 2.6Q). Concordantly, we observed decreased coverage of PAR1, a GZMK target whose cleavage induces barrier cell dysfunction and alterations in epithelialization, in young mice with increased systemic GZMK by IHC (Figure 2.6R). Taken together, these behavioral and transcriptomics data suggest that cognitive impairments driven by aged CD8⁺ T cell-derived circulating GZMK are likely mediated, in part, through interactions with brain-barrier cells.

Lastly, we investigated the rejuvenating potential of targeting circulating GZMK at old age. For this, we utilized a GZMK inhibitor previously shown to rescue lung function in the context of airway inflammatory disease mouse models *in vivo*. Aged (22 months) mice were given intraperitoneal injections of the GZMK inhibitor or vehicle control (Figure 2.6S), and hippocampal-dependent learning and memory was assessed by RAWM. Following GZMK inhibitor treatment, aged mice committed significantly fewer errors

locating a hidden platform during RAWM testing compared to vehicle treated aged control mice (Figure 2.6T). These behavioral data indicate that systemically targeting extracellular GZMK in an aged animal can restore cognition.

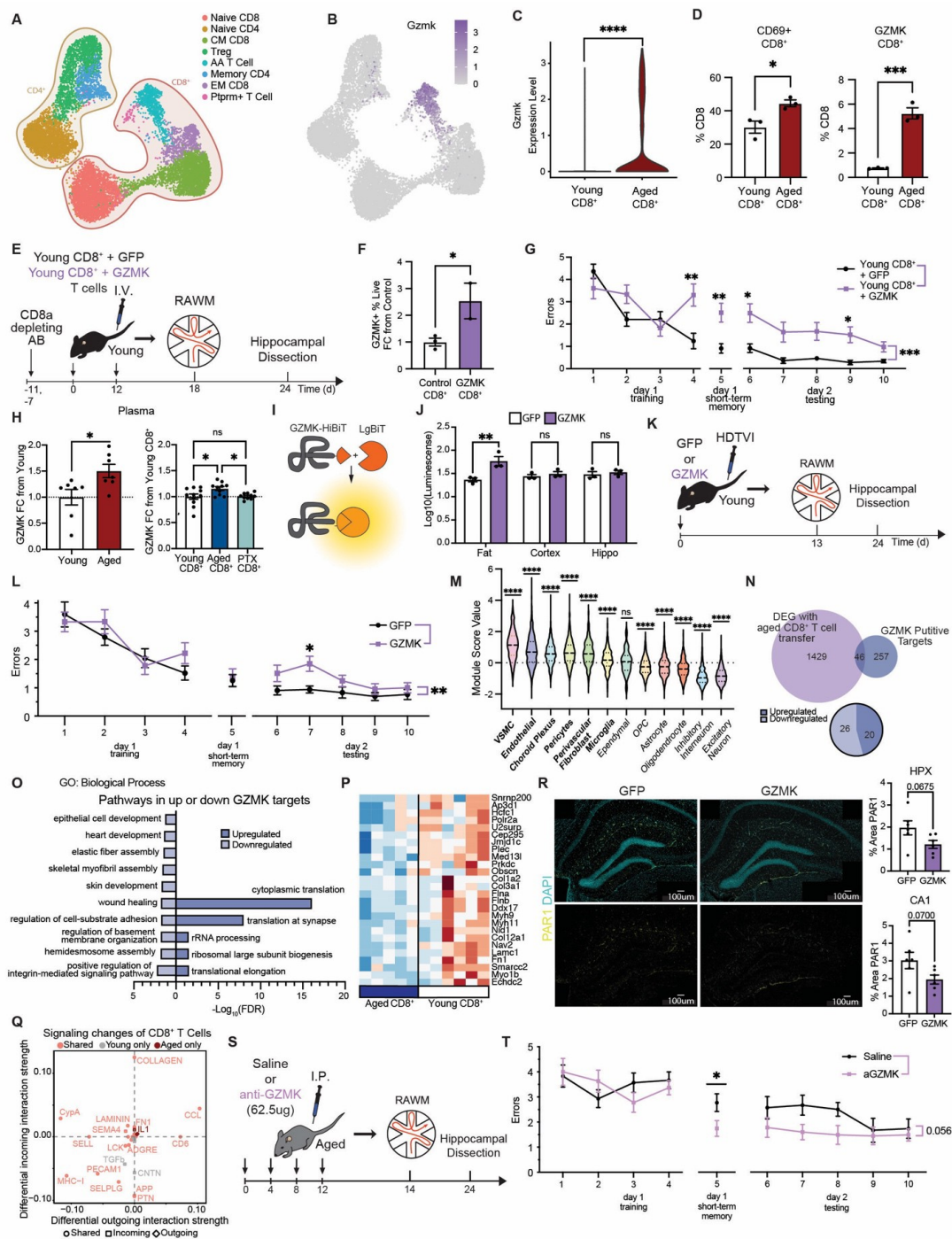


Figure 2.6. Aged CD8⁺ T cell-derived systemic GZMK promotes cognitive decline.
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- A**, UMAP projection of identified T cell types.
- B**, Expression of Gzmk in T cell types.
- C**, Violin plot of Gzmk expression in young and aged isochronic CD8⁺ T cells.
- D**, Percentage of activated (CD69⁺) young and aged CD8⁺ T cells and activated cells expressing GZMK following 3 days in culture, as measured by flow cytometry.
- E**, Schematic illustrating the timeline of adoptive transfer of 3e⁵ young (4 months) GFP or GZMK retrovirus treated CD8⁺ T cells to young mice (4 months, BoyJ) after treatment with 200ng CD8a depletion antibody, i.p., followed by cognitive testing.
- F**, Percent of cells expressing GZMK d5 post retroviral treatment versus control, as measured by flow cytometry.
- G**, Hippocampal-dependent learning and memory as evaluated by RAWM (n=11/group).
- H**, ELISA of GZMK levels, as normalized to control in aged (22 months) versus young (4 months) plasma and plasma of mice receiving young vehicle treated versus mice receiving vehicle or PTx treated aged CD8⁺ T cells.
- I**, Schematic illustrating HiBit reaction.
- J**, Levels of HiBit tagged GZMK in fat, cortex, and hippocampus 24 hours post hydrodynamic tail vein injection (HDTV).
- K**, Schematic illustrating the timeline of young mice (4 months) following systemic overexpression of GZMK or GFP via HDTV followed by cognitive testing.
- L**, Hippocampal-dependent learning and memory as evaluated by RAWM (n=14-15/group).
- M**, Module score of putative GZMK targets, overlain onto single nucleus vessel RNA sequencing.
- N**, Overlap of differentially expressed genes (p<0.05) in aged versus young CD8⁺ transfer hippocampi identified by bulk sequencing with putative GZMK targets expressed in the hippocampi (average expression>1), and directionality of overlapped targets.
- O**, Top GO terms of biological processes associated with differentially expressed up or downregulated overlapped targets.
- P**, Heatmap of differentially expressed downregulated GZMK targets.
- Q**, Predicted differential signaling pathways between young or aged CD8⁺ T cells and hippocampal cells by Cell Chat analysis.
- R**, Representative image and quantification of Par1 (yellow) in the full hippocampi and the CA1 region as measured by immunohistochemistry (n=6/group).
- S**, Schematic illustrating the timeline of treatment of aged mice (22 months) with 62.5ug anti-GZMK antibody or saline control, i.p., followed by cognitive testing.
- T**, Hippocampal-dependent learning and memory as evaluated by RAWM (n=9-10/group).

Statistical analysis was performed using DESeq2 (C,N,P), t-test (D,R), one-way ANOVA with Tukey's post-hoc (H), one-tailed t-test (F), two-way ANOVA with Šídák's post-hoc (G,J,L,T), one-sample t-test vs 50% (M), and Fisher's exact-tests (O).

Discussion

Cumulatively, our data show that activated aged circulating CD8⁺ T cells and their secreted factors are drivers of hippocampal-dependent cognitive decline. From a translational perspective, these data further suggest significant therapeutic potential to ameliorate age-related cognitive decline by targeting aged peripheral CD8⁺ T cell-derived factors in blood circulation at old age.

While a prominent role is emerging for CD8⁺ T cells as regulators of brain aging, thus far a focus has been placed predominantly on the role of CD8⁺ T cell infiltration and clonal expansion within the brain. Perplexingly, infiltrating CD8⁺ T cells in the brain have been ascribed contrasting detrimental and protective roles in mouse models of Alzheimer's disease pathology, underscoring the need to delineate the role of infiltrating versus non-infiltrating CD8⁺ T cells in brain aging. Outside of the brain parenchyma, activation and immune surveillance of CD8⁺ T cells via the meningeal lymphatic network is disrupted in aging. Moreover, single cell RNA-sequencing analysis identified associations between altered cytokine signaling in effector memory CD8⁺ T cells in the cerebrospinal fluid of elderly humans and age-related cognitive impairments, although no functional studies accompanied transcriptomics analysis. While we do not exclude a role for infiltrating CD8⁺ T cells in the aged hippocampus, our data define an important mutually inclusive functional role for non-infiltrating aged circulating CD8⁺ T cells and their secreted factors in driving cognitive decline in the aging hippocampus.

We further identify age-associated effector memory CD8⁺ T cell-derived circulating factors, such as secreted GZMK, as systemic mediators of hippocampal aging. It has been suggested that GZMK, alone or in combination with Interferon- γ , can enhance age-

related inflammatory processes. As such, age-associated CD8⁺ T cell derived factors in blood may work in concert with infiltrating CD8⁺ T cells to promote localized Interferon- γ mediated pro-aging effects across diverse brain regions. Of note, infiltrating GZMK⁺ CD8⁺ T cells have been shown to be detrimental in an Ab-based Alzheimer's model by promoting neurotoxic inflammation yet protective in a Tau-based Alzheimer's model by targeting microglia. In this context, targeting CD8⁺ T cell-derived GZMK in circulation, rather than infiltrating GZMK⁺ CD8⁺ T cells themselves, provides a promising alternative therapeutic approach that is agnostic to brain region and disease state.

Mechanistically, we link aged circulating CD8⁺ T cells to changes in the brain vasculature; in line with the emerging role of extracellular, secreted GZMK in affecting border-associated cells. Recently, we demonstrated that manipulations selectively to the brain vasculature drive both age-related decline and rejuvenation of hippocampal-dependent cognitive function. Moreover, previous studies indicate that vascular manipulations regulate neuronal and regenerative functions in the brain. In this context, our findings position aged circulating CD8⁺ T cells and their secreted factor GZMK as an upstream mechanism that induces age-related changes in the brain vasculature and subsequently leads to synaptic alterations and cognitive decline.

Taken together with this rapidly growing body of work, our data advance complementary strategies to rejuvenate cognition in old age by either targeting trafficking of infiltrating CD8⁺ T cells into distinct regions of the aged brain or systemically inhibiting age-associated CD8⁺ T cell-derived factors such as GZMK in blood circulation. More broadly, age-related changes in circulating CD8⁺ T cells have been linked to multimorbidity and premature senescence in a variety of peripheral tissues, posing an

important therapeutic target for broad age-associated disorders that extend beyond deficits in neurological functions.

Limitations of the study

The field of brain-body communication has shown that the effects of young and old blood are driven by multitudinous peripheral factors that work in concert to drive the full effects of aging. In this context, the aim of our study was to identify novel, age-specific factors that contribute to CD8⁺ T cell driven cognitive decline, specifically in aging. As such, our data build on this emerging body of work showing that targeting a single factor in circulation through a pharmacological approach is sufficient to promote functional recovery at old age. While we acknowledge mechanistic limitations in this pharmacological approach, such as a lack of cell specificity, our focus on a pharmacological GZMK inhibitor approach was prompted by the emphasis on the translational potential of our research.

While our work suggests that age-associated CD8⁺ T cells are sufficient to promote cognitive impairments, in part through GZMK, we note that we do not investigate whether other peripheral CD8⁺ T cell populations promote age-related cognitive decline. Additionally, while increased GZMK is a hallmark of age-associated CD8⁺ T cells, innate-like lymphocytes and NK cells have been shown to express GZMK^{52,53}. Notwithstanding, by functionally demonstrating the effect of CD8⁺ T cell-derived GZMK, we provide proof of concept for the role of peripheral CD8⁺ T cells in age-related cognitive decline. Mechanistically, our transcriptomics data suggest interactions between aged CD8⁺ T cells and their secreted factor, GZMK, and brain barrier cells as a potential mechanism by which circulating aged CD8⁺ T cells impact the aged hippocampus to promote cognitive

decline. However, which barrier cells are involved in this process, as well as the molecular mechanisms downstream of GZMK on the vasculature are unknown. Lastly, we highlight that the effects of aged circulating CD8⁺ T cells are likely the result of multiple pro-aging secreted factors and anticipate that our findings will prompt further mechanistic investigations identifying aged CD8⁺ T cell-derived pro-aging circulating factors.

Materials and Methods

Mouse strains. The following mouse lines were used: C57BL/6J mice (Jackson Laboratory line 000664), C57BL/6 aged mice (National Institutes of Aging), C57BL/6-Tg(UBC-GFP)30Scha/J mice (Jackson Laboratory line 004353), B6.SJL-Ptprca Pepcb/BoyJ mice (Jackson Laboratory line 002014), and B6;129S2-Tap1tm1Arp/J mice (Jackson Laboratory line 002458). All studies were done in young (4-5 month) or aged (22-24 month) male mice. The numbers of mice used to result in statistically significant differences were calculated using standard power calculations with $\alpha = 0.05$ and a power of 0.8. We used an online tool to calculate power (<https://www.stat.uiowa.edu/~rlenth/Power/index.html>) and sample size based on experience with the respective tests, variability of the assays and inter-individual differences within groups. All other studies were performed with male mice. Mice were housed under specific pathogen-free conditions under a 12h light-dark cycle, and all animal handling and use was in accordance with institutional guidelines approved by the University of California San Francisco Institutional Animal Care and Use Committee (IACUC) and the Stanford University Administrative Panel on Laboratory Animal Care (APLAC).

Tissue collection. Mice were anesthetized with 87.5 mg per kg ketamine and 12.5 mg per kg xylazine and transcardially perfused with ice-cold phosphate-buffered saline. To process the brains, the whole brain was sectioned in half along the sagittal plane. The hippocampus from one hemisphere was subdissected and snap-frozen and the other was fixed in phosphate-buffered 4% paraformaldehyde, pH 7.4 at 4 °C for 48 h before cryoprotection with 30% sucrose.

Lymphocyte isolation. For flow cytometry analysis, the hippocampus was subdissected and dissociated with the Neural Tissue Dissociation Kit (P) (Miltenyi Biotech) according to manufacturer instructions, then filtered through a 70 µm cell strainer and washed with 10 mL of MACS buffer (PBS with 2% FBS). For collection of splenocytes, spleens were removed, mechanically dissociated with a syringe plunger over a 70 µm cell strainer and washed with 10 ml of MACS. Cells were centrifuged and RBC lysis was performed (155 mM NH₄Cl, 1 mM KHCO₃ and 0.1 mM EDTA) with 2 ml for 5 minutes room temperature (RT). Subsequently, cells were washed, spun 500 RCF for 5 minutes, and resuspended in MACS. For lymph node isolation, cervical, axillary, brachial, and inguinal lymph nodes were removed and mechanically dissociated with a syringe plunger over a 70 µm cell strainer and washed with 10 mL of MACS. PBMCs were isolated from blood through RBC lysis (2 mL, 5 minutes RT) followed by washing with MACS and repeated lysis until sample was mainly colorless or with a 2:1 Ficoll gradient.

CD8a Antibody Depletion. Aged (22-24 months) mice were treated i.p. with 200 µg of *InVivoMAb* anti-mouse CD8a (2.43, BioXCell, BE0061) or *InVivoMAb* rat IgG2b isotype control (LTF-2, BioXCell, BE0090) in 200 µL PBS every 4 days until time of sacrifice. Depletion of circulating CD8⁺ T cells was confirmed with flow cytometry.

GZMK Inhibition. Aged (22-24 months) mice were treated i.p. with 62.5 µg of GZMK inhibitor D-Pro-Phe-Arg-Chloromethylketone (MedChemExpress, HY-P4245) or saline control in 100 µL PBS every 4 days for a total of 4 injections, followed by behavioral testing.

Adoptive transfer of CD8⁺ T cells. B6.SJL-Ptprca Pepcb/BoyJ (4 months) mice were treated i.p. with 200 µg of *InVivoMAb* anti-mouse CD8a in 200 µL PBS day -11 and -7 to

deplete resident CD8⁺ T cells. CD8⁺ T cells were isolated from single cell suspensions of splenocytes and PBMCs from young (3-4 months) or aged (22-28 months) C57BL/6J mice using the CD8a⁺ T cell Isolation Kit (Miltenyi Biotech, 130-104-075) according to manufacturer instructions. For adoptive transfer of young or aged CD8⁺ T cells, cells were immediately resuspended at concentrations of 3e5 (first injection) or 3e6 (second injection) in 100 μ L saline and injected via tail vein. For pertussis toxin treatment (PTx) (Thermo Fisher Scientific, PHZ1174), cells were incubated at a concentration of 2e6 cells/mL in T Cell Media (RPMI + 10% FBS + 1% p/s, NAA, L-glut, NaPyr + 50 μ M BME) with IL-2 (1:2000, stock 1000 U/ μ L, Thermo Fisher, 212-12) and 0.5 μ g/mL PTx or vehicle control. For Tofacitinib (Selleckchem, S5001) and InVivoMAb anti-mouse/human VLA-4 (CD49d) (BioXCell, BE0071), cells were incubated at a concentration of 2e6 cells/mL in T Cell Media (RPMI + 1.25% FBS + 1% p/s, NAA, L-glut, NaPyr + 50 μ M BME) with IL-2 (1:2000, stock 1000 U/ μ L, Thermo Fisher, 212-12) and 10 μ M Tofacitinib, 20 μ g/mL anti-VLA-4, or vehicle (DMSO) control. Cells were incubated for 24 hours, then were washed three times with saline before resuspension at concentrations of 3e5 cells/100 μ L saline (first injection) or 3e6 cells/100 μ L saline (second injection) and injection via tail vein. Each mouse was injected i.v. with 100 μ L of cells in saline (3e5-3e6 cells/injection).

Single nucleus RNA sequencing. Neuronal nuclei were isolated based on the demonstrated protocol by 10x Genomics with modifications and performed on nuclei isolated from three mice per group. Briefly, flash-frozen dissected hippocampi were dounce homogenized (Wheaton, Cat# 357538) in 500 μ L of NP40 lysis buffer (10mM Tris-HCl (pH = 7.4), 10mM NaCl, 3mM MgCl₂, 0.1% Nonidet P40 Substitute), 1mM DTT, 1x RNase Inhibitor, Nuclease-free H₂O) with 20 strokes of the loose pestle and 25 strokes

of the tight pestle. 500 μ L of NP40 lysis buffer was added and samples incubated for 7 minutes on ice. Samples were filtered through a 40 μ m filter and centrifuged at 500 RCF for 5 min at 4°C. Samples were resuspended in 1ml of Wash Buffer (PBS, 1%BSA, 1xRNAse Inhibitor) and incubated for 5 min on ice. Samples were centrifuged at 500 RCF for 5 min at 4°C, supernatant was removed, and samples were resuspended in 400 μ L of Wash Buffer with 1:10,000 dilution of Hoechst 33342 and incubated for five minutes before filtering through a 35 μ m FACS tube filter and sorting on a BD FACSAria II. Nuclei were sorted on a BD FACSAria Fusion with a 70 μ m nozzle and with a flow rate of 1–2.5. Nuclei were first gated by forward and side scatter, then gated for doublets with height and width. Nuclei that were Hoechst+ were sorted and samples were combined per group. Isolated nuclei were given to the UCSF-IHG Genomics Core for analysis with the 10x Genomics Chromium Single Cell Expression Solution 3' kit. The Genomics Core prepared cells for 10x Genomics Chromium single-cell capture. 30,000 nuclei were loaded per sample. cDNA libraries were prepared according to the standard 10x Genomics protocols. The final library pool was sequenced on the NovaSeq 6000 S2 system (CD8⁺ depletion) or the NovaSeq X 10B system (CD8⁺ adoptive transfer) at the UCSF CAT Core. The raw base sequence calls were demultiplexed into sample-specific cDNA files with bcl2fastq/mkfastq sample sheet using Cell Ranger 7.1 (10x Genomics). We note that due to technical limitations our sequencing approach did not capture robust numbers of astrocytes.

Single cell and single nucleus RNA sequencing analysis. Raw FASTQ files were processed and aligned to mm10 using the Cell Ranger software package (10x Genomics) for the RNA expression matrix, including introns in analysis for single nucleus analysis.

Outputs from adoptive transfer snRNAseq were integrated with Cell Ranger Aggr (10x Genomics) to normalize for sequencing depth. For CD8⁺ adoptive transfer sequencing, a total of 52,221 cells were sequenced at a depth of 37,013 reads per cell and approximately a 68% sequencing saturation of 34.1%. Further QC and analysis were performed on R 4.2.2. Lastly, for CD8⁺ depletion sequencing, 15,237-16,194 cells were sequenced at a sequencing depth of 23,213 reads per cell and 37.6% sequencing saturation (IgG2b) or a sequencing depth of 32,910 reads per cell and 47.4% sequencing saturation (CD8a). Ambient RNA was removed with the SoupX package and data from the four samples was integrated using Seurat (2000 variable features, 1:20 dimensions). For all experiments, downstream analysis and data visualization was performed using Seurat, as well as packages DropletUtils, ggplot2, knitr, WriteXLS, RColorBrewer, data.table, stringr, ggplot2, forcats, dplyr, Nebulosa, and htmlwidgets. Data were processed to remove doublets and unwanted sources of variation by removing cells with more than 3,500 and fewer than 300 genes per cell or nuclei with more than 5,000 and fewer than 300 genes per cell and regressing on number of UMIs. Genes expressed in fewer than three cells or nuclei were filtered out. Cells with a percentage of mitochondrial genes of higher than 10% or nuclei with a percentage higher than 0.1% were removed. Final nuclei counts of 14,589 (Young CD8), 10,510 (Aged CD8), 24,463 (Ptx CD8), 12,079 (IgG2b), and 12,062 (CD8a) were used for gene expression analysis. The matrices of data were log-normalized in a sparse data matrix and PCA was applied to reduce dimensionality. The first 20 (CD8 depletion) or 30 (adoptive transfer) PCA components were used to cluster cells by Louvain clustering implemented in Seurat while UMAP plots were independently generated to aid in 2D representation of multidimensional data

independent of the clustering. Cell types were identified using known markers, as well as the Allen Mouse Brain Atlas and clusters containing more than one cell type specific marker were excluded. Differential gene expression was performed with a minimum of 10% of cells or 5% nuclei expressed, log fold change threshold of 0.15, and a pseudocount of 0.1. Log-normalized gene expression data were used for visualizations with violin plots and UMAP feature plots. Average expression matrices were used for heatmap visualization. Volcano plots were created using the EnhancedVolcano package and UpSet plots with the ComplexHeatmap package. Pseudotime analysis was performed with monocle3. Predicted interactions were identified with CellChat.

Bulk RNA isolation and sequencing. RNA was isolated from previously flash frozen hippocampi from one hemisphere. Total RNA was isolated from samples by lysis using TRIzol Reagent (Thermo Fisher Scientific), separation with chloroform and precipitation with isopropyl alcohol, according to the manufacturer's instructions. Isolated RNA was sent to GENEWIZ for library construction and sequencing. Alignment of RNA-seq reads to the mouse mm10 transcriptome was performed using STAR using the ENCODE standard options, read counts were generated using RSEM and differential expression analysis was performed in R 3.6.1 using the DESeq2 package (detailed pipeline v.2.0.1 and options are available at GitHub (<https://github.com/emc2cube/Bioinformatics/>)). Genes significantly changed after treatment were determined using a nominal $p < 0.05$. GO term enrichment analysis was performed using PANTHER (v.18.0), with statistical enrichment-test over the Mus musculus reference genome (FDR < 0.05). GO terms with fully overlapping genes were narrowed to one term. Heat maps were generated using ggplot2. Module score analysis was performed by identifying cells expressing a dataset

of z-scored differentially expressed genes ($p < 0.05$) with the Seurat AddModuleScore function with a single nucleus RNA sequencing dataset.

Flow Cytometry. Single cell suspensions of splenocytes, PBMCs, lymph nodes, and hippocampal cells were isolated as stated above, then approximately 1×10^6 cells per sample were stained on a round-bottom 96-well plate with a live/dead marker (GhostDye UV 450, Tonbo, 13-0868) 1:100 in PBS for 20 minutes RT. Cells were subsequently washed in FACS buffer (PBS with 2% FBS, 2 mM EDTA, 2mM NaN₃) and spun for 2 minutes at 300 RCF. Samples were blocked with 2.4G2 (1:100 in FACS) for 10 minutes on ice. Cells were washed with FACS, then resuspended in an antibody cocktail in FACS buffer with Brilliant Stain Buffer (BD, 56379) at a 1:1 ratio for cell surface staining and incubated on ice for 30-60 minutes. After washing, cells were either fixed with 4% paraformaldehyde, washed and resuspended in FACS buffer for storage until analysis or followed with intracellular staining with the Foxp3 / Transcription Factor Staining Buffer Set (eBioscience, 00-5523-00) according to manufacturer instructions. Cells were analyzed using the BD LSR II Flow Cytometer at the UCSF Parnassus Flow Cytometry CoLab and Flow Jo software (TreeStar). Fluorescence minus one (FMO) controls were used for the initial antibody tests, then thereafter for certain stains. Immune cell populations after parabiosis were analyzed according to published protocols, with the same antibodies and concentrations used⁵⁸. For T cell populations, antibodies used were as follows: CD45-BV711 (1:200, BD Biosciences, 563709), CD45-PE (1:200, ThermoFisher Scientific, 5010370), CD45.2-FITC (1:200, eBioscience, 11-0454-82), CD45.1-PE (1:200, eBioscience, 12-0453-83), CD3-APC (1:200, Tonbo Biosciences, 20-0031-U025), CD8a-PB (1:200, ThermoFisher Scientific, MCD0828), CD4-PE-Cyanine7

(1:200, ThermoFisher Scientific, 25-0041-82), CD44-APC-eFluor 780 (1:100, ThermoFisher Scientific, MCD0828), CD69-AF700 (1:100, ThermoFisher Scientific, 56-0691-82), CD62L-PerCP-Cyanine5.5 (1:100, ThermoFisher Scientific, 45-0621-82), GZMK-FITC (1:100, LSBio, LS-C692079-200), or CCL5-PE-Cyanine7 (1:100, BioLegend, 2308-03). For CD45-PE labeling injections, mice were injected i.v. with 200 μ L of CD45-PE (1 μ L/100 μ L) in saline 3 minutes before sacrifice.

RT-qPCR Analysis. To quantify mRNA expression levels, equal amounts of cDNA were synthesized using the High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, 4368813), then mixed with SYBR Fast mix (Kapa Biosystems) and primers. *Gapdh* was amplified as an internal control. RT-qPCR was performed in the CFX384 Real Time System (Bio-Rad). Each sample and primer set were run in triplicates and relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Immunohistochemistry. Tissue processing and immunohistochemistry was performed on free-floating sections according to standard published techniques. Cryoprotected brains were sectioned coronally at 40 μ m with a cryomicrotome (Leica Camera). Free-floating coronal sections (40 μ m) were permeabilized with pre-treatment buffer (0.2% TritonX-100 in TBST) for 30 minutes RT, then washed 3x with TBST and blocked with TBST + 5% NDS. Sections were then incubated overnight at 4 degrees C with anti-Iba1 (1:1,000, Wako, 01919741), anti-Iba1 (1:1000, Synaptic Systems, 234-004), anti-CD68 (1:250, FA-11, Bio-Rad, MCA1957T), anti-cFos (1:100, 96F, Cell Signaling, 2250), anti-CD31 (1:200, R&D, AF3628), anti-CD8 (1:250, 4SM15, eBioscience, 14-0808-82), anti-Collagen1 (1:500, Abcam, ab21286), or anti-Acta2 (1:500, ThermoFisher Scientific, 14-9760-82), or anti-Par1 (1:200, Invitrogen, PA5-116040 in TBST + 5% NDS. Labelling was

revealed using secondary antibodies at 1:500 in TBST + 3% NDS for 1 hour room temperature. Sections were imaged using confocal microscopy (Zeiss LSM800 or Zeiss LSM900) or bright-field microscopy (Keyence). Individual cell numbers and intensity in the dentate gyrus was quantified using ImageJ.

Western Blot Analysis. For Western blot analysis, samples were combined with RIPA lysis buffer (Abcam, ab156034) with complete protease inhibitor (4693116001, Sigma-Aldrich) and phosphatase inhibitor (Thermo Fisher Scientific, 78420). Subsequently, the samples were mixed with 4× NuPage LDS loading buffer (Invitrogen, NP0008), loaded onto an SDS polyacrylamide gel (Invitrogen) and transferred onto a nitrocellulose membrane. Equal loading of samples was confirmed using Ponceau S solution (Sigma-Aldrich, P7170) and membranes were imaged with the ChemiDoc System (Bio-Rad). The blots were blocked in 5% milk in Tris-buffered saline with Tween-20 and incubated with anti-GAPDH (6C5, 1:5,000, Abcam, ab8245), anti-beta-Tubulin (1:1000, Eurogentec, MMS-435P-200), anti-Synapsin1 (1:250, Abcam, ab18814), anti-NR2A (1:1000, Sigma Millipore, 07-632), anti-NR2B (1:4000, Abcam, ab65783), anti-PSD95 (1:500, Cell Signaling, 2507S), anti-Homer1 (1:500, Invitrogen, PA5-21487), or anti-ZO1 (TJP1) (1:500, ThermoFisher Scientific, 40-2200). Horseradish-peroxidase-conjugated secondary antibodies (donkey anti-goat conjugated HRP (1:2,000, Invitrogen, A15999), goat anti-mouse conjugated HRP (1:2,000, Millipore, AP124P), and donkey anti-rabbit conjugated HRP (1:2,000, GE Healthcare, NA934V)) and an ECL kit (GE Healthcare) were used to detect protein signals. Developed membranes were imaged using the ChemiDoc System (Bio-Rad). Selected images were exported and quantified using ImageJ (v.2.0.0).

Cell Culture. CD8⁺ T cells were isolated from single cell suspensions of splenocytes and PBMCs from young (3-4 months) or aged (24 months) mice using the CD8a⁺ T cell Isolation Kit according to manufacturer instructions. Cells were cultured in T Cell Media with IL-2 (1:2000, stock 1000 U/ μ L) at a concentration of 2e6 cells/mL. Three days post culture, media was changed, and cells were incubated with GolgiStop Protein Transport Inhibitor (BD, 554724) at 4 μ L/6 mL cell culture for 6 hours to block intracellular protein transport. Cells were then washed and processed for flow cytometry.

GZMK ELISA. Mouse blood was collected by intracardial bleed at time of euthanasia. Blood was collected with EDTA, followed by centrifugation at 1,000g for 10 minutes for plasma separation. For ELISA analysis, plasma was aliquoted and stored at -80 °C until use. GZMK plasma protein levels were measured by ELISA (MyBioSource, MBS453082) according to the manufacturer's protocol.

HDTVl. GZMK (OriGene, #MR215106) and GFP control plasmids were used for HDTVl-mediated systemic overexpression. Hydrodynamic tail vein injections were performed as previously described^{29,60}. Endotoxin-free plasmids were prepared using the Qiagen Maxi-Prep Plus Kit (Cat# 12963, VWR). GZMK or GFP plasmid DNA (50 μ g) was suspended in 3 mL saline and injected in the tail vein in 5-7 seconds in mice. All coding plasmid sequences were verified by Sanger sequencing. To confirm systemic overexpression, mice were injected with HiBiT-tagged GZMK or GFP. The NEBaseChanger tool was used in combination with the Q5 Site-directed Mutagenesis kit (NEB # E0554S) to generate the C-terminally tagged GzmK construct. The 33 nucleotides coding for the 11 amino acid HiBiT tag were inserted between the last coding and stop codon. Briefly, the HiBiT coding sequence was split between the forward and reverse primers, the plasmid amplified,

ligated and transformed according to the manufacturer's instructions. The resulting plasmid was sequence verified using whole plasmid sequencing and amplified using an endotoxin free maxiprep kit. To perform HDTVl of constructs, plasmid DNA (50 µg) was suspended in 3 ml saline and injected in the tail vein in 5–7 seconds in mice. At 24 hours after HDTVl, the mice were euthanized, and plasma was collected by intracardial bleed. A total of 20 µg of protein from each sample was loaded in duplicate in an opaque 96-well plate (Corning, 353296). HiBiT luminescence was measured on the Cytation 5 (BioTek) using the Nano-Glo HiBiT Lytic Detection System (Promega, N3030) according to the manufacturer's instructions.

Retrovirus production. Retroviral GZMK and GFP expression transfer plasmids were generated using the NEBuilder HiFi DNA Assembly Kit (NEB #E5520S), following the manufacturer's recommended design considerations and protocol. Briefly, the MSCV vector backbone (Addgene #127890) was digested with the restriction enzymes EcoRI and BglII and gel purified. eGFP and murine GzmK coding sequences were PCR amplified with primer sets that included a 20 nucleotide overlap with the backbone and preserved the restriction sites. Additionally, a Kozak sequence was included in the forward primers. All plasmids were sequence verified using whole plasmid sequencing. HEK293T cells were lipotransfected with 40 µL Lipofectamine 3000 in 500 µL OptiMEM, mixed with equal amounts of 7.5 µg pCL-Eco, 7.5 µg retroviral GZMK or GFP, and 36 µL P3000 in 500 µL OptiMEM. 1 mL of transfection mix was added to 7 mL of 293T cells. After 48 and 72 hours, retrovirus-containing media was centrifuged for 5 min at 300 RCF and filtered through 45µm filter to remove cellular debris. Retrovirus was concentrated 100X using

lenti-X concentrator (Takara #631232), according to manufacturer instructions. Concentrated retrovirus was used at a dilution of 1:10.

Retroviral overexpression of GZMK and GFP. Isolated CD8⁺ T cells were cultured in T Cell Media + IL-2 (1:2000, stock 1000 U/μL) and activated for 24 hours with Mouse T-Activator CD3/CD28 beads (Gibco, 11452D). 24 hours post activation, cells were resuspended at 2e6 cells/mL in T Cell Media + IL-2 (1:1000) + polybrene (10 μg/mL) and seeded on a retronectin coated 12 well plate (15 μg/mL in PBS, Takara, #T100A). Equal volumes of retrovirus or vehicle in T Cell Media (1:10) was added per well. Cells were centrifuged at 2000 RCF for 60 minutes at 30 degrees C and incubated overnight. Media was changed the next day, then subsequent days to allow for proper expansion (reseeding cells at a density of 2e6 cells/mL). Transduction efficiency was analyzed by flow cytometry. 4 days post spinoculation, ½ the aged GFP⁺ CD8⁺ T cells were treated with PTx as described above. 24 hours post treatment, cells were washed and resuspended at a density of 3e5 cells/100 μL in saline for adoptive transfer via tail vein injection.

NOR. The NOR task was performed as previously described. On day one (the habituation phase), mice performed open field testing by exploring an empty arena for 10 min. Infrared photobeam breaks were recorded and movement metrics were analyzed using the MotorMonitor software (Kinder Scientific). On day two (the training phase), two identical objects were placed into the habituated arena, and the mice were allowed to explore for 5 min. On day three (the testing phase), one object was replaced with a novel object, and the mice were allowed to explore for 5 min. The time spent exploring each object was quantified using the Smart Video Tracking Software (Panlab; Harvard

Apparatus). Two different sets of objects were used. To control for any inherent object preference, half of the mice were exposed to object A as their novel object and half to object B. To control for any potential object-independent location preference, the location of the novel object relative to the trained object was also counterbalanced. To determine the percentage of time with the novel object, we calculate $(\text{time with novel object}) / (\text{time with trained object} + \text{time with novel object}) \times 100$. Mice that did not explore both objects during the training phase were excluded from the analysis.

RAWM. Spatial learning and memory were assessed using the RAWM paradigm, according to an established protocol. In this task, the mouse was trained to the location of a constant goal arm throughout the training and testing phase. The start arm changed each trial. Entry into an incorrect arm was scored as an error, and errors were averaged over training blocks (three consecutive trials). During training (day 1), the mice were trained for 12 trials (blocks 1–4), with trials alternating between a visible and hidden platform. After an hour break, learning was tested for 3 trials (block 5) using only a hidden platform. During testing (day 2), the mice were tested for 15 trials (blocks 6–10) with a hidden platform. When scoring, investigators were blinded to treatment. All blocks were analyzed for significance via two-way ANOVA with Šídák's post-hoc, and only significant data points were annotated on figures. Brackets represent significance of area under the curve.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data, statistical analyses, and reproducibility. All experiments were randomized and blinded by an independent researcher. Researchers remained blinded throughout histological, biochemical and behavioral assessments. Groups were unblinded at the end

of each experiment on statistical analysis. Data are expressed as mean $\pm$ s.e.m. The distribution of data in each set of experiments was tested for normality using the D'Agostino–Pearson omnibus test or Shapiro-Wilk test. Statistical analysis was performed using Prism (GraphPad). Means between two groups were compared using two-tailed unpaired Student's *t*-tests. Comparisons of means from multiple groups with each other were analyzed using one-way ANOVA followed by the appropriate post hoc test, as indicated in the figure legends. Additional statistical details are indicated in the respective figure legends. All data generated or analyzed in this study are included in this article. All measurements were taken from distinct samples. Data files for bulk RNA-sequencing (Accession: GSE266454, Token code: yfehegswbbslxon), CITE-sequencing (Accession: GSE266467, Token code: ehejkuoghxfhkv), and single nucleus RNA-sequencing (Accession: GSE266468, Token code: kfoxkisqbtbxzel) have been uploaded to GEO repository for reviewer access.

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