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Metabolic reprogramming in memory CD4 T cell responses of old adults

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ABSTRACT

To determine whether aging affects the ability of T cells to undergo metabolic reprogramming upon activation, we compared CD4 T cell responses after polyclonal in vitro stimulation. Compared to younger adults, CD4 memory T cells from healthy older individuals exhibited a higher upregulation of oxidative phosphorylation with increased production of reactive oxygen species and intracellular and secreted ATP. Increased ATP secretion led to increased purinergic signaling and P2X7-dependent increases in cytoplasmic calcium. The increased mitochondrial activity was not due to a difference in activation-induced mitochondrial biogenesis. Expression of carnitine palmitoyl transferase 1 was higher, conversely that of fatty acid synthase was reduced in older T cells, resulting in increased fatty acid oxidation, while depleting intracellular lipid stores. The aged CD4 memory T cells therefore maintain a more catabolic state in lipid metabolism, while their ability to upregulate glycolysis upon activation is preserved.

1. Introduction

The ability to generate and maintain memory T cells is central to immune health throughout life. Upon antigen encounter, such as after infection or vaccination, antigen-specific T cells expand by several magnitudes and differentiate into effector cells. While most of these effector cells die after the peak response, a subset of the expended subpopulation survives as memory cells that are long-lived and provide protection upon re-infection. Generation and maintenance of these memory T cells is compromised with immune aging [1,2]. The most striking clinical example for fading immune memory is the latent varicella zoster virus (VZV) infection. Reactivation of the virus manifesting as shingles increasingly occurs after the age of 50 years culminating in about 50% risk to experience shingles by the age of 80 years [3]. The failed protection has been related to a decline in VZV-specific memory cells in spite of the ongoing presence of the latent virus suggesting that at least some memory cells generated during childhood infection have limited lifespan [4]. Moreover, generation of long-lived memory cells, both, in a primary response as well as in a recall response, is impaired in late adulthood. Naïve CD4 T cells from older individuals have a propensity to differentiate into short-lived effector rather than memory cells due to sustained activation of the mTORC1 signaling pathway. As well, restimulated CD4 memory cells differentiate preferentially into CD39-expressing short-lived effector cells [5]. These effector cells have gained the expression of regulatory molecules including the dual specific phosphatase 4 (DUSP4) that limit cell function and survival [6]. A similar preference to generate short-lived effector T cells is see in vivo in older individuals. After VZV vaccination, loss of the expanded antigen-specific T cell population after the peak response is accelerated [6]. Consequently, the vaccination-induced gain in VZV-specific CD4 memory T cell frequencies is attenuated.

One important regulatory determinant driving lineage differentiation after T cell activation and in particular the generation of short-lived effector versus long-lived memory T cells is metabolic reprogramming [7,8]. Resting naïve T cells that only infrequently divide by homeostatic proliferation, have limited need for biomolecule production and mainly use nutrients for ATP production by oxidative phosphorylation (OXPHOS). TCR signaling switches cell metabolism to anabolic pathways to support the proliferative expansion and activates genes involved in glucose, glutamine and amino acids uptake and processing [9–11]. Metabolism of effector cells is dominated by glycolysis, mainly funneling glucose into the pentose phosphate pathway, the hexosamine pathways, and glutaminolysis. Rather than feeding fatty acids into the tricarboxylic acid (TCA) cycle to support oxidative phosphorylation (OXPHOS), fatty acid synthesis is activated to meet the need to build new membrane structures. Developing into a memory T cell, cells again commit to OXPHOS. Indeed, interventions to drive fatty acid oxidation (FAO) enhance memory cell formation [12,13].

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Although both cell types share OXPHOS as their main metabolic pathway, naïve and memory T cells are distinct as memory T cells have several characteristics which distinguish them metabolically from naïve T cells. They possess an increased spare respiratory capacity (SRC), in part due to increased CPT1a expression that controls FAO [14,15]. Moreover, CD8 memory T cells have differences in mitochondrial structures. CD4 memory T cells also have higher cytosolic concentration of GAPDH conferring spare glycolytic activity [15]. Memory T cells are therefore metabolically fitter than naïve T cells which may enable their faster response to antigenic challenges.

Here we have examined whether the metabolic state of CD4 memory T cells changes with age and whether activation-induced changes in metabolic programming in part account for immune memory defects. We found that after TCR stimulation CD4 memory T cells from young as well as old individuals equally upregulated glucose consumption. In parallel in the first 48 h, oxygen consumption rates (OCR) reflecting OXPHOS was also upregulated, but significantly more so in cells from older individuals. Increased OXPHOS was associated with increased mitochondrial reactive oxygen species (ROS) production and increased generation and secretion of ATP. Activated memory T cells from older individuals expressed higher CPT1a suggesting that increased OXPHOS was driven by increased FAO. Conversely, they expressed less fatty acid synthase. Taken together, memory CD4 T cells from older individuals maintain a lipid catabolic state after activation with increased production of ATP and depletion of lipid stores. Improving T cell responses in the elderly may therefore require the inhibition of mitochondrial energy production.

2. Materials and methods

2.1. Isolation of memory CD4 T cells

Peripheral blood was obtained from individuals who did not have an acute disease nor a history of immune-mediated diseases or cancer. Young adults were < 35 yrs. and old adults were > 65 yrs. old. In addition, LRS chambers and buffy coats from young and old donors were obtained from the Stanford Blood Center. The samples were diluted to 40 mL with 2% FBS/PBS and total CD4 T cells were negatively selected using the RossetteSep Kit from StemCell Technologies (Cat#:15062). After total T cell isolation, naive and memory CD4 T cells were separated using CD45RO magnetic beads from Miltenyi (Cat#: 130–046-001) and an AutoMacs instrument. After isolation, memory CD4 T cells were stimulated with Dynabeads (Gibco Cat#: 11132D) at a concentration of 1:4 beads to cells. Cultures from young and older adults were always run in parallel.

2.2. Seahorse metabolic assays

At indicated time points after Dynabeads activation (1–4 days), memory CD4 T cells were transferred to a Seahorse 96 -well plate at a confluency of 200,000 cells per well. The cells were attached to the bottom of the plate using CellTak from Corning (Cat#: 34240) at a dilution of 100× in water following manufacturer's protocol. T cells were incubated for at least 30 min in a CO_2 free incubator in unbuffered RPMI 1640 medium (Sigma R1383) supplemented with glucose (20 mM) and sodium pyruvate (1 mM) (Corning Cellgro 25-000 - CI). Measurements were performed at 37 °C using an XF96 extracellular analyzer (Seahorse Bioscience). The Seahorse XF Cell Mito Stress Test Kit was used to measure mitochondrial oxygen consumption rate. The cells were treated with oligomycin (1.5 μM), FCCP (1.5 μM), and Rotenone/Antimycin A (0.5 μM), respectively at time points indicated. Additional inhibitors used for Seahorse metabolic assays were UK5099 (2 μM), BPTES (3 μM), and Etomoxir (5 μM) (Seahorse Bioscience). All inhibitor concentrations are the final concentrations in the well of the Seahorse plate.

2.3. Simple western analysis

Cells were lysed with RIPA buffer (Santa Cruz Biotechnolgy Cat#: sc - 24,948) and sonicated for complete cell lysis. Lysates were centrifuged at 4 °C for 10 min at 10,000 RPMs and the supernatant was collected. Protein concentration was measured using Bradford Dye (BioRad Cat#: 5000205) and reading absorbance at 595 nm wavelength. Simple western was performed following manufacturer's instructions at a protein concentration of 0.2 μg/uL per sample. The samples were run on a Peggy Sue instrument (ProteinSimple Inc). The high sensitivity protocol was applied by increasing the stacking loading time to 20 s, the sample loading time to 12 s, and the separation time to 45 min. Primary antibodies were as listed: CPT1a (Cell Signaling, Cat#: 12252), SIRT1 (Novus Biologicals, Cat#: NBP1–49540), AMPK (R&D, Cat#: MAB3197), phospho-AMPK (Cell Signaling, Cat#: 2535), Cytochrome c (Cell Signaling, Cat#: 11940).

2.4. Western blot

Cell lysates produced with RIPA buffer were mixed with 4× Laemmli sample buffer and 10 μg of protein were loaded to a 4–12% PAGE gel. The gel was run at 120 V until proper protein separation was achieved. The proteins were transferred to a PVDF membrane and then incubated with primary antibody (1:1000) in 5% milk overnight at 4 °C. The next day, the membrane was washed with PBST and incubated with secondary HRP-linked antibody for 1 h. After this incubation, the membrane was washed and developed with ECL detection system. Primary antibodies were as listed: PDK1 (Novus Biologicals, Cat#: NBP2–22171), MPC1 (Cell Signaling, Cat#: 14462).

2.5. qPCR

RNA was isolated using RNeasy Micro Plus kit from Qiagen (Cat#: 74034) following manufacturer's instructions. Reverse transcription was done using VILO master mix (Cat#: 11755050). The generated cDNA was used for qPCR using power SYBR Green Master Mix (Cat#: 4367659). Primers are as listed:

SNAT1 (5′-CTGGCGTACCCAAAATGCAG-3′, 5'-TGCAGCTGGAGAA TGAGAGC-3′),

SNAT2 (5′-GCGATTGTGGGCAGTGGAAT-3′, 5′-ACCCTCCTTCATTG GCAGTCT-3′),

GLS (5′-TCCCCAAGGACAGGTGGAATA-3′, 5′-CCTTGAGGTGTGTA CTGGACT-3′),

AMPK (5′-GGACTCCTTTAAACCGCTTG-3′, 5′-CTTGGACATGAACT CTGGCT-3′),

CPT1a (5′-GACCGGGAGGAAATCAAACC-3′, 5′-CATGTGCTGGATG GTGTCTGT-3′).

2.6. ATP measurement

For intracellular ATP measurement, cells were lysed using RIPA buffer. Cell lysate was diluted 1:100 in ultrapure water and CellTiter-Glo from Promega (Cat#: G7570) was used to measure ATP levels following manufacturer's instructions. Briefly, 50 μL of cell lysate dilution was mixed with 50 μL of CellTiter-Glo reagent and incubated for 10 min at room temperature. Bioluminescence was measured using a GloMax 20/20 luminometer. Values obtained were compared to a standard curve to determine ATP concentration. ATP values were normalized to protein levels for each sample.

For extracellular ATP measurements, supernatant from each sample was collected and incubated at 95 °C in a heat block for 5 min to inactivate any ATPases present. Supernatants were mixed with CellTiter-Glo reagent; measurements were performed the same way as for intracellular ATP measurements described above.

2.7. Cytoplasmic calcium measurement

Dynabeads were removed from cells using a magnetic rack and cells were washed twice with calcium-free Hank's Balanced Salt Solution (HBSS ThermoFisher cat#: 14175095). Cells were stained with Fluo-8 AM (1:500) and probenecid (1:250) diluted in HBSS, 500 µL of staining solution was added to 1 million cells. Cells were incubated for 30 min at room temperature in the dark. After staining, cells were washed two times with HBSS and immediately analyzed in a flow cytometer.

For the inhibitory assays, cells were activated with Dynabeads for 24 h. After activation, cells were treated with the P2X7 inhibitor A438079 (150 nM) and incubated for an additional 24 h, when intracellular calcium levels were measured as described above.

2.8. Caspase 1 measurement

FLICA (fluorescence-labeled inhibitor of caspases, ImmunoChemistry Technologies Cat#:97) was used to measure the intracellular, active caspase-1. After two days of Dynabeads activation, the FAM-FLICA–caspase-1 probe was reconstituted in 50 µL DMSO and added directly to the cell culture medium at a ratio of 1:150, followed by incubation for 1 h at 37 °C. Cells were washed three times with the washing buffer supplied together with FLICA probe and then analyzed by flow cytometry on a BD Fortessa Cell Analyzer.

2.9. Immunohistochemical staining

Day 2 in vitro activated CD4 + CD45RO- T-cells were incubated with 3 µM BODIPY 493/503 (ThermoFisher Scientific, Cat: #D3922) for 1 h at 37 °C, washed with 1XPBS twice, and fixed with 4% paraformaldehyde (Affymetrix, Cat#: 199431LT) for 15 min at RT. Subsequently, BODIPY labeled cells were incubated with 2.5% normal horse serum blocking solution (Vector Laboratories, Cat#: S-2012) for 45 mins at RT. Subsequently, samples were stained with 10 µg/ml FAS monoclonal antibody, clone 3F2-1F3 (Abnova, Cat#: H00002194-M01) in 1% BSA overnight at 4 °C. Cells were washed twice in 1XPBS and incubated with 2 µg/ml anti-mouse Ig secondary antibody, labeled with Alexa Fluor 594 (Thermo Fisher Scientific, Cat#: A-11032) in 1% BSA for 1 h at RT in the dark. Cells were washed twice in 1XPBS and cytospun onto slides (Leica, Cat#: 75806–640) and mounted using ProLong Diamond Antifade Mountant with DAPI (ThermoFisher Scientific, Cat#: P36962). Sections were imaged with a LSM710 system (Carl Zeiss) using an EC Plan-Neofluar 40 × /1.30 Oil DICIII objective lens (Carl Zeiss).

3. Results

3.1. Increased activation-induced mitochondrial respiration in CD4 memory T cells with age

T cells undergo metabolic reprogramming upon activation that is closely tied to their ability to clonally expand in an immune response and acquire effector functions. To determine how age influences their ability to metabolically respond to stimulation, we first established the in vitro time course of mitochondrial respiration and glycolysis using Seahorse XF analyzer measurements at different time points post-activation. Mitotracker Green staining reflecting mitochondrial mass was unchanged on day 1, to then increase over the next two days (Fig. 1A). Parallel CFSE dilution experiments showed that cells did not divide within the first 48 h, while > 50% of the cells were cycling on day 3 (Fig. S1). Oxygen consumption rates (OCR) started to increase within the first 18 h, peaked at about 48 h with an about 10-fold increase and then dropped off again, but stayed elevated (Fig. 1B). Similar patterns were seen for CD4 naïve and memory T cells. Extracellular acidification rate (ECAR) paralleled the kinetics of OCR with a brisk increase over the first 48 h post-activation; in contrast to OCR, the increase in ECAR was sustained over the subsequent 48 h (Fig. 1C). Again, the patterns seen in naïve and central memory cells were similar.

Initial studies of unstimulated naïve and memory CD4 T cells did not show an age-associated difference. Subsequent experiments comparing the metabolic behavior between CD4 T cells from young and older individuals after activation were performed at the time of the peak response (48 h post-activation) to minimize the impact of confounding variables such as cell division. In all assays, samples from a young and an old individual were run in parallel to control for inter-assay variability. No aging-associated difference was seen for naïve CD4 T cells, and we therefore focused on memory CD4 T cells that in our previous studies have shown differences in the phosphorylation of the metabolic master regulator AMPK and the downstream AMPK-dependent expression of DUSP4 [16]. ECAR was not dependent on age (Fig. 1D), suggesting that glycolysis and therefore the production of metabolites necessary to support cell proliferation was not compromised with age. In contrast, mean basal OCRs of activated CD4 memory T cells from 14 individuals older than 65 years of age was significantly higher than those from 14 individuals younger than 35 years of age ($P = .007$, Fig. 1E and F). ATP synthase inhibition with oligomycin showed that this difference in mitochondrial respiration was entirely ATP coupled ($P = .008$, Fig. 1G); the extra-mitochondrial consumption was equally low and independent of age (Fig. 1E). Maximal OCR, determined after FCCP uncoupling, was only slightly higher in cells from older individuals and no longer significant. Also, SRC was not increased in T cells from older individuals (Fig. 1H).

Consistent with increased upregulation of mitochondrial activity in older CD4 memory T cells, production of mitochondrial reactive oxygen species (ROS) was higher in older T cells when compared to those from young individuals ($P = .01$, Fig. 2A). Intracellular ATP levels in old memory CD4 T cells were higher than in young cells, attesting that the mitochondria in older cells are functional and have increased activity ($P = .02$, Fig. 2B). ATP is not only an energy source for cellular functions, but it is also secreted by the cells and then serves as a mediator of purinergic signaling; in particular ATP stimulates P2X calcium channels [17,18]. Consistent with increased ATP production, supernatants of activated CD4 memory T cells from older individuals had higher ATP levels than those from young adults (P = .02, Fig. 2C). Cytoplasmic Ca^{2+} concentrations in activated CD4 T cells are sensitive to inhibition of the P2X7 receptor, indicating the contribution of purinergic signaling (Fig. 2D). We compared intracellular Ca^{2+} concentrations and found them to be increased in cells from older individuals ($P = .039$, Fig. 2E). Moreover, inhibition of ATP secretion and the P2X7 receptor, respectively reduced the frequencies of cells with increased active caspase 1, suggesting that this pathway contributes to the increased caspase 1 activation and cell death in activated T cells from older individuals (Fig. S2). Taken together, CD4 memory T cells from older individuals exhibit increased upregulation of mitochondrial respiratory activity resulting in increased ROS and ATP production and increased cytoplasmic Ca^{2+}.

3.2. Metabolic pathways driving respiratory activity in activated CD4 memory T cells with age

Mitochondrial mass similarly increased after activation for both young and old memory CD4 T cells, likely contributing to the observed activation-induced increase in OCRs. No aging-associated difference in Mitotracker Green staining was seen (Fig. 3A). The results by flow cytometry were confirmed by Western blot for cytochrome *c* (Fig. 3B). Since changes in mitochondrial mass did not account for the aging-associated differences observed in mitochondrial respiration, we set out to investigate whether differences in pyruvate metabolism, glutaminolysis or fatty acid oxidation were responsible for this finding. Seahorse assays were performed with excess in exogenous pyruvate and the activated CD4 T cells were treated with UK5099 to inhibit transport of pyruvate into the mitochondria. The inhibitory effect on OCR was

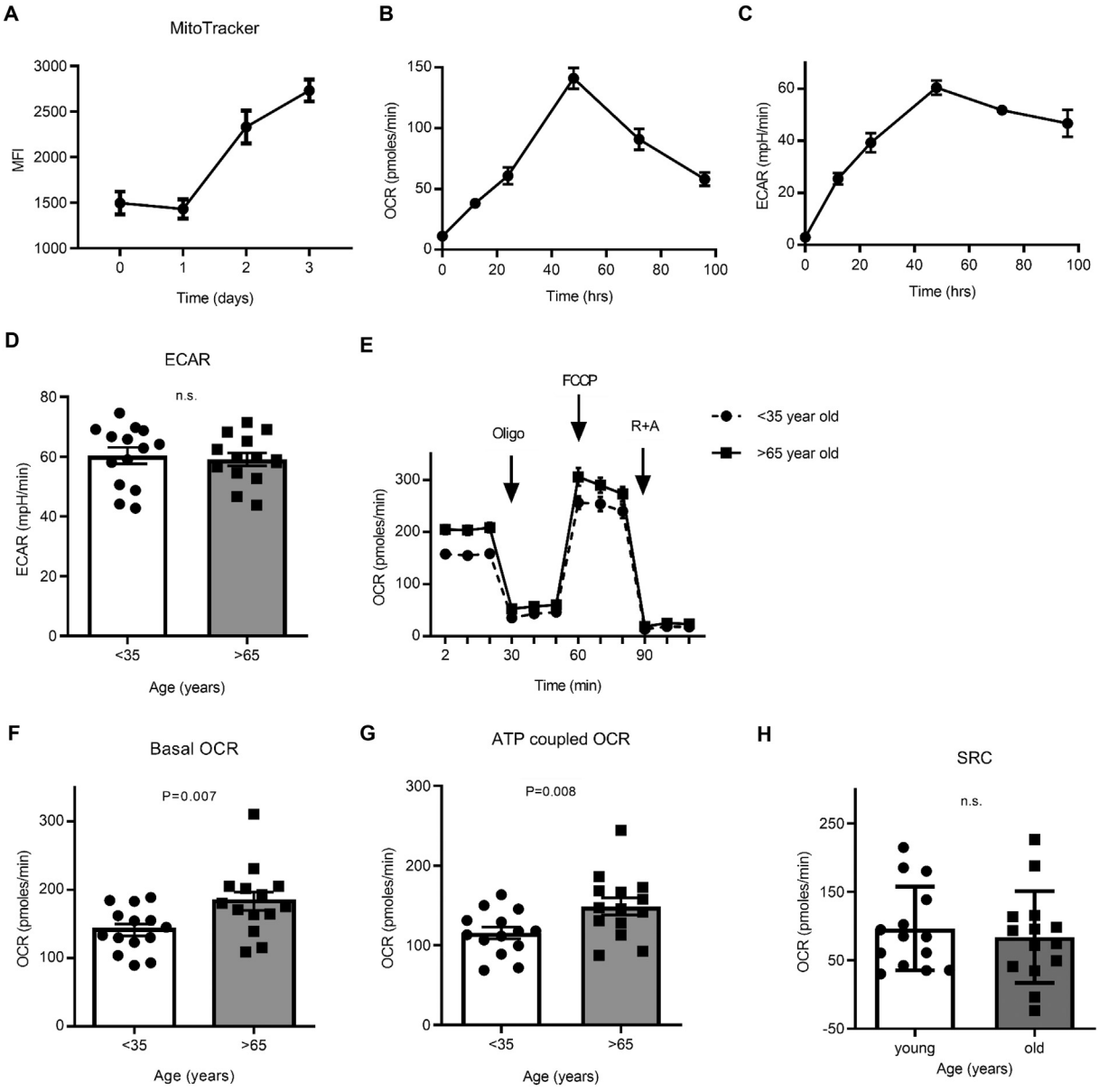

Fig. 1. Increased activation-induced mitochondrial respiration in CD4 memory T cells with age. **(A)** MitoTracker staining at indicated time points after Dynabead activation of memory CD4 T cells are shown. Results from day 0 to day 2 are shown as mean ± SEM from 10 to 11 adults; day 3 is mean ± SEM from 5 adults. **(B and C)** Memory CD4 T cells from healthy adults were activated with anti-CD3/CD28 Dynabeads. Basal O_2 consumption rates (OCR, B) and extracellular acidification rates (ECAR, C) were determined at indicated time points using Seahorse Analyzer. Kinetic data at 0 h (n = 3), 12 h (n = 5), 24 h (n = 8), 48 h (n = 14); 72 h (n = 11); 96 h (n = 4) after stimulation are shown as mean ± SEM. **(D)** Mean ± SEM ECAR from day 2 activated memory CD4 T cells from young and old individuals. Statistical analysis by unpaired two-tailed t-test. **E)** Real-time OCR from CD4 memory T cells two days after activation are shown under basal conditions and in response to indicated mitochondrial inhibitors. Mean ± SEM of triplicate measurement of cells from one young and one old individual run in parallel. **(F-H)** Mean ± SEM basal OCR (F), ATP-coupled OCR (G) and SRC (H) of samples in (D).

minimal both for activated young and older T cells (Fig. 3C), suggesting that pyruvate is not an important substrate for the mitochondrial respiration at this time point after activation. Furthermore, we measured pyruvate dehydrogenase kinase 1 (PDK1), an important regulator of pyruvate metabolism, and the mitochondrial pyruvate carrier 1 (MPC1) and there was no difference in expression for either of these proteins between young and old memory CD4 T cells (Fig. S3A and B). Together with the ECAR data, these data suggest that a difference in glycolysis or pyruvate transport did not account for the increased respiratory activity in activated CD4 memory T cells with age.

Glutaminolysis is the step-wise conversion of glutamine to glutamate and then to α-ketoglutarate, which in turn enters the tricarboxylic acid (TCA) cycle and can fuel mitochondrial respiration. In order to determine if glutaminolysis accounts for the increased mitochondrial respiration observed for CD4 memory T cells from older individuals, BPTES, an inhibitor of glutaminase, was added during the Seahorse assay. Again, the effect of glutaminase inhibition on mitochondrial respiration was minimal and independent of age (Fig. 3D). Furthermore, transcript levels of glutaminase and of the glutamine transporters SNAT1 and SNAT2 in activated CD4 memory T cells did not change with age (Fig. S2C), suggesting that glutaminolysis did not contribute significantly to the increased mitochondrial activity observed in old activated memory CD4 T cells.

Fatty acids are an important substrate source for the mitochondria as they are broken down into acetyl-CoA and fuel the TCA cycle. Fatty acid oxidation (FAO) is the major pathway of ATP synthesis in memory

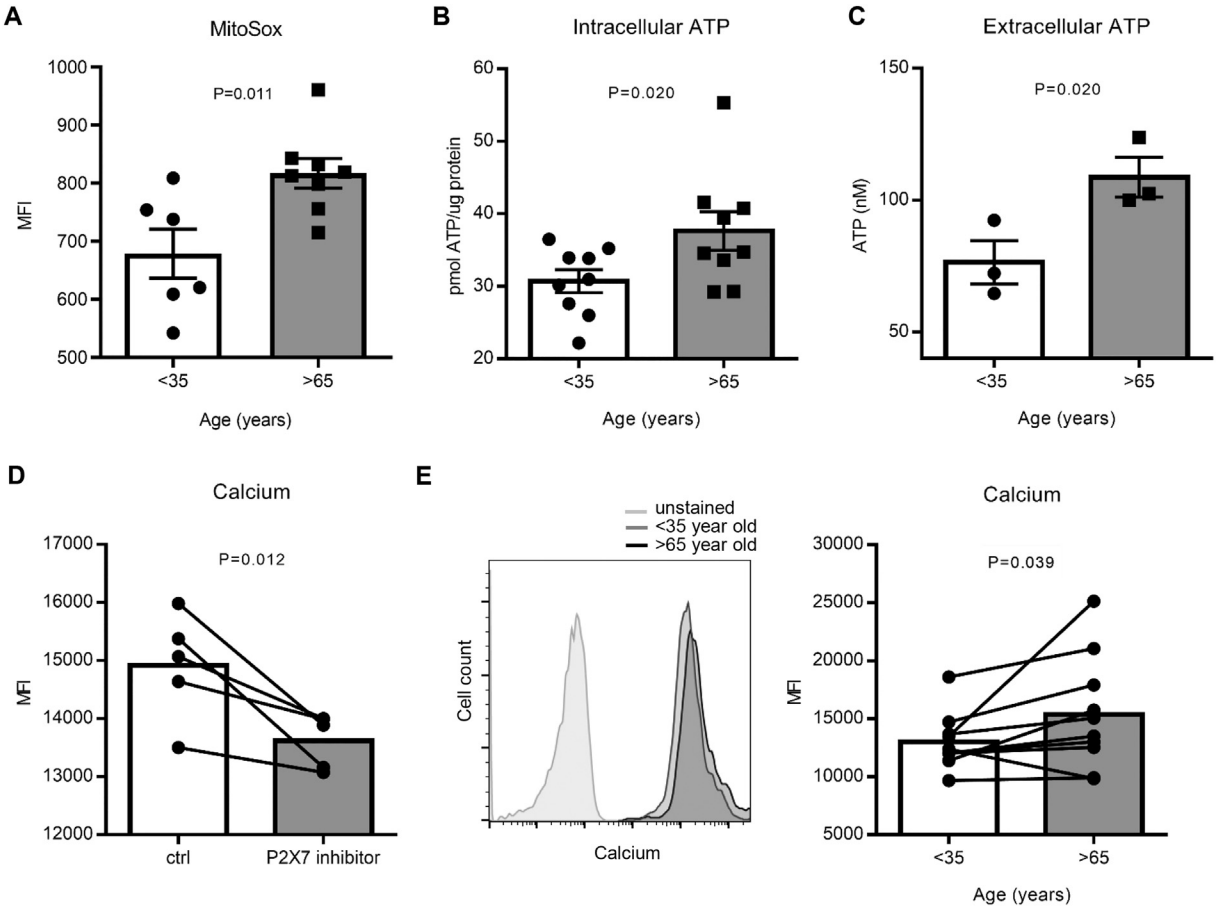

Fig. 2. Increased activation-induced mitochondrial ROS and ATP production by CD4 memory T cells with age. **(A)** MFI of MitoSox indicative of mitochondrial ROS in CD4 T cells from 6 young and 8 old adults two days after activation. Statistical analysis by unpaired two-tailed *t*-test. **(B)** Intracellular ATP in day 2-activated memory CD4 T cells ($n = 9$ pairs). **(C)** Extracellular levels of ATP from supernatants of activated young and old memory CD4 T cells ($n = 3$ pairs). Comparisons were done by unpaired one-tailed *t*-test. **(D)** Cultures with CD4 memory T cells were performed in the presence of vehicle or the P2X7 receptor inhibitors. Intracellular calcium concentrations were determined by Fluo8 staining and cytometric analysis of memory CD4 T cells two days after activation. **(E)** Calcium staining is performed as described in (D) and results from 10 young and 10 older adults, always run in pairs are shown (right). One representative flow cytometry staining graph of one young and one old is shown on left.

cells, but switches to fatty acid synthesis after activation to provide lipid substrate needed for cell proliferation [12]. Young and old activated memory CD4 T cells were treated with etomoxir to inhibit carnitine palmitoyltransferase 1A (CPT1a) while recording OCR. T cells from both age groups significantly decreased OCR reaching similar levels suggesting that the aging-associated increase in mitochondrial respiration was dependent on FAO (Fig. 3E). In summary, the activation-induced upregulation of OCR is mainly dependent on FAO with minor contribution of pyruvate metabolism and glutaminolysis. FAO coupled OCR is significantly higher in memory CD4 T cells from older individuals than those from young ones (Fig. 3F).

To determine whether increased FAO is associated with depletion of lipid stores, we activated memory CD4 T cells from young and old individuals by TCR crosslinking for 48 h and stained cytoplasmic neutral lipids with Bodipy. Bodipy staining was significantly higher in cells from young than those from old individuals ($P = .002$, Fig. 3G).

3.3. Catabolic lipid metabolism in old memory CD4 T cells after activation

To further characterize the influence of age on lipid metabolism in activated CD4 memory T cells, we quantified the expression of fatty acid synthase (FAS) and of CPT1. FAS synthesizes acetyl-CoA into long-chain fatty acids in the presence of NADPH provided by the pentose phosphate pathways. CPT1a plays an important role in the transport of fatty acids into the mitochondria and thus is a major regulator of FAO. Expression of the FAS enzyme complex was significantly reduced with age, as shown by immunohistochemistry ($p < .0001$, Fig. 4A). Correspondingly, we found increased expression levels of CPT1a in old activated memory CD4 T cells ($P = .026$, Fig. 4B). Knockdown of CPT1a resulted in a significant decrease in mitochondrial OCR in cells that had been activated for 2 days ($P = .003$, Fig. 4C). Furthermore, knocking-down CPT1a expression decreased the levels of secreted ATP ($P = .013$, Fig. 4D), indicating that a large amount of secreted ATP was generated by FAO. These data not only confirm that CD4 T cells upregulate FAO in the first two days after activation, but also that increased expression of CPT1a is an important contributor to the increased mitochondrial respiration observed with age.

3.4. Regulation of increased CPT1a transcription with age

We set out to investigate which molecule upstream of CPT1a could account for the increased expression observed in memory CD4 T cells. AMPK is a metabolic regulator known to be upstream of CPT1a. We found a small but significant increase in phosphorylated AMPK in old memory CD4 T cells when normalized to β-actin ($P = .028$, Fig. 5A). However, knockdown of AMPKα expression in memory CD4 T cells did not have any effect on CPT1a levels (Fig. 5B). SIRT1 is known to regulate CPT1a expression by deacetylating PGC1α. Expression levels in older individuals appeared to be bimodal, with SIRT1 expression trending to be increased in memory CD4 T cells of the larger subset of

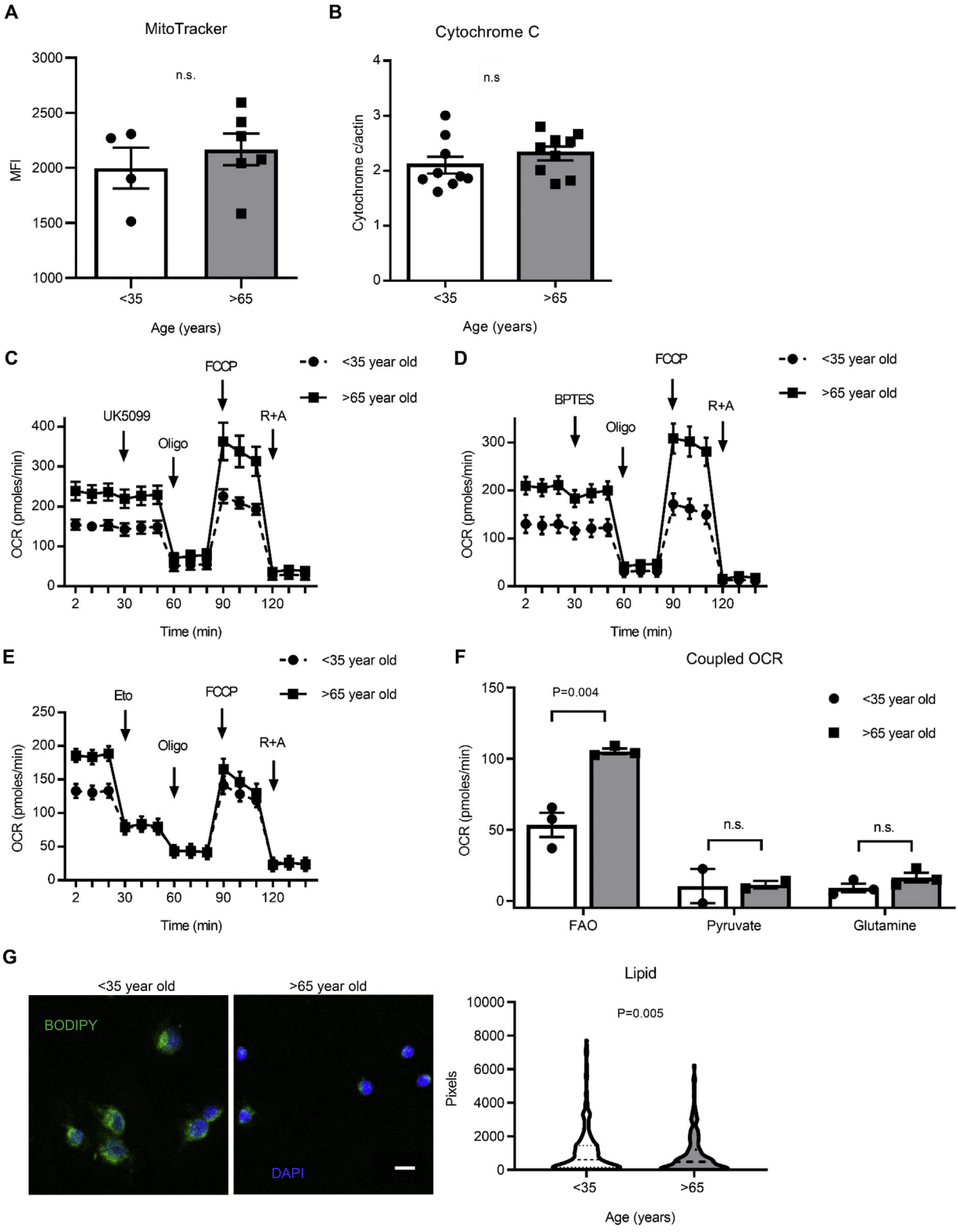

Fig. 3. Metabolic pathways driving respiratory activity in activated CD4 memory T cells with age. **(A)** MitoTracker staining of two-day activated CD4 memory T cells from 4 young and 6 older adults. **(B)** Western blot of cytochrome C expression by memory CD4 T cells from 9 young and 9 older adults on day 2 after activation. Comparisons were done by two-tailed unpaired *t*-test. **(C, D and E)** Real-time OCR from CD4 memory activated for 2 days. UK5099 to inhibit pyruvate transport into the mitochondria was added as indicated (C). Glutaminolysis was inhibited by adding BPTES (D). Fatty acid oxidation was inhibited by adding Etomoxir (E). Results are shown as mean ± SEM of triplicate cultures. **(F)** Summary for FAO-, pyruvate-, and glutamine-coupled mitochondrial OCR as shown in (C, D and E) from 3, 2 and 3 pairs of young and old individuals, respectively. Comparison by unpaired two-tailed *t*-test. **(G)** Representative images of BODIPY staining of activated CD4 memory T cells from one young and one old individual (left) and summary from three experiments are shown (right). Scale bars, 10 μm.

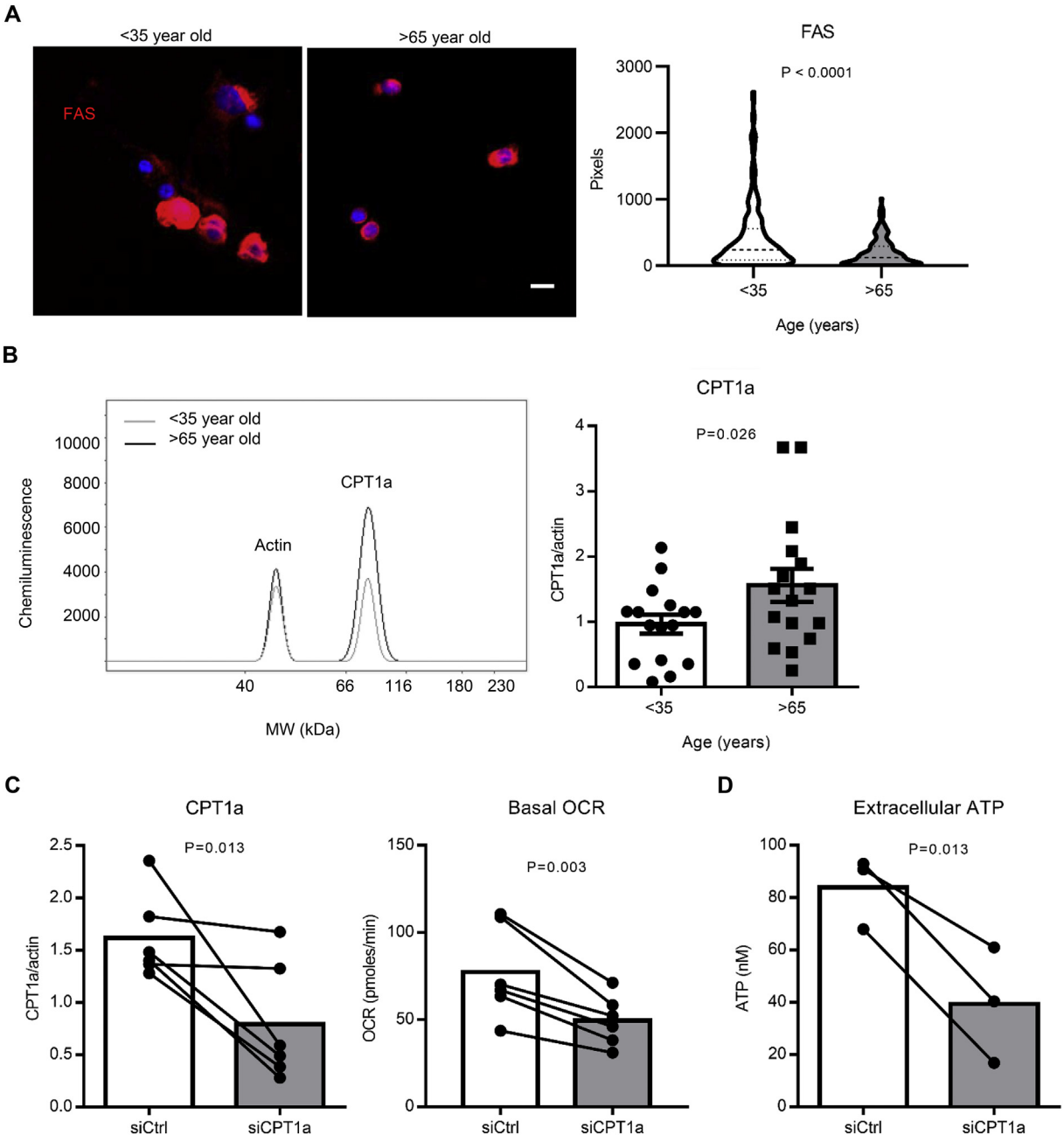

Fig. 4. Decreased FAS and increased CPT1a expression in activated CD4 memory T cells with age. **(A)** Representative images of FAS staining in activated CD4 memory T cells from one young and one old individual (left) and summary data from three experiments are shown (right). Scale bars, 10 μm. **(B)** CPT1a expression levels were determined by Peggy Sue western. A representative blot of signal intensities from activated CD4 memory T cells from a young and an old adult (left) and the ratio of CPT1a to actin from 16 pairs (right) are shown. Comparisons by unpaired one-tailed t-test. **(C)** Memory CD4 T cells were transfected with siRNA for CPT1a and activated for two days. Knockdown efficiency by Peggy Sue western (left) and basal OCR in five control and siCPT1a-silenced cells (right) are shown. Comparisons were done by paired one-tailed t-test. **(D)** Extracellular ATP concentrations in supernatants from activated CD4 memory T cells transfected with control or CPT1a-siRNA (n = 3). Comparison by one-tailed paired t-test.

older individuals (Fig. 5C). In contrast to AMPKα, knockdown of SIRT1 decreased CPT1a expression both at the transcript as well as the protein level (Fig. 5D). These results suggest that the age-related increase in SIRT1 expression contribute to increased CPT1a levels and ultimately increases FAO mitochondrial respiration in aged CD4 memory T cells after activation.

4. Discussion

Function of T memory cell is critically determined by their metabolic state and their ability to dynamically regulate metabolic pathways in response to activation signals [19]. Here we show that CD4 memory T cells from older individuals maintain a more catabolic state in the first 48 h after TCR stimulation as indicated by an increased OCR to ECAR ratio due to increased OXPHOS. T cell activation induces mitochondrial biogenesis. Moreover, activation of the mTORC pathway, generally increased with aging, reduces DRP1-dependent mitophagy [20,21]. However, we did not find a difference in mitochondrial mass that could account for the increased OCR in older activated memory CD4 T cells. Instead, we found that the increased OCR was caused by higher CPT1a expression suggesting increased FAO and NADH production in the TCA driving OXPHOS. In parallel, FAS expression declined with age.

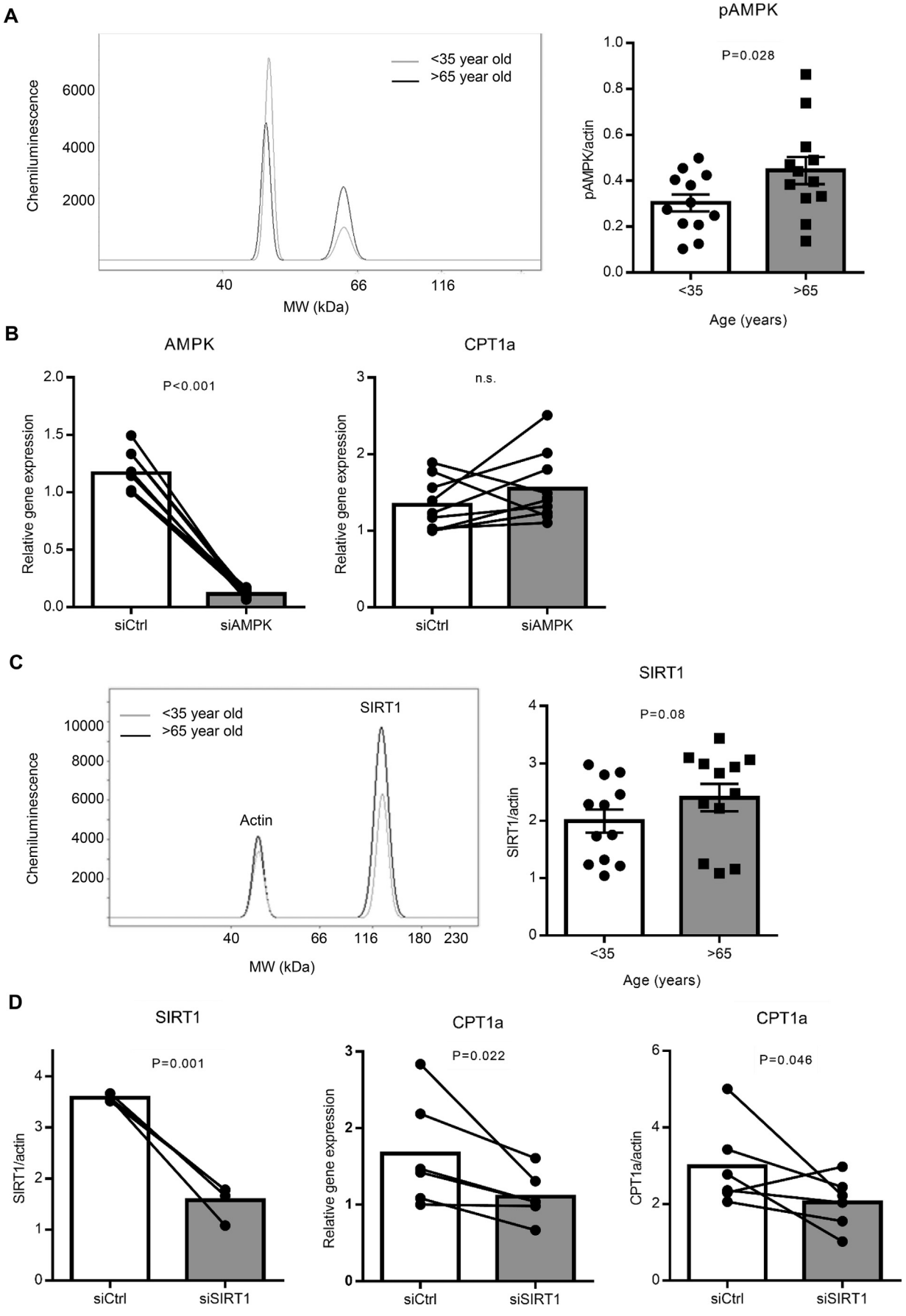

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Fig. 5. Increased SIRT1 expression in old memory CD4 T cells leads to increased CPT1a expression. **(A)** AMPK phosphorylation was compared by Peggy Sue western in day 2 activated CD4 memory T cells from 12 young and 12 older adults. Representative blot of signal intensities (left) and summary data (right). Comparison by unpaired one-tailed t-test. **(B)** Activated CD4 T memory cells were transfected with siRNA for AMPKα on day 1. Knockdown efficiency by qPCR (left) and CPT1a expression by Peggy Sue western (right) are shown on day 2 after activation ($n = 9$). **(C)** SIRT1 expression in day 2 activated CD4 memory T cells were determined by Peggy Sue western. A representative blot of signal intensities from a young and an old adult (left) and the ratio of SIRT1 to actin from the samples shown in (A) are shown. **(D)** Activated CD4 T memory cells were transfected with siRNA for SIRT1 on day 1. Knockdown efficiency by Peggy Sue western (left) and CPT1a expression by qPCR (middle) and Peggy Sue western (right) are shown on day 2 after activation ($n = 6$). Comparisons were done by paired one-tailed t-test.

Consistent with this metabolic state, cytoplasmic lipid stores were depleted. These results in healthy old humans are in contrast to previously published findings in mice where aged T cells had reduced mitochondrial biogenesis, reduced expression of enzymes of one-carbon metabolisms and lower respiratory capacity [22]. Instead, aged human CD4 memory T cells maintained a catabolic state with increased mitochondrial energy production, as is characteristic for non-proliferating T cells, for a longer time after activation than young T cells.

Increased OCR was an actively regulated process and not a response to energy deprivation. ATP levels in older T cells were increased and not decreased. AMPK is a key metabolic regulator of T cell metabolism that, in addition to energy deprivation, is activated by T cell activation and stimulate catabolic pathways [23,24]. We have previously shown increased AMPK activity in CD4 effector T cells that express CD39 and DUSP4 and that are increased in frequencies in T cell responses of older individuals [5,16]. Lanna and colleagues have described AMPK activation due to an increased expression of sestrin in $CD4^+CD28^-CD27^-$ terminally differentiated effector T cells [25,26]. In this T cell subset, considered to be senescent cells, AMPK activation induced the autophosphorylation of MAPKs and in particular the activation of the p38 pathway independent of TCR activation. In our studies of activated total CD4 memory T cells from older individuals, we confirmed elevated levels of phosphorylated AMPK, but the difference was small. Moreover, the catabolic state was not broad-based but very selective; only FAO-coupled OCR was increased but not pyruvate- or glutamine-coupled OCR. Aging-associated differences in OCR were no longer appreciated after pharmacological inhibition of CPT1a or after CPT1a silencing, clearly implicating fatty acid transport and FAO in this aging-associated difference. AMPKα silencing did not reduce CPT1a expression suggesting that AMPK activation was not solely responsible for the increased OCR.

In addition to AMPK, SIRT1 is an important regulator of fatty acid metabolism. SIRT1 is generally considered a longevity gene, at least in part accounting for increased lifespan with calorie restriction [27,28]. In aged naïve and memory T cells SIRT1 expression is increased, in part due to an age-related decline in miR181a that targets SIRT1 [29,30]. A notable exception are terminally differentiated effector CD45RA T cells that have decreased expression of SIRT1 [31]. SIRT1 has been shown to promote lipolysis. In animal models, SIRT1 activators as well as hepatic overexpression of SIRT1 has been shown to prevent hepatic steatosis [31]. SIRT1 and AMPK have a reciprocal relationship with SIRT1-mediated deacetylation of LKB1 activating AMPK and AMPK phosphorylating SIRT1 [32,33]. More important for the transcriptional regulation of CPT1a may be the ability of SIRT1 to deacetylate PGC-1α, thereby facilitating AMPK-mediated PGC-1α phosphorylation [34]. Phosphorylated PGC-1α interacts with PPARα to induce the transcription of CPT1a. In our studies, silencing of SIRT1 expression by about 50% reduced CPT1a transcription as well as protein expression. The emerging model is that increased expression of SIRT1 in memory T cells with age leads to increased CPT1a transcription and maintains a more lipid-catabolic state in the first 48 h after TCR stimulation due to increased FAO and OXPHOS and decreased fatty acid synthesis.

CPT1a expression has been found to be a characteristic hallmark of long-lived memory CD8 T cells induced by IL15 culture when compared to short-lived effector CD8 T cells cultured with IL2 [12]. Together with increased mitochondrial biogenesis and mitochondrial fusion [14], the higher CPT expression accounted for increased spare respiratory capacity that equips memory T cells with extra capacity to produce energy on demand and improved resistance to stress. In vivo, antigen-specific CPT1a overexpressing antigen-specific T cells showed improved survival as memory T cells after infection [12]. These data raise the possibility that the increased expression of CPT1a with age in human CD4 memory T cells is an adaptive mechanism that is beneficial for the host. However, in contrast to the murine study, increased expression of CPT1a in activated human memory T cells from older individuals was not associated with increased spare respiratory capacity, implicated in the mouse studies as driving cell resistance to stress.

While studies on the metabolic programming induced by T cell activation emphasize the switch from OXPHOS to aerobic glycolysis and anabolic metabolic metabolism in support of blast transformation and cell division, mitochondrial activity is clearly important [35]. In our in vitro system of T cell activation, it takes up to 72 h for the OCR to ECAR ratio to change; in the first 48 h, ECAR and OCR increase in parallel. Complex III-derived ROS, which are generated as byproducts of mitochondrial respiration, are essential for T cell activation through several mechanisms [35]. Mitochondria move to the subsynaptic region of the TCR synapse and promote proximal TCR signaling, in part by inactivating phosphatases. Optimal activation of nuclear factor of activated T cells (NFAT) requires ROS. At later stages of TCR activation, ROS dampens effector T cell differentiation while promoting memory cell survival. In response to elevated ROS, ATM activates the LKB1/AMPK metabolic pathway to phosphorylate the TSC2 tumor suppressor, repressing mTORC1 and inducing autophagy [36]. Inhibition of mTORC1 activation in T cell responses is known to favor generation of memory cells [36]. However, in spite of the aging-associated increase in ROS production and in AMPK phosphorylation, we have seen increased mTORC1 activation in CD4 memory T cell responses in older individuals that favored the generation of short-lived effector over long-lived memory T cells [5,37]. In conclusion, while increased ROS production in older memory CD4 T cells may be beneficial for memory cell development, it is not sufficient to correct the preferential commitment to effector cell differentiation in older individuals.

In addition to ROS activity, mitochondrial activity regulates T cell activation through secretion of ATP and purinergic signaling [36]. The increased OXPHOS in older memory T cells was entirely ATP-coupled and activated older T cells had higher cytoplasmic ATP concentration as well as secreted more ATP. T cells secrete ATP into the immune synapse where it activates ATP-gated P2X Ca^{2+} channels [38-40]. Indeed, we find increased cytoplasmic Ca^{2+} concentrations in activated T cells from older individuals. Activation of P2X channels by ATP can have beneficial as well as harmful effects depending on context and signal strength [41-44]. Age-dependent increased caspase 1 activity in activated CD4 memory T cells was at least in part dependent on extracellular ATP and could be reduced by inhibiting ATP secretion or blocking the P2X7 receptor.

In summary, CD4 memory T cells from older individuals are competent to upregulate oxidative phosphorylation as well as glycolytic activity upon activation. Considering that both naïve and memory T cells are characterized by FAO, but memory T cells are superior in oxidative phosphorylation due to increased CPT1a expression leading to increased fatty acid transport into the TCA cycle, the pattern observed with age may indicate an adaptive mechanism, consistent with the model that T cell aging and differentiation in part share the same pathways [2,45]. However, in contrast to naïve to memory

differentiation, the increased CPT1a expression with age is not linked to an increased mitochondrial mass and increased SRC. Together with reduced fatty acid synthesis, the increase in FAO induces a lipid-catabolic state in old CD4 memory T cells while they enter proliferation, which may harm their ability to survive.

4.1. Study approval

The study was approved by the Stanford Institutional Review Board and all participants gave written informed consent.

4.2. Statistics

Statistical analysis was performed using GraphPad Prism 8.0 employing paired and unpaired one- or two-tailed *t*-test; a p value of < 0.05 was considered significant. All data are presented as mean ± SEM.

Author contributions

REY, HZ, CMW and JJG designed research and analyzed data. REY, HZ and YS performed the experimental work. REY, HZ, CMW and JJG wrote the manuscript.

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Declaration of Competing Interests

The authors have declared that no conflict of interest exists.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2019.07.003.

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