Impaired nutrient uptake by CD8+ EMRA T cells reinforces the senescent state observed during type 2 diabetes.

Lauren A Callender[1,2], Elizabeth C Carroll[1,3], Conor Garrod-Ketchley[1], Johannes Schroth[1], Jonas Bystrom[1], Victoria Berryman[4], Melanie Pattrick[4], Desiree Campbell-Richards[4], Gillian A Hood[5], Graham A Hitman[6], Sarah Finer[4,5], Sian M Henson[1*]

[1]William Harvey Research Institute, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, Charterhouse Square, London, UK

[2]Current address: Translational Science, Achilles Therapeutics Ltd, Stevenage Bioscience Catalyst, Stevenage, UK

[3]Current address: Department of Life Sciences, Institute of Technology Sligo, Sligo, F91 YW50, Ireland

[4]Barts Health NHS Trust, London, UK

[5]Institute of Population Health Sciences, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, London, UK

[6]Blizard Institute, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, London, UK

*Corresponding author:

Dr. Sian M. Henson. s.henson@qmul.ac.uk

Abstract

Mitochondrial health and cellular metabolism can heavily influence the onset of senescence in T cells. CD8$^+$ EMRA T cells exhibit mitochondrial dysfunction and alterations to oxidative phosphorylation, however, the metabolic properties of senescent CD8$^+$ T cells from people living with type 2 diabetes (T2D) are not known. We show here that mitochondria from T2D CD8$^+$ T cells had a higher oxidative capacity together with increased levels of mtROS, compared to age-matched control cells. While fatty acid uptake was increased, fatty acid oxidation was impaired in T2D CD8$^+$ EMRA T cells, which also showed an accumulation of lipid droplets and decreased AMPK activity. Increasing glucose and fatty acids in healthy CD8$^+$ T cells resulted in a fragmented mitochondrial morphology, similar to CD8$^+$ EMRA T cells in T2D. The resulting mitochondrial changes are likely to have a profound effect on T cell function. Consequently, a better understanding of these metabolic abnormalities is crucial as metabolic manipulation of these cells may restore correct T cell function and help reduce the impact of T cell dysfunction in T2D.

Introduction

Senescent T cells have been implicated in a number of different chronic inflammatory diseases, such as cancer [1], obesity [2] and rheumatoid arthritis [3]. Type 2 Diabetes (T2D), which is characterised by progressive metabolic imbalances and chronic inflammation, is also more prevalent with increasing age [4]. Senescence has previously been implicated in the pathogenesis of T2D. With senescent cell burden being shown to increase in tissues that undergo diabetes induced damage, such as the kidneys [5] and pancreas [6]. Interestingly, $CD4^+CD28^-$ T cells, which are a late stage differentiated population, have been shown to accumulate in T2D [7]. A similar finding was observed in obese mice, where senescent $CD4^+$ T cells were found to accumulate in the visceral adipose tissue (VAT) and caused the induction of chronic VAT inflammation [2]. More recently, $CD8^+CD28^-CD57^+$ T cells have also been shown to increase the risk of hyperglycaemia in humans, highlighting a potential role for this subset in the pathogenesis of T2D [8]. Collectively, these studies show an association between T2D and terminally differentiated T cells.

T cells rely on glycolysis to initiate effector function and are extremely sensitive to changes in glucose concentration, hyperglyceamia alone is able to impair T cell activation and migration [9; 10]. Despite this reliance on glycolysis, mitochondrial function is also important for T cells playing a crucial role in the oxidative stress response and the provision of key metabolic intermediaries [11]. Alterations in mitochondrial function accompanies numerous chronic inflammatory diseases, and increasing evidence shows that mitochondrial dysfunction contributes to immunosenescence and inflammation [12]. Both ageing and T2D share important features that include oxidative stress and low-grade inflammation. In ageing, as in the early stages of T2D there is a persistent accumulation of oxidative damage, caused by increased ROS production in all cells [13]. Mitochondria are the main producers and also the main targets of ROS as it damages mtDNA, leading to a vicious cycle

of increased ROS production that accelerates both ageing and T2D [14]. These findings support the notion that impaired T cell metabolism plays a pathogenic role in the development of human T2D.

We explore this idea using functional analysis of mitochondrial metabolism coupled with the assessment of hyperglycaemia and inflammation on mitochondrial fitness. We find that T2D causes significant changes in the metabolism of senescent CD8+ T cells: increased lipid storage and mtROS production, together with a reduction in fatty acid oxidation and AMPK activity. Increasing glucose and fatty acid levels in healthy CD8+ T cells resulted in a fragmented mitochondrial morphology, similar to CD8+ EMRA T cells in T2D. Furthermore we show that increasing the inflammatory milieu resulted in an environment that drives an increased rate of CD8+ T cell senescence.

Materials and Methods

Ethics and donor recruitment

Our clinical protocol was approved by the NRES Committee North East (16/NE/0073) and all subjects provided written informed consent. We recruited healthy volunteers (Age range: 20 – 75 years, n = 48) and people aged >18 years living with T2D (Age range: 50 – 77 years, n = 52), identified through the Diabetes Alliance for Research in England (DARE) database, with preferential sampling from people aged 50 – 60 years. Exclusion criteria for all participants were: inability to provide written informed consent, infection or immunisation in the month prior to blood collection, any known immunodeficiencies or a history of chemotherapy/radiotherapy, on any immunosuppressive medications within the last 6 months, significant comorbidity or had a history of neoplasm in the last 10 years. Peripheral blood was obtained using heparinised tubes, and peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll hypaque (Amersham Biosciences). In addition to a blood sample, the participants age, gender, length of time since T2D diagnosis and medications were documented (Table 1).

Flow cytometric analysis and cell sorting

Flow cytometric analysis was performed using the following antibodies: anti-KLRG1 PE (MAFA) from Miltenyi Biotec, anti-CD8 PerCP (SK1), anti-CD45RA BV605 (HI100), anti-CD45RA APC (HI1000), anti-CD27 BV421 (O323), anti-CD27 FITC (O323), anti-CD28 BV785 (CD28.2), and anti-CCR7 PECy7 (G043H7) from BioLegend. anti-Ki67 PE (B56; BD Bioscience) and anti-pAMPK (T172; 40H9) were used for intracellular staining using solution AB (ThermoFisher). All samples were analysed using a LSR Fortessa (BD Biosciences) and the resulting data examined using FlowJo software (BD Bioscience). UMAP analysis was performed using FlowJo on down sampled CD8 T cell populations (5000 events), with standard parameters (nearest neighbours = 15, minimum distance = 0.5) and clustered via flowSOM.

Magnetic beads were used to isolate CD8$^+$ T cells by positive selection according to the manufacturer's instructions (Miltenyi Biotec). The purity of T cell subsets was assessed by flow cytometry.

Mitochondrial measurements

Mitochondrial mass was assessed by incubating antibody labelled PBMCs with 100 nM of MitoTracker Green FM (ThermoFisher) for 30 minutes at 37°C, 5% CO_2. Mitochondrial ROS was measured in labelled PBMCs using 2μM MitoSOX (ThermoFisher) incubated for 20 minutes at 37°C, 5% CO_2. Unfixed samples were immediately analysed using a LSR Fortessa (BD Bioscience).

Transmission electron microscopy

CD27/CD45RA defined CD8$^+$ EMRA T cell subsets were isolated and fixed using 2% paraformaldehyde, 1.5% glutaraldehyde in 0.1 m phosphate buffer at pH 7.3. The EMRA T cells were processed as previously described [12] and were examined using a Jeol 1010 transmission electron microscope (Jeol) with image capture using a Gatan Orius CCD camera (Gatan).

Metabolic assays

Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were measured in bead sorted CD8$^+$ T cells following 15 minute stimulation with 1 μg/ml anti-CD3 and 5 ng/ml IL-2. The assay was performed using RPMI without phenol red and carbonate buffer (Sigma) supplemented with 25 mM glucose, 2 nM L-glutamine and 1 mM pyruvate. The metabolic stress test was performed using 1 μM oligomycin, 1.5 μM fluorocarbonyl cyanide phenylhydrazone (FCCP), 100 nM rotenone and 1 μM antimycin A (Sigma) using a XF-96 Extracellular Flux Analyzer (Agilent).

Fatty acid oxidation (FAO) was performed in substrate limited media: DMEM (Gibco), 0.5 mM glucose, 1 mM Lglutamine, 0.5 mM carnitine, and 1% FCS). 45 minutes prior to the assay cells were transferred to warm Krebs-Henseleit KH Buffer (KHB) (111 mM NaCL, 4.7mM KCL, 1.25 mM CaCl2, 2 mM MgSO4, 1.2 mM NaH2PO4) supplemented with 2.5 mM glucose, 0.5 mM carnitine, 5 mM HEPES, pH 7.4. Subsequently, 15 minutes prior to the assay, 40 µM Etoximir was added to selected groups. Finally, the cells were provided with Palmitate:BSA (Aglient) as a substrate and subjected to a Mitochondrial Stress Test as described above.

Nutrient uptake experiments

Lipid uptake was measured in PBMCs that had been stimulated for 1 hour at 37°C with 1 µg/µL of anti-CD3 in complete RPMI-1640 medium. 1 nM of the fluorescent palmitate analogue BODIPY FL C16 (Invitrogen) was then added and the cells incubated in the dark for 10 minutes. Cells were subjected to surface staining prior to flow cytometric analysis.

Sera culture experiments

PBMCs isolated from a young donor were cultured in either low (5 mM) or high glucose (25 mM) RPMI-1640 medium with or without 10% sera from either T2D patients or healthy age matched control participants. Antibody staining was carried out on samples at day 0, 3 and 7 and the cells analysed by flow cytometry in order to phenotype the samples.

Cytokine array

The Proteome Profiler Human Cytokine Array kit (R&D Systems; ARY0058) was used to quantitate the cytokines present in sera taken from people living with T2D or age matched individuals according to the manufacturer's instructions.

Confocal microscopy

To assess mitochondrial morphology, CD8$^+$ T cells were incubated in either high (25 mM), low glucose (5 mM) or with 200 μM palmitate overnight prior to plating onto 12 well microscope slides at a density of 0.05 x 10^6 per well. Cells were fixed in 2% PFA and 100 nM of MitoTracker Green FM (ThermoFisher) added for a duration of 30 minutes at 37°C, 5% CO_2. 4',6-diamidino-2-phenylindole (DAPI) (ThermoFisher Scientific) was then added for 10 minutes at room temperature in the dark. Samples were imaged on a Zeiss LSM 880 confocal microscope with a X63 oil immersion objective lens. Excitation was at 488 nm from an argon-ion laser. Fluorescence detection was in the green, 488 nm and UV, 405 nm channel.

Cellular Senescence RT2 Profiler PCR Arrays

Unstimulated CD8$^+$ EMRA T cells from T2D and healthy age-matched control participants were isolated using MACS sorting for CD8$^+$ T cells and then FACS sorted using anti-CD27/anti-CD45RA for EMRA isolation. RNA was extracted using the RNAeasy Micro Kit (Qiagen) and preamplified using the RT2 PreAMP cDNA Synthesis Kit (Qiagen) and RT2 PreAMP Pathway Primer Mix (Qiagen). Resulting gene-specific cDNA was then analysed using the Cellular Senescence RT2 Profiler PCR Arrays according to the manufactures instructions (Qiagen).

Statistical analysis

GraphPad Prism was used to perform statistical analysis. Statistical significance was evaluated using the paired Student *t*-test or a two-way ANOVA with Bonferroni correction used for post-hoc

testing. Data was expressed as mean ± SEM and P values are represented using the following

notation: p* = <0.05, p** = <0.01, p*** = <0.005 and p**** = < 0.001.

Results

CD8+ T cells display a premature aged phenotype in T2D

The cell surface markers CD45RA, CD28, CD27, CCR7 and KLRG1 were used to quantify the frequency of CD8+ T cell subsets in people living with T2D compared with healthy age-matched controls and young participants using UMAP (Uniform manifold Approximation and Projection) analysis (Figure 1A). UMAP is an unbiased manifold learning technique for dimension reduction. The plot represents each CD8+ T cell subset as a point, which when combined with heat map expression data allows the properties of each cluster to be determined (Figure 1B). T2D participants had a specific increase in the number of CD8+ EMRA T cells, which was mirrored by a loss in the number of CD8+ naïve T cells, demonstrating that people living with T2D have a prematurely aged T cell phenotype. All people living with T2D were on medication, with the majority taking 3 – 6 different types of medication to control their condition (Table 1). To assess the potential impact of medications on CD8+ EMRA numbers, we compared the percentages of the T cells with the total number of drugs used, however no relationship was found (Figure 1C). This led us to conclude that the rise in CD8+ EMRA T cells was a consequence of T2D and not the medications taken by the participants.

T2D alters the mitochondrial and metabolic properties of CD8+ EMRAs

Mitochondrial health and cellular metabolism can heavily influence the onset of senescence in T cells [15] . CD8+ EMRA T cells exhibit mitochondrial dysfunction and impaired OXPHOS [12]; however, the metabolic properties of CD8+ EMRA T cells from people living with T2D have not been assessed. The mitochondrial mass of CD8+ T cell subsets was determined using Mitotracker green staining. While T2D CD8+ EMRA T cells showed a tendency toward decreased mitochondrial content it was not significantly different from the age-matched controls (Figure 2A). When the mitochondria were observed by electron microscopy, mitochondria from T2D CD8+ EMRA cells

were found to exhibit a different morphology compared to participant without T2D. The mitochondria from participants with T2D they were typically smaller, rounder with little or no cristae (Figure 2B).

Data obtained from a cellular senescence RT2 profiler array indicated that mitochondria from T2D EMRAs may have the propensity to produce more ROS than the EMRA from healthy individuals. NADPH oxidases 4 (NOX4) was greatly upregulated in the T2D CD8+ EMRA T cells compared with the control EMRAs (Figure 2C). NOX enzymes, including NOX4, are major contributors to cellular ROS [16]. The mitochondrial gene; superoxide dismutase 2 (SOD2), which converts superoxide by-products of OXPHOS into hydrogen peroxide and diatomic oxygen [17], was also elevated. This is likely an attempt to reduce the damaging effects of elevated oxidative stress. Despite the elevated SOD2 expression, examination of mtROS levels using the mitochondrial superoxide indicator MitoSOX revealed that mtROS levels were higher in T2D EMRA subsets compared with the age matched controls or EMRAs isolated from younger individuals (Figure 2D).

T2D CD8+ EMRA T cells have an altered bioenergetic profile

The consequence of a reduction in mitochondrial content in CD8+ EMRA T cells isolated from people living with T2D was investigated further. Due to the limited cell numbers obtained from T2D blood donations; it was not possible to acquire enough CD8+ EMRA T cells to run flux analysis on this subset. Instead, the bioenergetic profiles of whole CD8+ T cells from young, healthy age-matched controls and T2D participants were compared (Figure 3A). Interestingly, CD8+ T cells from people living with T2D had an elevated basal and spare respiratory capacity (Figure 3B) compared to age-matched or younger individuals. This may be a consequence of metformin usage as it has been demonstrated to increase oxidative capacity in mitochondria [18]. However, despite this

apparent increase in respiratory function, CD8^{+} T cell from people living with T2D displayed a far higher proton leak indicative of mitochondrial damage (Figure 3B).

The energy source fuelling the increased oxidative respiration was then investigated. We have shown previously that CD8^{+} EMRA T cells taken from people living with T2D displayed a lack of glucose uptake [19] but here we have assessed fatty acid usage. In contrast to glucose uptake, CD8^{+} EMRA T cells from T2D participants significantly increased palmitate uptake (Figure 4A). Consequently, we used the Seahorse XF Palmitate assay to investigate fatty acid oxidation (FAO). A significant decrease in FAO was observed in CD8^{+} T cells taken from people living with T2D (Figure 4B). As palmitate was not being utilised, we subsequently used Nile Red to assess the accumulation of lipid droplets. This revealed that CD8^{+} EMRA T cells from T2D participants were indeed accumulating lipid (Figure 4C). Unfortunately, these experiments were unable to determine what energy source was being utilised for mitochondrial respiration by the EMRA T cells from people living with T2D (Figure 3A).

The observed alterations in nutrient usage in T2D CD8^{+} EMRA T cells led us to examine the levels of intracellular phosphorylated AMP-activated protein kinase (pAMPK), a master regulator of both glucose and lipid metabolism [20]. pAMPK was found to be reduced in the T2D CD8^{+} EMRA T cells compared to EMRAs from people without a diagnosis of T2D (Figure 4D).

Inflammation together with high serum glucose induce the senescent phenotype

Finally we assessed whether the senescent phenotype was induced by high concentrations of glucose or by inflammatory mediators present in the sera of those with T2D. We first cultured young PBMCs in either low (5mM) or high glucose (25mM), however we found no difference in the percentage of CD8^{+} EMRA T cells observed between the two conditions (Figure 5A). We then

mimicked the environment of T2D, culturing young PBMCs with either 10% T2D or age-matched control sera. We found no difference in CD8^{+} T cell phenotype between the two conditions at day 3. However, by day 7 both the control sera and T2D sera resulted in an increase in the CD8^{+} EMRA T cell population (Figure 5B). Despite both conditions driving an increase in EMRA T cells, the higher level of inflammatory mediators present in T2D sera pushed this process significantly further than the age-matched control sera (Figure 5C).

While inflammation was found to drive the appearance of the senescent phenotype, altered nutrient levels, both increased concentrations of glucose and palmitate, did result in the fragmentation of mitochondria (Figure 5D). These observed changes in mitochondrial morphology resemble the mitochondrial dysfunction seen via electron microscopy in CD8^{+} EMRA T cells taken from people living with T2D (Figure 2B).

Taken together these data imply that the low grade chronic inflammatory state observed in people living with T2D drives the differentiation of CD8^{+} T cells, the build-up of senescent CD8^{+} EMRA T cells leads to changes in nutrient usage promoting the accumulation of dysfunctional mitochondrial. These poorly functioning mitochondria generate high levels of mtROS, further adding to the inflammatory burden in a detrimental feed-forward loop.

Discussion

The impact of immunosenescence during ageing is well established, however it is now recognised to be a central finding in a plethora of syndromes including type 2 diabetes [4]. Immunosenescence is accompanied by alterations to T cell immunity and also by a low-grade chronic inflammatory state [21]. We show here a rise in the presence of senescent CD8$^+$ T cells in people living with T2D. Despite the use of medication to manage their disease, T2D participants exhibited greater levels of inflammatory cytokines in their blood serum when compared to the age-matched control participants. It is important to note that it is hard to assess what the impact of these medications themselves are; however, it would be extremely challenging to find a large enough cohort of people living with T2D that did not manage their disease (or related conditions such as hypertension or hypercholesterolaemia) through medication to test this. Nevertheless, we found no correlation between the number of medications people were on and the number of CD8$^+$ EMRA T cells. As a result, we find it unlikely the increase in CD8$^+$ EMRA T cells is influenced by the medications used.

Inflammation plays a key role in the pathogenesis of many age-related and chronic diseases. Numerous studies have shown a correlation between elevated levels of inflammatory markers, such as IL-6 and C-reactive protein, and the risk of cardiovascular events [22]. TNFα plays a crucial role in insulin resistance and the onset of T2D [23]. Moreover, in vitro studies have shown that incubation with TNFα is enough to drive the onset of T cell senescence. However, no TNFα was detected in the sera of the T2D participants. We propose that this is due to both the short-lived nature of TNFα and the fact that our study participants had been living with T2D for on average 15 years and were managing their disease through numerous medications. As a result, the chronic inflammatory cytokine signatures of these participants are likely to be different to the acute inflammatory cytokine signatures present during the onset of disease. However more

inflammatory cytokines were found in the sera of people living with T2D and these were able to differentiate CD8^{+} T cells to a greater extent than sera from age matched individuals, but further experiments are needed to fully appreciate the contribution of each cytokine to EMRA T cell generation.

Metabolic regulation plays an important role during T cell senescence, with mitochondrial dysfunction and elevated aerobic glycolysis now identified as key features of senescent CD8^{+} T cells [12]. Since T2D is also characterised by metabolic imbalances we began to investigate T cell metabolism in the context of T2D. Mitochondria from T2D CD8^{+} T cells displayed a higher oxidative capacity, a phenomenon also observed in PBMCs isolated from people with T2D [24]. This increased oxygen consumption from T2D CD8^{+} T cells did not equate to improved function, mitochondrial morphology showed T2D EMRA cells to be fragmented and the increased proton leak suggests more mitochondrial damage in T2D CD8^{+} T cells. mtROS was also elevated in T2D EMRA CD8^{+} T cells compared to healthy age-matched control cells. Oxidative stress in T2D is known to contribute to vascular complications such as atherosclerosis [25; 26]. Additionally, mtROS can also result in DNA damage and senescence [27]. Therefore, elevated mtROS levels in T2D CD8^{+} EMRA T cells could result in damage to the extracellular environment in which they sit and may also act to reinforce the senescent phenotype of these cells.

In activated T cells, insulin stimulates glucose uptake and enhances T cells responsiveness and energy requirements necessary for appropriate T cell function [28]. It is therefore unsurprising that CD8^{+} EMRA T cells in the context of T2D, characterised by insulin resistance and deficiency, displayed alterations in glucose and fatty acid uptake. We show here that elevated levels of glucose and fatty acids are capable of generating the mitochondrial dysfunction seen during T2D. pAMPK, which is usually activated in response to low glucose and positively regulates FAO [29; 30],

was reduced in T2D CD8^{+} EMRA T cells. Decreased AMPK could provide an explanation for the lack of FAO in these cells. Furthermore, if AMPK levels were increased in these cells FAO may be restored and the accumulation of FA in lipid droplets avoided. As glucose uptake and FAO are both impaired, it remains unclear what energy source T2D CD8^{+} T cells are metabolising. One potential candidate is glutamine. Glutamine mediated metabolism has been shown to be important during T cell activation and proliferation [31]. It is used by many cell types in order to fuel oxidative metabolism under stress conditions [32; 33]. Furthermore, a recent report has demonstrated a role for glutamine in maintaining the viability of senescent cells and the senescence-associated secretory phenotype [34]. However, as glutamine uptake is also positively regulated by pAMPK [35], the reduced pAMPK levels in T2D EMRAs mean this hypothesis is unclear. To be certain, specific glutamine uptake assays need to be conducted.

In summary, the impaired nutrient uptake and usage in CD8+ EMRA T cells in T2D has a profound effect of their phenotype and function. Consequently, a better understanding of these metabolic abnormalities is crucial as metabolic manipulation of these cells may restore correct T cell function and help reduce the impact of T cell dysfunction in T2D.

Acknowledgements

References

[1] C. Zelle-Rieser, S. Thangavadivel, R. Biedermann, A. Brunner, P. Stoitzner, E. Willenbacher, R. Greil, and K. Jöhrer, T cells in multiple myeloma display features of exhaustion and senescence at the tumor site. J Hematol Oncol 9 (2016) 116.

[2] K. Shirakawa, X. Yan, K. Shinmura, J. Endo, M. Kataoka, Y. Katsumata, T. Yamamoto, A. Anzai, S. Isobe, N. Yoshida, H. Itoh, I. Manabe, M. Sekai, Y. Hamazaki, K. Fukuda, N. Minato, and M. Sano, Obesity accelerates T cell senescence in murine visceral adipose tissue. J Clin Invest 126 (2016) 4626-4639.

[3] C.M. Weyand, Z. Yang, and J.J. Goronzy, T-cell aging in rheumatoid arthritis. Curr Opin Rheumatol 26 (2014) 93-100.

[4] B.B. Duncan, M.I. Schmidt, J.S. Pankow, C.M. Ballantyne, D. Couper, A. Vigo, R. Hoogeveen, A.R. Folsom, and G. Heiss, Low-grade systemic inflammation and the development of type 2 diabetes: the atherosclerosis risk in communities study. Diabetes 52 (2003) 1799-805.

[5] D. Verzola, M.T. Gandolfo, G. Gaetani, A. Ferraris, R. Mangerini, F. Ferrario, B. Villaggio, F. Gianiorio, F. Tosetti, U. Weiss, P. Traverso, M. Mji, G. Deferrari, and G. Garibotto, Accelerated senescence in the kidneys of patients with type 2 diabetic nephropathy. Am J Physiol Renal Physiol 295 (2008) F1563-F1573.

[6] H. Sone, and Y. Kagawa, Pancreatic beta cell senescence contributes to the pathogenesis of type 2 diabetes in high-fat diet-induced diabetic mice. Diabetologia 48 (2005) 58-67.

[7] S. Giubilato, G. Liuzzo, S. Brugaletta, D. Pitocco, F. Graziani, C. Smaldone, R.A. Montone, V. Pazzano, D. Pedicino, L.M. Biasucci, G. Ghirlanda, and F. Crea, Expansion of CD4+CD28null T-lymphocytes in diabetic patients: exploring new pathogenetic mechanisms of increased cardiovascular risk in diabetes mellitus. Eur Heart J 32 (2011) 1214-26.

[8] Y.-h. Lee, S.R. Kim, D.H. Han, H.T. Yu, Y.D. Han, J.H. Kim, S.H. Kim, C.J. Lee, B.-H. Min, D.-H. Kim, K.H. Kim, J.W. Cho, W.-W. Lee, E.-C. Shin, and S. Park, Senescent T Cells Predict the Development of Hyperglycemia in Humans. Diabetes 68 (2019) 156.

[9] M.E. Stegenga, S.N. van der Crabben, M.C. Dessing, J.M. Pater, P.S. van den Pangaart, A.F. de Vos, M.W. Tanck, D. Roos, H.P. Sauerwein, and T. van der Poll, Effect of acute hyperglycaemia and/or hyperinsulinaemia on proinflammatory gene expression, cytokine production and neutrophil function in humans. Diabet Med 25 (2008) 157-64.

[10] P. Bogdanski, D. Pupek-Musialik, J. Dytfeld, P.P. Jagodzinski, A. Jablecka, A. Kujawa, and K. Musialik, Influence of insulin therapy on expression of chemokine receptor CCR5 and selected inflammatory markers in patients with type 2 diabetes mellitus. Int J Clin Pharmacol Ther 45 (2007) 563-7.

[11] G. Desdín-Micó, G. Soto-Heredero, and M. Mittelbrunn, Mitochondrial activity in T cells. Mitochondrion 41 (2018) 51-57.

[12] S.M. Henson, A. Lanna, N.E. Riddell, O. Franzese, R. Macaulay, S.J. Griffiths, D.J. Puleston, A.S. Watson, A.K. Simon, S.A. Tooze, and A.N. Akbar, p38 signaling inhibits mTORC1-independent autophagy in senescent human CD8+ T cells. J Clin Invest 124 (2014) 4004-16.

[13] P. Alonso-Fernández, and M. De la Fuente, Role of the immune system in aging and longevity. Curr Aging Sci 4 (2011) 78-100.

[14] J. Moura, P. Madureira, E.C. Leal, A.C. Fonseca, and E. Carvalho, Immune aging in diabetes and its implications in wound healing. Clin Immunol 200 (2019) 43-54.

[15] L.A. Callender, E.C. Carroll, E.A. Bober, A.N. Akbar, E. Solito, and S.M. Henson, Mitochondrial mass governs the extent of human T cell senescence. Aging Cell 19 (2020) e13067.

[16] F. Chen, S. Haigh, S. Barman, and D. Fulton, From form to function: the role of Nox4 in the cardiovascular system. Front Physiol 3 (2012).

[17] M.C. Velarde, J.M. Flynn, N.U. Day, S. Melov, and J. Campisi, Mitochondrial oxidative stress caused by Sod2 deficiency promotes cellular senescence and aging phenotypes in the skin. Aging 4 (2012) 3-12.

[18] Y. Wang, H. An, T. Liu, C. Qin, H. Sesaki, S. Guo, S. Radovick, M. Hussain, A. Maheshwari, F.E. Wondisford, B. O'Rourke, and L. He, Metformin Improves Mitochondrial Respiratory Activity through Activation of AMPK. Cell Reports 29 (2019) 1511-1523.e5.

[19] E.Y.M. Lau, E.C. Carroll, L.A. Callender, G.A. Hood, V. Berryman, M. Pattrick, S. Finer, G.A. Hitman, G.L. Ackland, and S.M. Henson, Type 2 diabetes is associated with the accumulation of senescent T cells. Clin Exp Immunol 197 (2019) 205-213.

[20] D.G. Hardie, F.A. Ross, and S.A. Hawley, AMPK: a nutrient and energy sensor that maintains energy homeostasis. Nat Rev Mol Cell Biolol 13 (2012) 251-62.

[21] R. Macaulay, A.N. Akbar, and S.M. Henson, The role of the T cell in age-related inflammation. Age 35 (2013) 563-72.

[22] C-Reactive Protein, Fibrinogen, and Cardiovascular Disease Prediction. N Engl J Med 367 (2012) 1310-1320.

[23] D.E. Moller, Potential Role of TNF-α in the Pathogenesis of Insulin Resistance and Type 2 Diabetes. Trends Endocrinol Metabol 11 (2000) 212-217.

[24] D. Nicholas, E.A. Proctor, F.M. Raval, B.C. Ip, C. Habib, E. Ritou, T.N. Grammatopoulos, D. Steenkamp, H. Dooms, C.M. Apovian, D.A. Lauffenburger, and B.S. Nikolajczyk, Advances in the quantification of mitochondrial function in primary human immune cells through extracellular flux analysis. PLoS One 12 (2017) e0170975.

[25] J.W. Baynes, and S.R. Thorpe, Role of oxidative stress in diabetic complications: a new perspective on an old paradigm. Diabetes 48 (1999) 1-9.

[26] P. Dandona, K. Thusu, S. Cook, B. Snyder, J. Makowski, D. Armstrong, and T. Nicotera, Oxidative damage to DNA in diabetes mellitus. Lancet 347 (1996) 444-5.

[27] P. Davalli, T. Mitic, A. Caporali, A. Lauriola, and D. D'Arca, ROS, Cell Senescence, and Novel Molecular Mechanisms in Aging and Age-Related Diseases. Oxid Med Cell Longevy 2016 (2016) 3565127.

[28] F.B. Stentz, and A.E. Kitabchi, Activated T lymphocytes in Type 2 diabetes: implications from in vitro studies. Curr Drug Targets 4 (2003) 493-503.

[29] D.G. Hardie, The AMP-activated protein kinase pathway--new players upstream and downstream. J Cell Sci 117 (2004) 5479-87.

[30] J. Xu, J. Ji, and X.H. Yan, Cross-talk between AMPK and mTOR in regulating energy balance. Crit Rev Food Sci Nutr 52 (2012) 373-81.

[31] E.L. Carr, A. Kelman, G.S. Wu, R. Gopaul, E. Senkevitch, A. Aghvanyan, A.M. Turay, and K.A. Frauwirth, Glutamine Uptake and Metabolism Are Coordinately Regulated by ERK/MAPK during T Lymphocyte Activation. J Immunol 185 (2010) 1037.

[32] C. Yang, J. Sudderth, T. Dang, R.G. Bachoo, J.G. McDonald, and R.J. DeBerardinis, Glioblastoma Cells Require Glutamate Dehydrogenase to Survive Impairments of Glucose Metabolism or Akt Signaling. Cancer Res 69 (2009) 7986.

[33] A. Le, A.N. Lane, M. Hamaker, S. Bose, A. Gouw, J. Barbi, T. Tsukamoto, C.J. Rojas, B.S. Slusher, H. Zhang, L.J. Zimmerman, D.C. Liebler, R.J.C. Slebos, P.K. Lorkiewicz, R.M. Higashi, T.W.M. Fan, and C.V. Dang, Glucose-independent glutamine metabolism via TCA cycling for proliferation and survival in B cells. Cell Metab 15 (2012) 110-121.

[34] Y. Johmura, T. Yamanaka, S. Omori, T.W. Wang, Y. Sugiura, M. Matsumoto, N. Suzuki, S. Kumamoto, K. Yamaguchi, S. Hatakeyama, T. Takami, R. Yamaguchi, E. Shimizu, K. Ikeda, N. Okahashi, R. Mikawa, M. Suematsu, M. Arita, M. Sugimoto, K.I. Nakayama, Y. Furukawa, S. Imoto, and M. Nakanishi, Senolysis by glutaminolysis inhibition ameliorates various age-associated disorders. Science 371 (2021) 265-270.

[35] J. Blagih, F. Coulombe, E.E. Vincent, F. Dupuy, G. Galicia-Vázquez, E. Yurchenko, T.C. Raissi, G.J. van der Windt, B. Viollet, E.L. Pearce, J. Pelletier, C.A. Piccirillo, C.M. Krawczyk, M. Divangahi, and R.G. Jones, The energy sensor AMPK regulates T cell metabolic adaptation and effector responses in vivo. Immunity 42 (2015) 41-54.

Figure legends

Figure 1. Phenotypic characteristics of CD8$^+$ T cells taken from young, older and people living with T2D. (A). UMAP dimensionality reduction and clustering of CD8$^+$ T cells using CD27, CD28, CD45RA, CCR7 and KLRG1, n = 6. (B). Heatmap to show protein levels of cell surface markers CD27, CD28, CD45RA, CCR7 and KLRG1 in each cluster, n = 6. (C). The relationship between T2D CD8$^+$ EMRA T cells and medication shown as the percentage of CD8$^+$ EMRA T cells defined as percentage of the total CD8$^+$ T cell population against the total number of medications, n = 22. Line of best fit generated using linear regression.

Figure 2. Mitochondrial changes to CD8$^+$ EMRA T cells from people living with T2D.

(A) Representative histograms and quantification of Mitotracker Green in CD8$^+$ T cell EMRAs isolated from young, older individuals and people living with T2D. Data expressed as mean ± SEM, n = 7. (B) Electron microscope images of CD8+ EMRA T cells from young, older individuals and people living with T2D, scale bars measure 0.5 μM. (C) Cellular senescence RT2 profiler array data showing the change in mRNA expression in genes associated with oxidative stress from T2D CD8$^+$ EMRA T cells compared to age matched individuals. Fold change cut-off < 2, n = 3. (D) Graphs and quantification of MitoSOX staining in CD8$^+$ T cell EMRAs isolated from young, older individuals and people living with T2D. Data expressed as mean ± SEM, n = 7. P values were determined using a two-way ANOVA with Bonferroni analysis used for post-hoc testing, p* = < 0.05.

Figure 3. CD8$^+$ T cells from people living with T2D show increased oxidative metabolism.
(A). OCR of the CD8$^+$ T cells was measured after 15-minute stimulation with 0.5 μg/ml anti-CD3 and 5 ng/ml IL-2, cells were subjected to a mitochondrial stress test using the indicated mitochondrial inhibitors. n= 4. (B). Basal and maximal OCR along with proton leak following 15-minute stimulation with 0.5 μg/ml anti-CD3 and 5 ng/ml IL-2. Data expressed as mean ± SEM, n =

4. P values were determined using a two-way ANOVA with Bonferroni analysis used for post-hoc testing, $p^* = < 0.05$, $p^{**} = < 0.01$, $p^{****} = < 0.001$.

Figure 4. CD8+ T cells from people living with T2D show increased lipid uptake but decreased fatty acid oxidation. (A) Lipid uptake assessed using BODIPY C16. Representative dot plots and cumulative data. Data expressed as mean ± SEM, young n = 5, old n = 8, T2D n = 6. (B). FAO determined OCR of the CD8+ T cells was measured after 15-minute stimulation with 0.5 μg/ml anti-CD3 and 5 ng/ml IL-2 and incubation with palmitate. Cells were subjected to a mitochondrial stress test using indicated mitochondrial inhibitors. n = 2. (C). Plots and quantification of lipid droplets visualised using Nile Red in young, older individuals and T2D CD8+ EMRAs. Data expressed as mean ± SEM, young n = 5, control n = 5, T2D n = 6. (D). Flow cytometry plots and cumulative data of pAMPK in young, control and T2D CD8+ EMRAs. Data expressed as mean ± SEM, young n = 7, old n = 8, T2D n = 7. P values were determined using a two-way ANOVA with Bonferroni analysis used for post-hoc testing, $p^* = < 0.05$

Figure 5. Altering nutrient content and inflammatory mediators drives the senescent phenotype. (A) Plots and quantification of CD8+ EMRA T cells following 7 days in culture with 10% FCS and either low (5mM) and high (25mM) glucose. Data expressed as mean ± SEM, n = 4. (B) Plots and quantification of CD8+ EMRA T cells at day 0, 3 and 7 of culture with 10% sera from age-matched control and T2D participants. Data expressed as mean ± SEM, n = 3. (C) Cytokine levels quantified from a cytokine array using T2D serum or sera from aged matched controls. n = 4. (D) Representative images of Mitotracker green staining in CD8+ T cell incubated either in low glucose (5mM) or high glucose (25mM) and palmitate (200μM). n = 4. P value was determined using a paired T-test, $p^* = < 0.05$.

Figure 1.
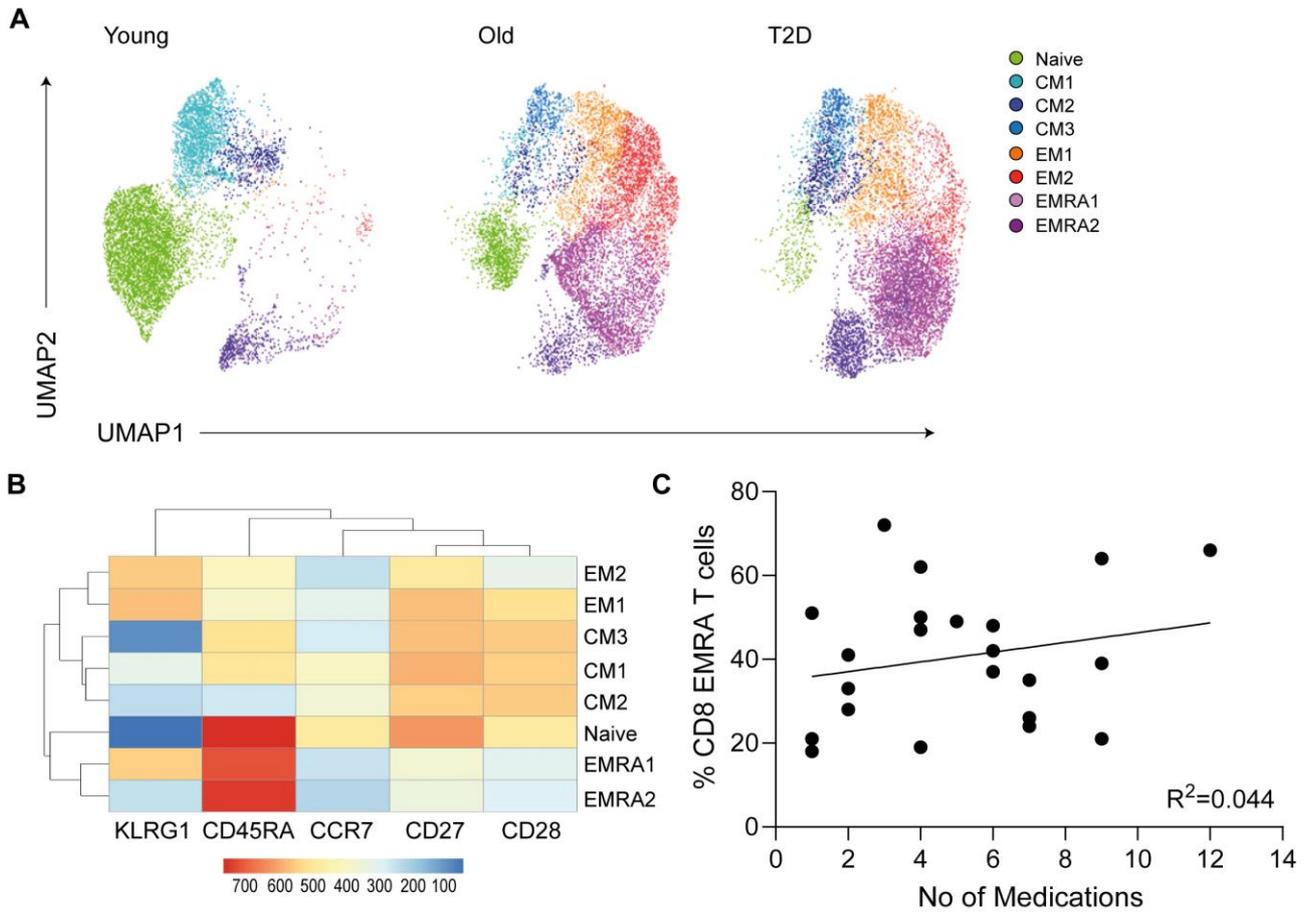
Figure 2.
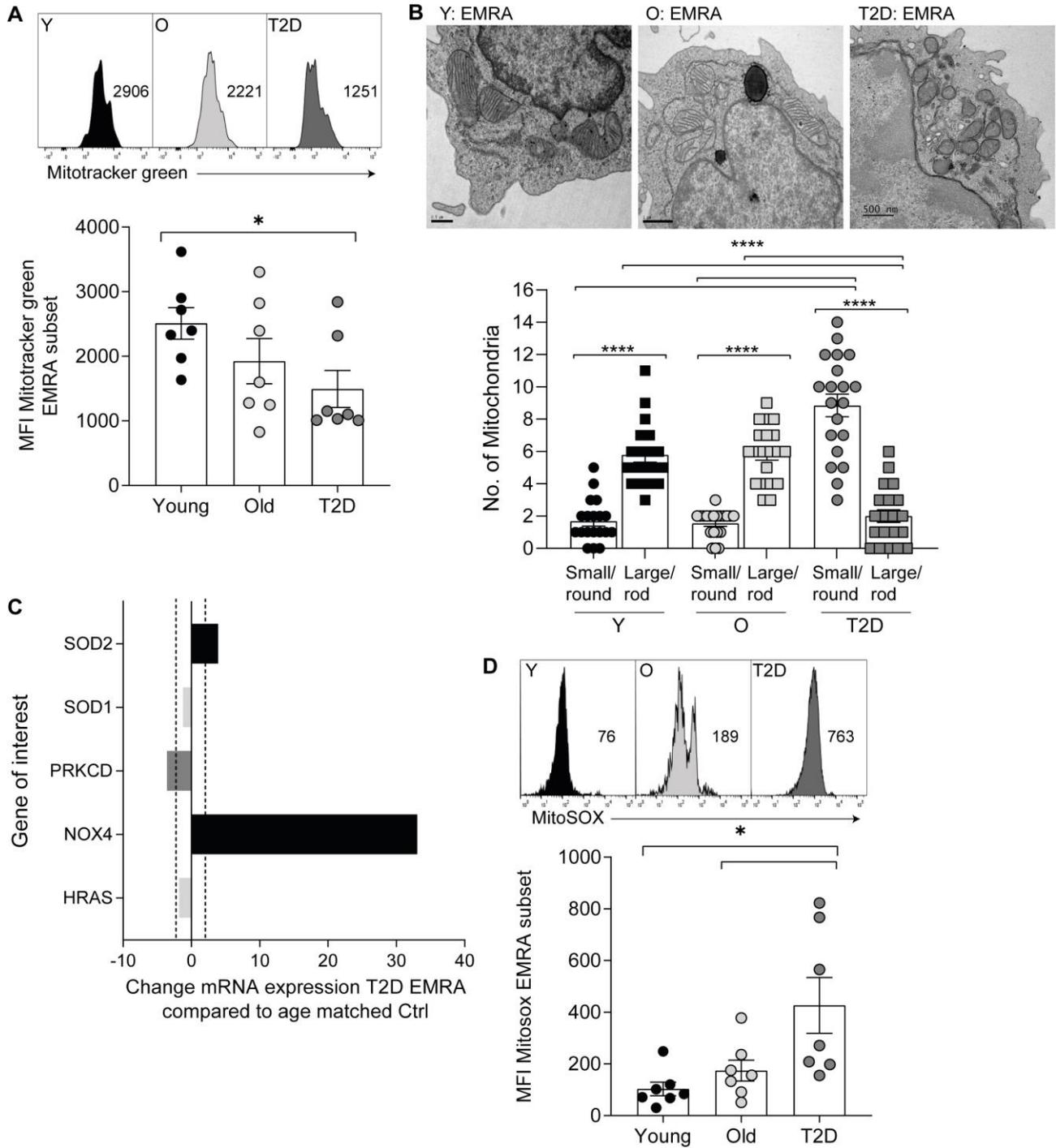
Figure 3.
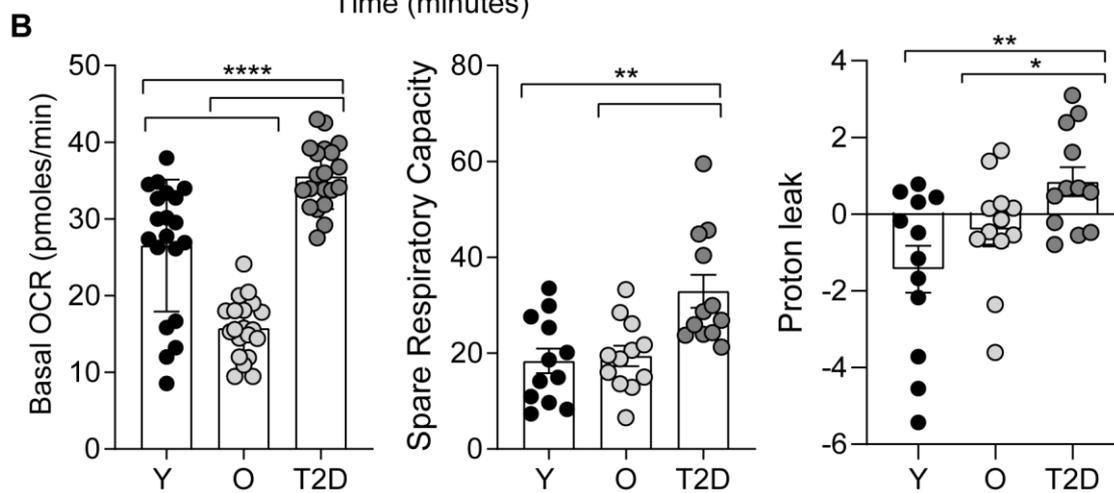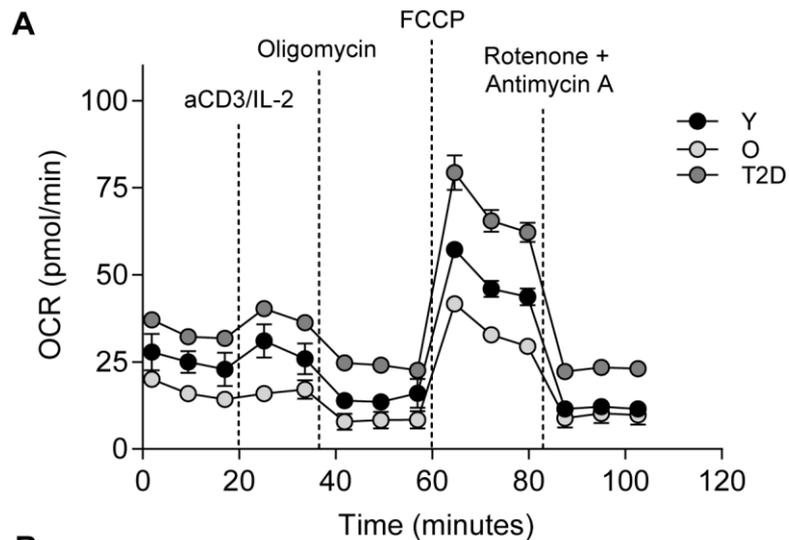
Figure 4.
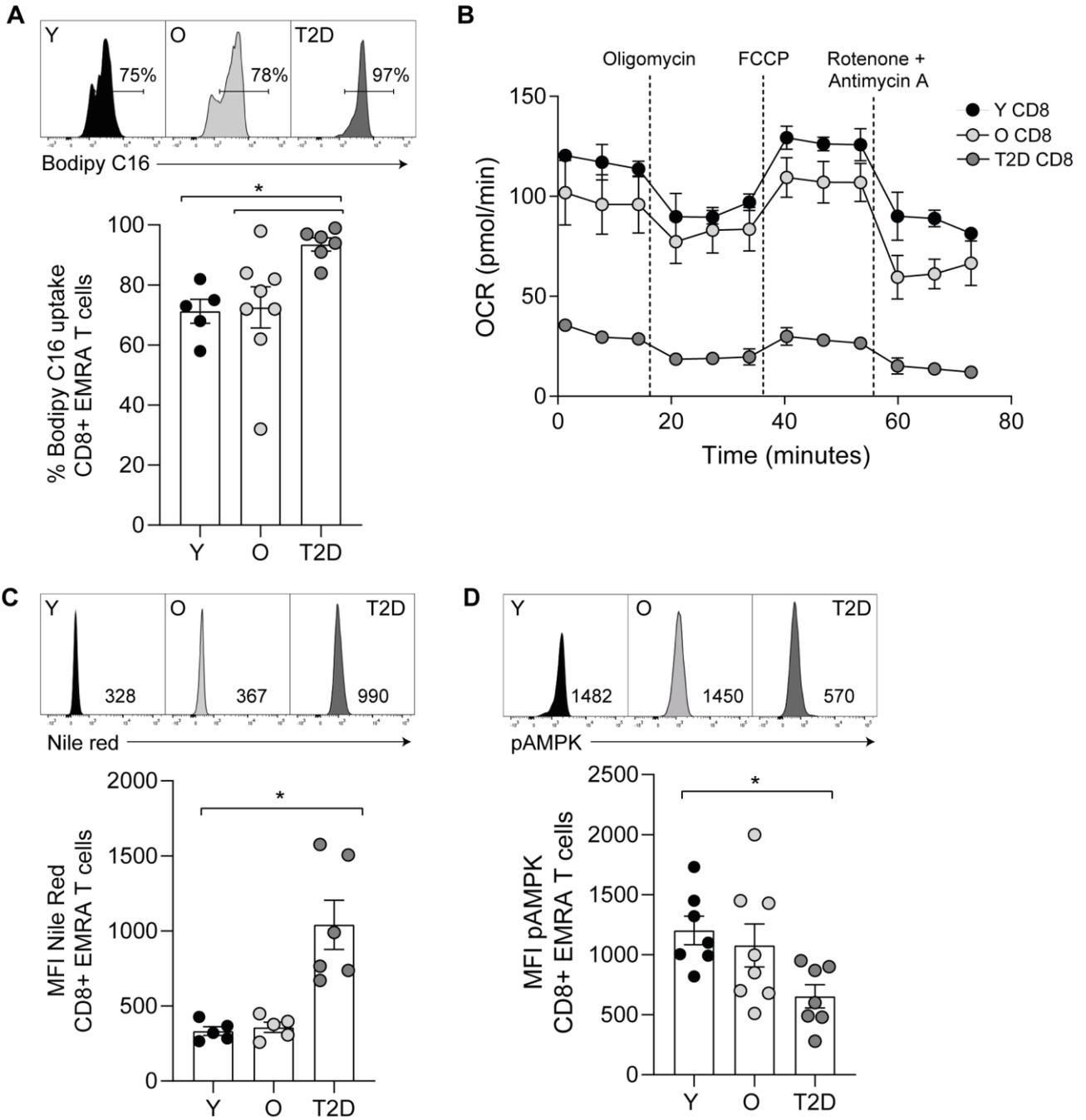
Figure 5.
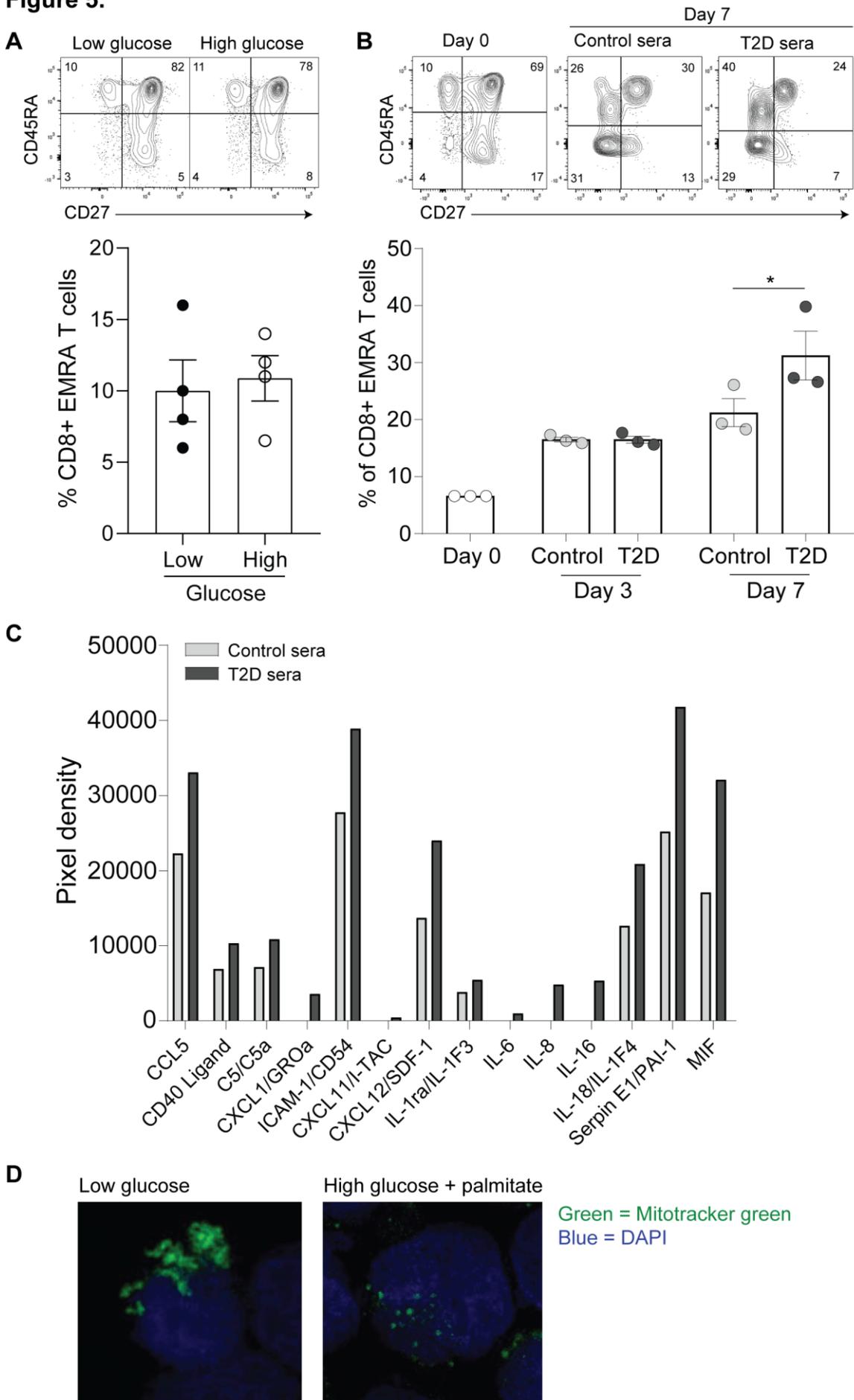
Table 1. Donor characteristics

Donor characteristics	T2D participants	Older participants	Young participants
Total number of participants	52	23	25
Age ranges	50-77 years	49-75 years	20-36 years
Median age	64	62	31
Gender (Male	65%	48%	49%
Mean length of time since diagnosis	15 years	N/A	N/A
Medication(s): 1-2 3-6 7+	23% 42% 35%	N/A N/A N/A	N/A N/A N/A
Medication category: Diabetes Statins Other	Metformin, Dulaglutide, Glizcazide, Canagliflozin, Glargine, Humalog, Liraglutide, Gliptin, Levemir Simvastatin, Atrovastatin, Pravastatin Rampril, Amlodipine, Bisoprolol, Pregabalin, Morphine, Quinninesulphate, Solifenacin, Colecalciferol, Taddalafil, Travopost, Doxazosin, Bendro, Citalopram, Fenofibrate, Spironolactone, Bendroflumethiazide, Beclomethasone dipropionate, Salamol, Trimethoprim, Paroxetine, Tramadol, Isosorbide mononitrate, Omeprazole, Edoxaban, Candesartan, Levothyroxine, Tamsulosin, Aspirin	N/A	N/A