

Aus der III. Medizinischen Klinik und Poliklinik
der Universitätsmedizin der Johannes Gutenberg-Universität Mainz

Deep characterization of the cellular profile and metabolic phenotype of human senescent
CD8⁺ T Cells

Umfassende Charakterisierung des zellulären Profils und des metabolischen Phänotyps
humaner seneszenten CD8⁺ T-Zellen

Inauguraldissertation
zur Erlangung des Doktorgrades der
Medizin
der Universitätsmedizin
der Johannes Gutenberg-Universität Mainz

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Mainz, 2026

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List of Abbreviations

2-NBDG	2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose
ACT	Adoptive Cell Transfer
ADP	Adenosine Diphosphate
AML	Acute Myeloid Leukemia
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
BAFF	B-cell Activating Factor
BCMA	B-cell Maturation Antigen
BSA	Bovine Serum Albumin
cADPR	Cyclic ADP-Ribose
CAR	Chimeric Antigen Receptor
CD	Cluster of Differentiation
CDK	Cyclin-dependent Kinase
CLL	Chronic Lymphocytic Leukemia
CMV	Cytomegalovirus
CTLA-4	Cytotoxic T-lymphocyte Associated Protein 4
DAMP	Damage-Associated Molecular Pattern
DC	Dendritic Cell
DDR	DNA Damage Response
DLBCL	Diffuse Large B Cell Lymphoma
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DSB	Double-strand breaks
EBV	Epstein-Barr Virus
ECAR	Extracellular Acidification Rate
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
ETC	Electron Transport Chain
FACS	Fluorescence-Activated Cell Sorting
FAD	Flavin Adenine Dinucleotide
FAO	Fatty Acid Oxidation
FCCP	Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
FCS	Fetal Calf Serum

FDA	Food and Drug Administration
GLUT-1	Glucose Transporter 1
HCV	Hepatitis C Virus
HEPES	2-(4-(2-Hydroxyethyl)-1-piperazinyl)- ethanesulfonic acid
HIV	Human Immunodeficiency Virus
HLA	Human leucocyte antigen
HSV	Herpes simplex Virus
ICI	Immune Checkpoint Inhibitor
IFN α	Interferon alpha
IFN β	Interferon beta
IFN γ	Interferon gamma
IL	Interleukin
ILC	Innate Lymphoid Cell
KLRG-1	Killer cell lectin-like receptor subfamily G member 1
LAG-3	Lymphocyte-activation gene 3
LDH	Lactate Dehydrogenase
LDHA	Lactate Dehydrogenase subunit A
LDHB	Lactate Dehydrogenase subunit B
MACS	Magnetic-activated Cell Sorting
MDR	MitoTracker Deep Red
MDSCs	Myeloid-derived Suppressor Cells
MFI	Mean Fluorescence Intensity
MG	MitoTracker Green
MHC	Major Histocompatibility Complex
MM	Multiple Myeloma
NAD	Nicotinamide adenine dinucleotide
NF- κ B	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NK	Natural Killer cell
NSCLC	Non-small-cell Lung Cancer
OCR	Oxygen Consumption Rate
OXPHOS	Oxidative Phosphorylation
PAMP	Pathogen-associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate-buffered Saline

PD-1	Programmed Cell Death Protein 1
PD-L1	Programmed Cell Death 1 Ligand 1
PFA	Paraformaldehyde
PI	Propidium Iodide
ROS	Reactive Oxygen Species
SA- β -GAL	Senescence-associated- β -Galactosidase
SASP	Senescence Associated Secretory Phenotype
SRC	Spare Respiratory Capacity
TCA cycle	Tricarboxylic Acid Cycle
TCM	Central Memory T Cell
TCR	T Cell Receptor
TEM	Effector Memory T Cell
TEMRA	T-cell Effector Memory Re-expressing CD45RA
TIGIT	T cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif (ITIM) domain
TILs	Tumor Infiltrating Lymphocytes
TIM-3	T Cell Immunoglobulin Mucin 3
TME	Tumor Microenvironment
TMRE	Tetramethylrhodamine ethyl ester
TN	Naïve T Cell
TNF α	Tumor Necrosis Factor α
Treg	Regulatory T Cell
uPAR	Urokinase-type plasminogen activator receptor
VZV	Varicella Zoster Virus

1 Zusammenfassung

Die Beziehung zwischen Krebserkrankungen und dem alternden Immunsystem, bekannt als Immunoseneszenz, ist angesichts einer alternden Weltbevölkerung zu einer bedeutenden neuen Herausforderung in der Hämatologie und Onkologie geworden. Es ist gut belegt, dass die Inzidenzen sowohl solider als auch hämatologischer Krebserkrankungen mit dem Alter zunehmen (1). Daher ist ein besseres Verständnis darüber erforderlich, wie seneszente Immunzellen, insbesondere CD8⁺ T-Zellen, die tumorgerichtete Immunreaktion sowie die Wirksamkeit von T-Zell-basierten Therapien, wie Immuncheckpoint-Inhibitoren, bispezifischen Antikörpern und CAR-T-Zellen, beeinflussen. Der zentrale Inhalt dieser Studie ist eine detaillierte phänotypische Analyse seneszenten CD8⁺ T-Zellen, insbesondere jener, die durch CD28⁻CD57⁺ gekennzeichnet sind. Diese Zellen reichern sich mit zunehmendem Alter an und zeigten klassische Zeichen der Seneszenz, wie eine verringerte Proliferationsfähigkeit. Dennoch behielten sie starke Effektorfunktionen bei, einschließlich hoher Spiegel von Granzyme B und Perforin. Dieses Paradoxon der reduzierten Teilungsfähigkeit bei gleichzeitig erhaltener zytotoxischer Aktivität unterstreicht die komplexe Rolle, die diese Zellen in der Tumorüberwachung des Immunsystems spielen könnten. Kurzfristig könnten sie von Vorteil sein, langfristig jedoch schädlich, wenn sie dysreguliert sind oder nicht auf zusätzliche Stimulation reagieren.

Aus translationaler Sicht ist ein wichtiger Aspekt dieser Arbeit die Identifikation und Verfeinerung von Oberflächenmarkern, die den seneszenten T-Zell-Phänotyp charakterisieren. Zusätzlich zu den etablierten Markern CD28⁻ und CD57⁺ erweitert diese Arbeit das Seneszenz-Panel um KLRG1, die Re-Expression von CD45RA, den Verlust von CD27 sowie eine reduzierte Expression wichtiger Homing- und Zytokinrezeptoren. Bemerkenswerterweise waren klassische Erschöpfungsmarker wie PD-1 und TIM-3 auf seneszenten Zellen nicht stark exprimiert. Dies weist darauf hin, dass Seneszenz und Erschöpfung zwar beide mit einer Dysfunktion von T-Zellen zusammenhängen, jedoch unterschiedliche immunologische Zustände darstellen. Diese Unterscheidung ist relevant für die Entwicklung von Immuncheckpoint-Therapien, die hauptsächlich auf Erschöpfungspfade abzielen, aber möglicherweise die Rolle seneszenten T-Zellen bei Therapieresistenzen übersehen.

Ebenfalls ließ sich beobachten, dass der Überstand aus der Zellkultur von Tumor-Zelllinien seneszenzähnliche Merkmale wie eine erhöhte SA-β-Gal- und uPAR-Expression in T-Zellen hervorrufen konnte, was nahelegt, dass das Tumormikromilieu aktiv zur Immunalterung beiträgt, um einer Immunantwort zu entgehen.

Die Untersuchung der metabolischen Dysfunktion seneszenten T-Zellen ist ein weiterer zentraler Aspekt dieser Arbeit. Es zeigte sich, dass seneszente T-Zellen eine verringerte mitochondriale Atmung und eine reduzierte Glycolyse aufwiesen. Interessanterweise zeigten seneszente T-Zellen trotz dieser funktionellen Beeinträchtigungen ex vivo eine hohe mitochondriale Masse und ein hohes Membranpotential, was darauf hinweist, dass die mitochondriale Masse nicht zwangsläufig mit metabolischer Funktionalität gleichzusetzen ist. Auffällig ist auch, dass diese Zellen keine signifikant erhöhte mitochondriale ROS-Produktion zeigten.

Ein besonders neuartiger und potenziell bedeutender Befund betrifft den Laktatstoffwechsel. Diese Studie gehört zu den ersten, die zeigten, dass seneszente CD8⁺ T-Zellen eine geringe Expression von LDHB aufwiesen und nicht in der Lage waren, nach Aktivierung LDHA hochzuregulieren. Da viele Tumore eine laktatreiche, immunsuppressive Umgebung schaffen, könnte diese Unfähigkeit, Laktat effizient zu metabolisieren, dazu führen, dass seneszente T-Zellen im Tumormikromilieu metabolisch beeinträchtigt sind. Diese Beobachtung könnte im Bereich der Krebsimmuntherapie zu neuen Entwicklungen führen. Insbesondere könnte die gezielte Veränderung metabolischer Signalwege, beispielsweise durch die Regulation von LDH-Isoenzymen, helfen, die Funktion seneszenten T-Zellen wiederherzustellen oder zu verbessern.

Zusammenfassend bietet diese Arbeit einen Überblick über die Seneszenz von CD8⁺ T-Zellen, indem sie Oberflächenphänotyp, Proliferation, Effektorfunktion und Metabolismus mit den größeren klinischen Fragestellungen der Immunalterung und Krebs verbindet. Die Arbeit schafft eine Grundlage für das langfristige Ziel, einen effektiveren und länger anhaltenden Effekt der T-Zell-basierten Therapie durch Behebung der metabolischen Dysfunktion seneszenten CD8⁺ T-Zellen zu erreichen.

2 Introduction

Aging is a gradual, irreversible physiological process leading to a progressive decline in tissue and cellular function. It is associated with several age-related diseases, such as neurodegenerative disorders, cardiovascular disease, metabolic diseases, osteoarthritis and cancer (1). According to the 14th population projection by the Federal Statistical Office of Germany, 24-30% of the population will be 67 or over by 2060, compared to 19% in 2018. Similarly, a marked increase in the percentage of citizens aged 80 or over is predicted, rising from 6% in 2018 to 9-13% in 2060 (2). A society with an increasingly aging population is faced with a rise in age-related diseases, which has profound implications for the healthcare system. In 2024, cancer was the second leading cause of death in the United States, following heart disease. Aging is a key risk factor for developing malignant diseases, as the prevalence of cancer rises with age. Most carcinomas, leukemias, lymphomas, melanomas, sarcomas and glioblastomas occur in patients over 50 and increase steadily until age 85 (3, 4). Aging and cancer share several mechanistic grounds, referred to as “hallmarks.” These include genomic instability, epigenetic alterations, chronic inflammation and intestinal dysbiosis. However, it is unclear whether there is a causative relationship between aging and cancer or whether they are driven independently by time (4). Like all organismal systems, the immune system is affected by aging. The different age-related changes impacting the innate and adaptive immune systems are defined as “immunosenescence”. It describes the compromised ability of the immune system to mount an effective immune response to new antigens, such as pathogens and malignant cells and its diminished capacity to sustain memory responses. This compromise further leads to a reduced vaccine effectiveness (5). Further cardinal features of immunosenescence include increased susceptibility to autoimmunity and a lingering, low-grade inflammation in the absence of infection, coined “inflammaging (6, 7). Dysfunctional, late-differentiated T cells accumulate with age. Due to persistent lifelong antigen stimulation, such as a chronic viral infection, T cells acquire a senescent phenotype (6). These dysfunctional T cells contribute to chronic inflammation, promote tumor progression and impair anti-tumor immunity. The central impact of a dysfunctional and dysregulated immune system in the initiation and progression of cancer is emphasized in Hanahan and Weinberg’s concept of “The Hallmarks of Cancer”, in which they included tumor-promoting inflammation and the avoidance of immune destruction as drivers of tumor progression (8, 9).

In recent years, cancer immunotherapy has emerged as a groundbreaking approach to treating advanced or refractory cancers. Cancer immunotherapy leverages the patient’s immune system to recognize and destroy malignant cells. Cancer immunotherapy includes immune checkpoint inhibitors, cancer vaccines, bispecific T-cell engagers (BiTEs) and adoptive cellular therapies (ACTs), such as tumor-infiltrating lymphocytes (TILs), chimeric antigen receptor T (CAR-T) cell therapy and T cell receptor-engineered T (TCR-T) cell therapy. However, these

treatment approaches are limited by various mechanisms affecting the T cell function, such as T cell differentiation state, exhaustion and senescence (10). T cells with a senescence profile fail to proliferate or expand after transfer, ultimately failing to elicit a durable anti-tumor response (11). Therefore, preventing or reversing T cell senescence might improve the endogenous tumor response and increase the response to immunotherapy, such as adoptive cell therapy (ACT).

2.1 Aim of the study

The overarching aim of this study is to investigate the metabolic alterations and mitochondrial dysfunction in human senescent CD8⁺ T cells. T cell senescence represents an essential aspect of immunosenescence, a gradual decline in immune system functionality that occurs with aging. Immunosenescence is associated with reduced cancer immunosurveillance and impaired efficacy of cancer immunotherapies, including immune checkpoint therapies, cancer vaccines and adoptive T cell transfer therapies such as CAR-T cell and TCR-T cell therapies (12). This is particularly significant in an aging society, where the incidence of cancer is increasing and there is an unmet need to develop novel strategies to enhance T cell function and improve therapeutic outcomes.

Several studies have identified CD28⁻CD57⁺ CD8⁺ T cells as a senescent subset based on their accumulation with age, impaired proliferation and altered functional properties (13-16). Therefore, we used these markers to identify senescent CD8⁺ T cells in the peripheral blood of healthy donors to further characterize the phenotype of these cells by establishing a flow cytometry-based panel of surface markers, incorporating markers of activation, differentiation and exhaustion, as well as the classic senescence marker Senescence-associated- β -Galactosidase (SA- β -GAL). This panel aimed to better define the unique properties of senescent T cells in a more comprehensive manner.

To better understand the functional implications of senescence, we investigated the effector functions of senescent CD8⁺ T cells by assessing their ability to degranulate and measuring the production of cytotoxic molecules, including Granzyme B and Perforin.

While metabolic dysregulation and mitochondrial dysfunction have been implicated in T cell senescence, few studies have explored these aspects in an integrated manner, particularly in the specific CD28⁻CD57⁺ CD8⁺ T cell subset. Therefore, we aimed to examine various parameters related to mitochondrial dysfunction and metabolic changes, linking these findings to T cell senescence. Mitochondrial dysfunction is a hallmark of senescent T cells, which is central to their altered metabolism and reduced functionality. To characterize mitochondrial dysfunction, we measured key parameters, including mitochondrial mass, mitochondrial membrane potential and the production of mitochondrial reactive oxygen species (ROS).

Additionally, we investigated the cells' Spare Respiratory Capacity (SRC), as the failure to maximize oxygen consumption during cellular stress is a robust indicator of mitochondrial impairment. Metabolic changes in senescent T cells also involves alterations in glucose metabolism. We evaluated glucose metabolism by measuring the expression of GLUT-1, glucose uptake and surrogate markers of NAD⁺ metabolism, known to regulate cellular energy balance.

The metabolic regulation by Lactate Dehydrogenase (LDH) has been extensively studied in cancer cells, however, the role of LDH is a rarely explored avenue in the context of T cell senescence. LDH mediates the conversion of pyruvate to lactate and consists of two subunits: LDHA (promoting glycolysis) and LDHB (favoring oxidative metabolism). Therefore, we measured the expression of LDHA and LDHB in senescent T cells using flow cytometry to investigate their potential contribution to altered cellular metabolism in these cells.

This study aims to define the key features of metabolic and mitochondrial dysfunction in senescent CD8⁺ T cells and link these findings to their phenotypic and functional characteristics. This study enhances our understanding of the metabolic factors involved in T cell senescence. It establishes a basis for future potential interventions to target these pathways to enhance T cell-based immunotherapies, especially for older adults with cancer.

3 Literature discussion

3.1 Immunosenescence and the innate immune response

The innate immune system is the body's first line of defense against pathogens, operating with a rapid, nonspecific response. It includes physical and chemical barriers, humoral components such as the complement system, cytokines, chemokines and cellular defenses. Cellular mediators involved are neutrophils, macrophages, monocytes, dendritic cells (DCs), mast cells, natural killer (NK) cells and innate lymphoid cells (ILCs) (17). The innate immune system relies on pattern recognition receptors to identify pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). Aging significantly impacts the innate immune system, increasing susceptibility to infection, chronic diseases and inflammatory conditions. DAMPs and PAMPs become more abundant with age and combined with an age-dependent impairment in the mucous barrier function, there is increased exposure to these molecular patterns, contributing to a chronic inflammatory state (18-20). With aging, there is a reduction and delay in the production of type I interferons (IFN α and IFN β) due to an altered signaling pathway and a reduced number of circulating plasmacytoid dendritic cells, which are critical sources of type I interferons in early infection stages (21-24). This impacts the innate immune system in multiple ways, as type I interferons recruit and activate NK cells and macrophages (25). Furthermore, dendritic cells in aged mice show defective migration to draining lymph nodes and weakened ability to stimulate a CD8⁺ T cell-mediated cytotoxic response (26, 27). Aged dendritic cells also produce higher amounts of IL-6 and TNF α , contributing to a pro-inflammatory phenotype (28). Additionally, aging is associated with increased NF- κ B activation, leading to a rise in myeloid-derived suppressor cells (MDSCs) that inhibit T cell function (29). NK cells, crucial for modulating antimicrobial and antitumor activity, undergo remodeling with age, resulting in a profile characterized by lower cytotoxicity and reduced cytokine production (30). Overall, aging impacts the innate immune system, leading to chronic inflammation, reduced type I interferon production and decreased effectiveness of dendritic cells and natural killer cells, thus heightening susceptibility to infections and chronic diseases.

3.2 Immunosenescence, the adaptive immune response and B cell aging

The adaptive immune response can recognize a large variety of antigens through the diverse repertoire of receptors on B cells and T cells. This specificity enables the immune system to precisely target and eliminate pathogens, infected cells and neoplastic cells. After an initial encounter with a pathogen, the adaptive immune system establishes an immunological memory, allowing a rapid and efficient response upon subsequent exposures. B cells play a pivotal role in adaptive immunity. Upon antigen activation, they differentiate into memory cells

and plasma cells, which produce antibodies that bind to specific antigens, neutralizing or marking them for elimination.

Additionally, B cells process and present antigenic peptides on MHC class II molecules, activating T helper cells (CD4⁺ T cells) (31). B cells are substantially affected by the aging process, mainly due to decreased replenishment and reduced responsiveness upon activation (32). Similar to T cells, naïve B cells differentiate into memory subsets through continuous antigen stimulation. Aging leads to a decrease in B cell activating factor (BAFF) levels, which play an important role in the maintenance of naïve B cells, thereby diminishing the output of newly generated B cells. This results in the expansion of memory B cells with a limited variety of B cell receptors (33).

Additionally, aging causes a redistribution of B cell subsets in the periphery, significantly increasing the numbers of age-associated B cells (ABCs), which are enriched in autoimmune and autoinflammatory diseases (34, 35). Furthermore, older B cells exhibit a deficiency in the induction of activation-induced deaminase (AID), resulting in impaired immunoglobulin class switching and reduced somatic hypermutation, ultimately resulting in lower antibody avidity (36). During the COVID-19 pandemic, studies have shown that older adults exhibited reduced SARS-CoV-2 neutralization and lower frequencies of memory B cells after the mRNA vaccine compared to younger adults (37).

3.3 Hallmarks of T cell aging

One of the manifestations of immunosenescence is T cell aging. Several hallmarks have been previously identified that play a role in the development, maintenance and characteristic features of T cell aging (6). Thymic involution is an important primary cause of age-related changes in T cells, which reaches its peak at puberty, transforming the thymic lymphoid tissue into fatty tissue (38). Since the thymus is the organ responsible for generating a highly diverse yet selected T cell repertoire, thymic involution leads to a reduced de-novo generation of naïve T cells and a reduced peripheral T cell repertoire, favoring a compensatory clonal expansion of peripheral T cells (39). The clonal expansion of peripheral naïve T cells is associated with partial differentiation and contraction in the diversity of TCRs (40). Furthermore, the homeostasis of peripheral naïve T cells is also dependent on the recruitment to secondary lymphoid organs, where they are exposed to IL-7 produced by T zone fibroblastic reticular cells. Age-associated changes in the architecture of secondary lymphoid organs may compromise proliferation (41). The application of IL-7 to cancer patients for one week resulted in a significant rise in naïve T cells and an expansion of CD4⁺ and CD8⁺ T cells, showing an increased TCR repertoire in vivo (42).

T cell aging has furthermore been linked to the loss of proteostasis, which leads to insufficient destruction of misfolded proteins by proteolytic enzymes, promoting the development of senescent T cells (43). Autophagy, which describes the elimination of entire dysfunctional organelles, is also compromised in aged T cells. The accumulation of defective organelles, such as mitochondria, due to a decrease in autophagy contributes to a loss of mitochondrial quality control, linking autophagy and mitochondrial dysfunction in the context of T cell senescence (44). Further hallmarks of T cell aging are epigenetic alterations, affecting DNA methylation and the histone code. It has been shown that individuals with higher numbers of memory CD8⁺ T cells and reduced numbers of naïve CD8⁺ T cells display shorter leukocyte telomere lengths and an advanced DNA methylation age (45). A central element of immunosenescence is T cell senescence, characterized by an irreversible growth arrest in response to extensive replication and genotoxic, oxidative and inflammatory stress (46). Senescent T cells accumulate with increasing age, contributing to age-related diseases. Nevertheless, senescent T cells are not exclusively found in the elderly population. Research has shown that senescent T cells may also be identified in younger individuals, particularly in chronic viral infections, such as Cytomegalovirus, and in autoimmune diseases (47).

3.4 Molecular mechanisms of cellular senescence

Cellular senescence was first introduced in an article by Leonard Hayflick and Paul Moorhead in 1961, wherein they describe the phenomenon of an irreversible growth arrest in human fetal fibroblasts after about 50 replication cycles in cell culture (48). These findings were later coined as “replicative senescence” and have been causally linked to progressive telomere shortening since small amounts of telomeric DNA are lost with each round of replication, as the DNA replication apparatus cannot completely duplicate the ends of chromosomal DNA. While germline and certain hemopoietic stem cells possess the ability to extend telomeres by the activity of the enzyme telomerase, only low levels of telomerase are present in somatic cells (49). When shortened telomeres reach a critical length, telomere-capping structures are lost, triggering a DNA damage response (DDR) pathway, as the telomere ends resemble one-ended double-strand breaks (DSBs). In addition to the aging-associated and telomere-dependent replicative senescence, forms of stress-induced premature senescence have been described. Cells respond to oncogene activation by undergoing cellular senescence to halt tumorigenesis (50). Oncogene activation leads to hyperproliferation, DNA damage accumulation and reactive oxygen species (ROS) production, activating the DDR pathway (51, 52). Ultimately, persistent activation of the DDR pathway results in the activation and stabilization of p53 and transcriptional activation of the cyclin-dependent kinase (Cdk) inhibitor p21, which elicits a cell cycle arrest (53).

3.5 Development of senescent T cells in the context of T cell differentiation

Chronic antigen stimulation which occurs in the context of latent persistent infections (including cytomegalovirus (CMV), Epstein-Barr virus (EBV), varicella zoster virus (VZV), and herpes simplex virus (HSV)) and chronic persistent infections (including hepatitis C virus (HCV) and human immunodeficiency virus (HIV)) is described as a causal mechanism of T cell senescence. When T cells are induced to proliferate repeatedly throughout life, they differentiate and eventually reach a stage of telomere-dependent, replicative senescence (54). As T cells differentiate, their functional capacity and surface markers change. After positive and negative selection, naïve T cells are released from the thymus. They express the surface molecule CD45RA, yet they lack CD45RO, express the lymph-node homing markers CCR7 and CD62L and the co-stimulatory molecules CD27 and CD28, which interact with B cells and antigen-presenting cells (APCs) to initiate the activation of the T cell. Therefore, they can be described as CD45RA⁺, CD45RO⁻, CD27⁺, CD28⁺ and CCR7⁺ (55-58). Central and effector memory T cells are defined by their function and can be distinguished by their expression of CCR7, CD27 and CD28. Central memory T cells (T_{CM}) are defined as CD45RA⁻, CD45RO⁺, CD27⁺, CD28⁺ and CCR7⁺ (58). They can produce interleukin-2 (IL-2) and interferon γ (IFN γ), yet cannot produce effector and cytotoxic molecules mediating direct killing activity (59, 60). The cells are named “central memory” T cells for their ability to home to secondary lymphoid organs (55). Effector memory T cells (T_{EM}) can produce high levels of effector cytokines such as IL-4 and IL-5, tumor necrosis factor α (TNF α), IFN γ and cytotoxic molecules such as perforin and granzymes, allowing a rapid effector response (59, 60). Previous studies suggested a precursor-product relationship between T_{CM} and T_{EM} cells since T_{CM} cells can generate T_{EM} cells in vitro, yet not vice versa (59). Furthermore, a subset of T_{EM} cells is re-expressing CD45RA, called terminal effector (T_{TE}) or T_{EMRA} cells. These cells are described as CD45RA⁺, CD45RO⁻, CD27⁻, CD28⁻ and CCR7⁻, displaying short telomeres, a low proliferative capacity and strong cytotoxic and pro-inflammatory activity (61, 62). When T cells enter a state of irreversible growth arrest, they are, by definition, senescent. It is, however, not yet fully understood which changes late differentiated T cells such as T_{EMRA} undergo to reach a fully senescent phenotype.

3.6 The senescent phenotype and biomarkers for T cell senescence

In addition to the “gold-standard” characteristic of growth arrest, several other features are used to identify senescent T cells. However, there are no markers to date that exclusively define senescence. Morphologically, senescent fibroblasts often exhibit a flattened and enlarged cell body and a vacuolated and granulated cytoplasm (63). Senescence-associated- β -Galactosidase (SA- β -GAL) was one of the first biomarkers to identify senescent cells. This

histochemical marker is found in most senescent cells but is usually not detectable in presenescent, quiescent, or transformed and immortalized cells. Since then, SA- β -GAL staining has been widely used to detect senescence in a broad range of cells (64). It has been demonstrated that most subsets of human PBMCs, especially CD8⁺ T cells, exhibit increased SA- β -GAL activity with advancing age (65). Another marker includes the urokinase-type plasminogen activator receptor (uPAR), a cell-surface protein that is upregulated in senescent cells (66). In T lymphocytes, uPAR has been described as an activation marker (67).

The cyclin-dependent kinase inhibitor p16^{INK4a} (CDKN2A) has also been used as a surrogate marker for identifying senescent cells (68, 69). However, the use of p16^{INK4a} as a single marker does not specifically identify senescence since it is also expressed during the normal differentiation into effector T cells (70). It was shown that T lymphocytes accumulate the G1-regulating proteins p15 and p16 when they enter a state of replicative senescence in culture (71). It was further demonstrated that p53, p21, and p16 were increased in regulatory T cell (Treg) -induced senescent T cells (72). Furthermore, telomere shortening, loss of telomerase activity, DNA damage and genomic instability were observed in senescent cells (73, 74). T cells that have reached a state of replicative senescence in culture after multiple rounds of antigen-specific stimulation are resistant to apoptosis (75). Other studies have also demonstrated a reduced apoptosis in T cells from older mice compared to younger mice (76, 77). Therefore, it was proposed that the inability to remove senescent cells through apoptosis is one reason for the buildup of senescent cells with age (78). A key feature of senescent cells is the senescence-associated secretory phenotype (SASP), which may explain the role of senescent cells in age-related pathologies. The SASP contains a large number of soluble signaling factors, such as interleukins, chemokines and growth factors, secreted proteases and extracellular matrix (ECM) components, which can be directly or indirectly involved in the promotion of inflammation, linking senescence to “inflammaging” (79). Senescent T cells show a secretory phenotype similar to the SASP described in other cell types, producing and secreting pro-inflammatory factors after stimulation, such as IL-2, IL-6, IL-8, TNF α and INF γ (80). It was found that the expression of CD57 correlated with the production of Granzyme A, Granzyme B and Perforin, showing a highly cytotoxic potential of these T cells (81). Furthermore, a positive correlation between IFN- γ -production, age and the frequency of CD8⁺CD28⁻CD57⁺ T cells has been previously observed (82).

3.7 Surface markers of senescent T cells

Identifying senescent T cells through surface marker expression poses a challenge, with different studies employing various combinations of markers in flow cytometry analysis. The absence of consistent markers leads to considerable variability in defining senescent T cells. A characteristic feature of T cell senescence is the gradual loss of the co-stimulatory molecules

CD27 and CD28 (83). As individuals age, the expression of CD28 on CD8⁺ T cells progressively declines, with CD8⁺ T cells being more affected by this decline than CD4⁺ T cells (13, 15, 84, 85). Furthermore, T cells that show a loss of CCR7 and a re-expression of CD45RA accumulate with age (86). Senescent T cells express the markers Killer cell lectin-like receptor subfamily G (KLRG-1) and CD57. The co-inhibitory receptor KLRG-1 is expressed in NK cells and antigen-experienced T cells. The expression of KLRG-1 can be induced on naïve CD8⁺ T cells during viral infections in vivo, having undergone many cell divisions, leading to a state of proliferative arrest (87). A study comparing the percentage of KLRG-1⁺ CD8⁺ T cells in older (mean age 92.4 years) and younger individuals (mean age 32.3 years) revealed an increase in KLRG-1 with age (88). CD57 expression in T cells is related to a lack of proliferative ability, a history of repeated cellular divisions, and shorter telomeres (89). It was repeatedly shown that CD57 expression on CD8⁺ T cells increases with age (13, 15, 84). Previous studies have characterized senescent T cells based on their expression of CD57 and CD28, describing CD28⁺CD57⁺ T cells as senescent (13, 15).

The co-inhibitory receptor T-cell immunoreceptor with Ig and ITIM domains (TIGIT) has emerged as a novel marker for T-cell senescence. TIGIT expression was shown to be upregulated in CD8⁺ T cells from adults aged over 61 years. These TIGIT⁺ T cells from elderly donors exhibited a downregulation of CD28 and a high co-expression of the inhibitory receptor PD-1. They were susceptible to apoptosis and showed functional defects, as indicated by low TNF α , IFN γ and IL-2 production levels. Additionally, they displayed increased degranulation and higher production of Granzyme B and Perforin (90). Whether the cell surface inhibitory markers PD-1, LAG-3, TIM-3, CTLA-4 and CD160 can also be seen as senescence markers is still unclear (91). Studies indicate that elderly adults (65 years and older) exhibit increased levels of PD-1 and LAG-3 on CD8⁺ T cells compared to younger individuals (21-40 years), after peptide stimulation. No differences were observed in the expression of CD160 between young and old individuals (92). A higher expression of PD-1 was found in T_{CM} and T_{EM} T cells. T_{EMRA} cells, however, displayed a lower expression of PD-1 compared to T_{CM} and T_{EM} cells (93). Interestingly, another study revealed no differences in PD-1, CTLA-4 and TIM-3 expression in unstimulated CD8⁺ T cells between older subjects (mean age 86 years) and younger ones (mean age 34 years). After stimulation for three days, the older group exhibited a lower percentage of PD-1 and TIM-3 and a higher percentage of CTLA-4-expressing CD8⁺ T cells (94).

3.8 Comparing senescent T cells to T_{EMRA} and exhausted T cells

While T_{EMRA} cells display some senescent features such as shortened telomeres, a low proliferative capacity, high levels of ROS, DNA damage and a secretome resembling the SASP, their cell cycle block is in part reversible, thus distinguishing them from truly senescent

cells (70, 95, 96). Like T cell senescence, T cell exhaustion represents a form of T cell dysfunction. While sharing overlapping characteristics, such as defective proliferation, increased cell cycle arrest and accumulation in the tumor microenvironment, T cell senescence and T cell exhaustion are distinct processes, each characterized by specific development and molecular signatures, with the key difference lying in their potential for recovery of function (97). T cell exhaustion could be demonstrated in humans during viral, bacterial and parasitic infections and cancer. T cell exhaustion is a result of continuous antigenic stimulation, leading to a poor effector function and the expression of inhibitory receptors, such as PD-1, TIM-3, LAG-3, CTLA-4, TIGIT, B- and T-Lymphocyte attenuator (BTLA), CD244 and CD160 (98-104). Exhausted T cells exhibit reduced production of IL-2, IFN γ and TNF α , revealing their reduced effector function, contrasting the pro-inflammatory and cytotoxic secretory phenotype of senescent T cells (105). Senescent and exhausted T cells show a reduced proliferation. However, in exhausted T cells, the cell cycle arrest is reversible, as the blockade of the PD-1/PD-L1 inhibitory pathway can restore the ability to undergo proliferation (106). The irreversibility of cell cycle arrest is considered a key feature of senescence. However, this idea has been recently challenged, as a study has shown that the proliferative arrest of senescent CD4⁺ T cells could be reversed by blocking AMPK-TAB1-dependent p38 activation (107).

3.9 Metabolic alterations in senescent T cells

3.9.1 Role of mitochondria in energy metabolism

Mitochondria are dynamic organelles found in the cells of most eukaryotes that perform diverse and interconnected functions in the cell. They are crucial for cellular metabolism, as they produce adenosine triphosphate (ATP), the primary source of chemical energy. In addition to their role in energy metabolism, mitochondria are involved in signaling, cellular differentiation, cell growth, cell cycle control and apoptosis. Mitochondria comprise an outer and inner membrane, which are structurally and functionally distinct, as larger ions and molecules can permeate the outer membrane. In contrast, the inner membrane is highly impermeable and requires membrane transporters to transfer ions and molecules. The primary role of mitochondria is the production of ATP by aerobic respiration. The central steps in ATP production are the citric acid cycle, known as the Krebs Cycle and oxidative phosphorylation. Glycolysis is the metabolic pathway converting glucose to pyruvate. Following glycolysis, pyruvate is transported across the inner mitochondrial membrane and oxidizes to acetyl-CoA, NADH and CO₂. Acetyl-CoA enters the citric acid cycle and is progressively oxidized to CO₂ and reduced cofactors. Three NADH molecules and one FADH₂ molecule are produced, which serve as electron sources for the electron transport chain.

Additionally, one molecule of GTP is produced, which can be converted to ATP. During oxidative phosphorylation, ATP is generated using the energy released by the electron

transport chain (ETC) reactions. As electrons pass through the protein complexes of the ECR, an electrochemical gradient is formed along the inner membrane of the mitochondrion, creating a form of potential energy. As protons flow back into the mitochondrial matrix, ATP synthase catalyzes the phosphorylation of adenosine diphosphate (ADP) to ATP (108-110).

3.9.2 T cell immunometabolism

Immune cells can adapt their metabolism to meet their energy demands in various stages of differentiation and activation. The significant pathways involved in controlling immune metabolism are glycolysis, the tricarboxylic acid (TCA) cycle, fatty acid oxidation (FAO), fatty acid synthesis, glutaminolysis, amino acid synthesis and the pentose phosphate pathway (111). The differentiation of naïve T cells into effector and memory T cells requires a distinct regulation of their energy metabolism. Resting cells, such as naïve T cells, generate energy through OXPHOS using glucose-derived pyruvate or fatty acid oxidation (112). When naïve T cells recognize an antigen and receive a proper co-stimulation, they rapidly proliferate and acquire specialized effector functions. Resting cells mainly utilize nutrients to maintain cellular homeostasis. Effector T cells, however, incorporate nutrients in biomass to generate daughter cells. This switch from naïve to effector T cells is energetically demanding and requires metabolic reprogramming, favoring glycolysis over OXPHOS (113, 114). It was observed that CD28 co-stimulation, acting through phosphatidylinositol 3'-kinase (PI3K), promotes the surface expression of the glucose transporter GLUT-1 and increases glycolysis (115).

During aerobic glycolysis, glucose is metabolized to lactate despite the presence of oxygen, a process known as the Warburg effect. While less efficient at generating ATP than oxidative phosphorylation, this process provides glycolytic intermediates for synthesizing amino acids, nucleotides and lipids. It supplies a carbon source necessary to sustain proliferation and the production of inflammatory molecules (114, 116). A possible explanation as to why effector cells favor glycolysis rather than efficiently producing ATP via OXPHOS could be linked to citrate production after the breakdown of glucose. Citrate moves from the mitochondria and is converted to acetyl-CoA by cytosolic ATP Citrate Lyase (ACL). This enzyme can translocate from the cytoplasm to the nucleus and directly acetylate the histones of effector gene promoters (117). Memory T cells predominantly exist in a quiescent state and depend on fatty acid oxidation and OXPHOS for their metabolism (118, 119). This metabolic transition is crucial for their long-term survival, similar to the pathway used by naïve T cells. In contrast to naïve and effector T cells, memory T cells possess a greater mitochondrial mass and increased spare respiratory capacity (120).

In summary, T cells switch from OXPHOS and FAO to mainly aerobic glycolysis and glutaminolysis (a series of biochemical reactions converting the amino acid glutamine into α -ketoglutarate, which enters the TCA cycle) after an antigenic challenge. After successfully

resolving the challenge, the formed memory T cells and regulatory T cells switch back to OXPHOS and FAO again. During aging, this metabolic switch is disrupted by mitochondrial dysfunction, causing aged T cells to remain mainly glycolytic, maintaining an inflammatory phenotype (121).

3.9.3 Altered energy metabolism of senescent T cells

A key aspect of cellular senescence is metabolic dysregulation, which reflects a disrupted balance between glycolysis and oxidative phosphorylation. Initial studies on senescence indicated that fibroblastic cultures develop a glycolytic phenotype upon reaching replicative senescence (122). A different study compared the metabolic phenotype of human CD8⁺ T_{EMRA} cells (CD27⁺CD45RA⁺), which largely exhibit features of senescence, such as the expression of KLRG-1 and CD57, to naïve (CD27⁺CD45RA⁺), effector memory (T_{EM}; CD27⁺CD45RA⁻) and central memory (T_{CM}; CD27⁺CD45RA⁻) T cells. A metabolic flux assay analysis using Seahorse technology revealed a lower basal metabolic rate, reduced oxygen consumption and a lower spare respiratory capacity in T_{EMRA} and naïve T cells compared to T_{EM} and T_{CM} subsets, indicating that T_{EMRA} and naïve T cells rely less on OXPHOS. The extracellular acidification rate (ECAR), an indicator of glycolysis and lactic acid production, was elevated in T_{EM} and T_{EMRA} CD8⁺ T cells compared to T_{CM} and naïve T cells. These findings indicate that T_{EMRA} cells predominantly depend on glycolysis. However, the same study also investigated the expression of the glucose transporter GLUT-1 and observed that CD8⁺ T_{EMRA} T cells had the lowest expression of GLUT-1, suggesting a reduced ability to access extracellular glucose (123).

3.9.4 NAD⁺/NADH Balance and the role of CD38

A balanced ratio of NAD⁺ and NADH and an adequate supply of mitochondrial NAD⁺ are crucial for optimal cell function. Reduced NAD⁺ levels are closely tied to a decline in cellular and mitochondrial function associated with aging.

NAD⁺ acts as a cofactor in various oxidation-reduction pathways and in addition to energy metabolism, it is an essential cofactor for a number of enzymes (124). ATP production and maintenance of the mitochondrial membrane potential depend on NAD⁺ as a cofactor. NADH originates from glycolysis and the citric acid cycle within the mitochondria, while NAD⁺ is regenerated through reactions such as the oxidation of NADH by complex I (125). NAD⁺ can be synthesized “de novo” from tryptophan via the kynurenine pathway and from vitamin precursors such as nicotinic acid through the Preiss-Handler pathway. Besides liver tissue, most cells cannot directly convert tryptophan to NAD⁺. Liver tissue mainly converts tryptophan to nicotinamide (NAM), which is then released into the serum. Peripheral cells take up NAM to convert it into NAD⁺ through the NAM salvage pathway (124). Several NAD⁺-consuming

enzymes (CD38, poly (ADP-Ribose) polymerases (PARPs) and Sirtuins) regulate the overall NAD⁺ levels in cells (126).

CD38 is the primary NADase in cells, playing a crucial role in regulating NAD⁺ levels. This enzyme is also responsible for synthesizing the second messenger molecules cyclic ADP-ribose (cADPR), ADP-ribose (ADPR) and, to a lesser degree, nicotinic acid adenine dinucleotide phosphate (NAADP) (127). In recent years, CD38 has also gained prominence as a regulator of T cell activation and function. It has been shown that mature T cells display low levels of CD38, and its expression is enhanced after lymphocyte activation, leading to the hypothesis that CD38 is a marker of T cell activation (128). Moreover, CD38 is described as a marker of terminally exhausted T cells refractory to PD-1 blockade (129).

Furthermore, it has been reported that CD38 is linked to mitochondrial function. A recent publication in immunocompromised patients with uncontrolled CMV infection revealed that CMV-specific CD8⁺ T cells with metabolic dysregulation and mitochondrial stress highly express CD38. CD38 inhibition recovered the metabolism of these dysfunctional T cells (130). HEK 293 cells transfected with a CD38 plasmid showed a marked decrease in total respiratory capacity and a higher release of lactate, indicating that CD38 leads to mitochondrial dysfunction and a reliance on glycolysis. Furthermore, it could be shown in this study that CD38 degrades the NAD⁺ precursor Nicotinamide Nucleotide (NMN) in mouse tissue (131).

NAD⁺-depleting enzyme CD38 also plays an important role in cellular senescence. Studies showed that the expression and activity of CD38 increase in aging cells, establishing a connection between cellular senescence and the decline of NAD⁺ (132, 133).

3.9.5 Mitochondrial dysfunction in senescent T cells

Mitochondrial dysfunction is widely recognized as a hallmark of the aging process. This condition is characterized by mutations in mitochondrial DNA, decreased respiratory capacity, heightened production of ROS and diminished mitochondrial membrane potential (125, 134). Such alterations significantly impact both the morphology and functionality of mitochondria (6). Furthermore, mitochondrial dysfunction strongly correlates with the decline in T cell functionality. Desdín-Micó et al. compellingly illustrated that deleting mitochondrial transcription factor A (TFAM) in T cells replicated the mitochondrial dysfunction commonly associated with aging. Animals deficient in TFAM exhibited numerous signs of aging, encompassing neurological, metabolic, muscular and cardiovascular impairments. This study emphasizes the significance of mitochondrial function in T cells concerning the overall aging process and the development of age-associated diseases (135).

3.9.6 Mitochondrial oxidative stress

The accumulation of ROS derived from mitochondria drives senescence and age-related pathologies. Low levels of mitochondrial ROS are deemed physiological, as they function as important signaling molecules during cell cycle regulation and immune response, facilitating the activation, proliferation and differentiation of T cells (121). High levels of ROS, however, damage cellular structures, compromising cellular survival. Oxidative stress leads to DNA damage by causing base oxidation, single- and double-strand breaks and shortened telomeres, leading to cell cycle arrest and senescence via the DDR pathway (125). ROS are primarily formed in mitochondria as a byproduct of the reactions in the respiratory chain, mainly by complexes I, II and III, whose stability diminishes with age (136). Moreover, complexes I, III and IV can form supercomplexes, which enhance the activity of complex I and the efficiency of the electron transfer (137). The stability of these supercomplexes also decreases with age, contributing to increased ROS production (138). Mitochondrial and cytoplasmic ROS production, measured by MitoSOX and Dihydroethidium (DHE), was higher in T_{EMRA} CD8⁺ T cells than in less-differentiated T cell subsets (123).

3.9.7 Mitophagy

The balance between mitochondrial biogenesis and degradation through mitophagy is altered in aged T cells. Mitophagy occurs in response to cellular damage, which may arise from oxidative stress, hypoxia, loss of mitochondrial membrane potential and accumulation of unfolded proteins. This process is crucial for preserving mitochondrial quality by eliminating damaged mitochondria through autophagosomes (139). Through the two primary mechanisms of mitophagy, the PINK1 (PTEN-induced putative kinase 1)/Parkin-dependent pathways and the receptor-mediated pathway, mitophagy safeguards the cell against dysfunctional mitochondria. The diminished mitochondrial membrane potential in compromised mitochondria stabilizes PINK1 on the outer mitochondrial membrane. This stabilization prompts the recruitment of the E3 ubiquitin ligase Parkin, guiding damaged mitochondria into autophagosomes (121). The receptor-mediated pathway is activated in hypoxic conditions and during cellular differentiation (136). Several studies have investigated the relationship between mitophagy and aging. One study noted decreased mitophagy efficiency in aged CD4⁺ T cells, evidenced by the persistence of dysmorphic mitochondria and an increased presence of undegraded autophagosomes in the CD4⁺ T cells from older donors (140). A decreased autophagic activity was observed in CD8⁺ T cells of old donors (>60y). In this study, senescent CD8⁺ T cells, defined by the expression of CD57, displayed low basal autophagy levels compared to non-senescent CD57⁻ T cells (141).

3.9.8 Mitochondrial mass in CD8⁺ T cell subsets

The morphology of mitochondria in senescent cells differs significantly from that of healthy cells. Replicative and oncogene-induced senescence was associated with an increase in

mitochondrial mass (142-144). This growth is attributed to the buildup of dysfunctional mitochondria stemming from impaired mitophagy, the process responsible for degrading damaged mitochondria. Some authors suggest that an increase in mitochondrial mass could offset a decline in mitochondrial function and restore cellular energy levels (144). However, this observation does not apply to all cell types. It was observed that CD8⁺ T_{EMRA} cells display a lower mitochondrial content compared to CD4⁺ T_{EMRA} cells, which was associated with a lower mitochondrial membrane potential, reduced OXPHOS, higher mitochondrial ROS per mitochondrion and a more rapid acquisition of a senescent phenotype in the CD8⁺ subset (145). A separate study investigated different facets of mitochondrial dysfunction in CD8⁺ T_{EMRA} cells compared to naïve, T_{CM} and T_{EM} CD8⁺ T cells. Following stimulation with anti-CD3 antibodies and IL-2, it was observed that the T_{EMRA} subset showed a reduced number of mitochondria. The assessment of mitochondrial mass using mitochondria-specific dyes indicated that the T_{CM} and T_{EM} subsets exhibited the highest mitochondrial mass. In contrast, the T_{EMRA} subset displayed a considerably lower mitochondrial mass. Additionally, the mitochondrial content in T_{EMRA} CD8⁺ T cells was evaluated by examining the mitochondrial to nuclear DNA ratio, revealing the lowest ratio in the T_{EMRA} subset (123).

3.9.9 Altered Mitochondrial Dynamics

Mitochondria are interconnected organelles with constantly changing structures that engage in fission and fusion, altering their size, shape and position within the cell. During aging, inadequate mitochondrial dynamics contribute to the accumulation of dysfunctional mitochondria (146). Mitochondria are connected through mitochondrial fusion at the outer and inner membranes, facilitating the exchange of mitochondrial DNA and metabolites, which enhances overall mitochondrial function. Mitochondrial fission refers to the process in which a single mitochondrion divides into two (or more). It is essential for partitioning organelles during cell division, ensuring proper distribution of mitochondria and facilitating the removal of damaged mitochondria through autophagy (147). Elongated, enlarged and hyperfused mitochondria were observed in different models of cellular senescence (142, 148-150). Research demonstrated that the knock-down of FIS1, a protein involved in mitochondrial fission, increased reactive oxygen species, inducing senescence. In contrast, over-expression of FIS1 prevented the elongation of mitochondria and reversed the senescent phenotype, highlighting the role of mitochondrial fission in the development of senescence (150). Variations in mitochondrial dynamics have been noted among distinct T-cell subsets. Memory T cells relying on OXPHOS show fused mitochondrial networks, whereas glycolytic effector T cells show small, fragmented mitochondria (151, 152). PD-1 signaling prevents mitochondrial fission by downregulating the fission protein DRP1 (Dynamin-related protein1) (153). In addition, DRP1 is reduced in the tissues of aged mice (154, 155).

3.9.10 Impaired Mitochondrial Membrane Potential

In addition to increased mitochondrial mass and decreased respiratory capacity, dysfunctional mitochondria exhibit a low mitochondrial membrane potential ($\Delta\Psi_m$). The mitochondrial membrane potential is generated by Complexes I, III and IV and along with the proton gradient ΔpH , contributes to the transmembrane potential of hydrogen atoms necessary for the production of ATP. While transient changes in mitochondrial membrane potential are physiological, prolonged disruptions adversely impact cell viability (156). Mitochondrial membrane potential is closely linked to ROS production. A low mitochondrial membrane potential correlates with heightened ROS production since inefficient electron transport causes electron leakage, forming superoxides and various ROS (157, 158). It was previously shown that CD8⁺ T_{EMRA} cells (CD27⁻CD45RA⁺), which were stimulated with an anti-CD3 antibody, exhibited a reduced mitochondrial membrane potential compared to naïve, T_{CM}, and T_{EM} T cells, as assessed by the presence of the monomeric form of JC-1 (a cationic, fluorescent carbocyanine dye used as an indicator of mitochondrial membrane potential) in depolarized mitochondria (123).

3.9.11 Lactate dehydrogenase – a key enzyme in cellular metabolism

The accumulation of lactate has been discussed in recent years as a marker for aging, for example in the context of age-related neurological diseases, aging fibroblasts, cancer and fibrosis (159-161). Lactate levels are regulated by the differential expression of Lactate dehydrogenase (LDH) subunits. While LDH has been extensively studied in cancer cells, not much is known about the regulation in aged or senescent T cells.

LDH is a ubiquitous enzyme that catalyzes the coupled interconversion of pyruvate to lactate and nicotinamide adenine dinucleotide hydrogen (NADH) to nicotinamide adenine dinucleotide (NAD⁺). Pyruvate, the final product of glycolysis, is fed into the citric acid cycle, ultimately resulting in the production of ATP by oxidative phosphorylation. In anaerobic conditions, LDH converts pyruvate to lactate in a process known as lactic acid fermentation. This reaction is essential for cellular homeostasis, as it regenerates the levels of NAD⁺, which are needed for reaction steps during glycolysis. LDH facilitates the reverse reaction when lactate is transported in the liver as part of the Cori cycle. During gluconeogenesis, lactate is converted to pyruvate and back to glucose (162).

While it is widely accepted that low intracellular pO₂ levels result in the production and accumulation of lactate, many evidences suggest that lactate is produced in various cell types and tissues, even in the presence of ample oxygen (163-165). Furthermore, it was shown that glucose contributes to the citric acid cycle in tissues mostly indirectly via circulating lactate, demonstrating a link between mitochondrial metabolism and lactate (166). This led to multiple

hypotheses claiming that lactate formation succeeds glycolysis even in aerobic conditions and is an important carbon source for the citric acid cycle (167, 168).

While LDH is primarily localized in the cytoplasm, studies have also shown some activity in mitochondria and the nucleus (169, 170). LDH can attach to the outer side of the inner mitochondrial membrane, where it converts lactate to pyruvate for entry into the mitochondrion and subsequent processing in the citric acid cycle (168).

There are five known isoforms of LDH (LDH-1 to LDH-5). LDH-1 to LDH-5 are assembled as hetero- or homotetramers of the two enzyme subunits, LDH-A (M $\triangleq$ "muscle") and LDH-B (H $\triangleq$ "heart"), encoded by the *LDHA* and *LDHB* gene (171). The six isoforms are differentially expressed in different cell types. LDH-1 is comprised of four LDHB subunits (4H) and is the major isoform expressed in heart tissue. LDH-2 consists of three LDHB and one LDHA subunit (3H1M) and is the main isoform of the reticuloendothelial system and red blood cells. The LDH-3 isozyme has two LDHA and two LDHB subunits (2H2M) and is mainly expressed in lymphatic, brain and lung tissue. Isozyme LDH-4 consists of one LDHB and three LDHA subunits (1H3M) and is the primary isoform expressed in the kidneys. The LDH-5 isozyme has four LDHA subunits (4M) and is mainly expressed in the liver and skeletal muscle (172, 173). The metabolic characteristics of the isoenzymes are determined by the number of LDHA and LDHB subunits in the tetrameric complex. The LDHB subunit kinetically favors the conversion of pyruvate to lactate. The higher the number of LDHB subunits in an LDH isoform, the less likely the enzyme is to catalyze the forward reaction. Conversely, LDHA has a high affinity for pyruvate, making it efficient in catalyzing the conversion of pyruvate to lactate (174).

3.9.12 The role of *LDHA* and *LDHB* in T cells

A recent study investigated the LDH isoenzyme spectrum in murine T cells isolated from different lymphoid organs. It was revealed that thymic T cells express LDH-1 to LDH-5, while the T cells isolated from the spleen and lymph nodes express LDH-3, LDH-4, and LDH-5. Peripheral T cells expressed LDH-3, LDH-4 and LDH-5, with LDH-5 emerging as the dominant LDH isoenzyme. Furthermore, in murine naïve T cells, LDHB is the predominant LDH subunit at both mRNA and protein levels. However, upon stimulation targeting CD3 and CD28, *LDHA* became the dominant LDH subunit (175).

Similarly, in human T cells, TCR activation of naïve CD8⁺ T cells upregulates *LDHA* expression at the mRNA level, while it does not influence LDHB expression (176). Deleting *LDHA* and/or *LDHB* in murine T cells altered the LDH isoenzyme spectrum, resulting in opposite effects on glycolysis. The deletion of LDHA and the combined deletion of *LDHA* and *LDHB* reduced glycolysis compared to control T cells, as indicated by reduced glucose consumption, lactate production, and ECAR. Conversely, the deletion of *LDHB* resulted in heightened glycolysis compared to control T cells, showing increased oxygen consumption, lactate production and

ECAR. It was further demonstrated in vivo that both the deletion of *LDHA* and *LDHB* in murine CD4⁺ T cells compromises proliferation and survival in an antigen-specific manner. This effect was more pronounced in the *LDHA* cKO (conditional knockout) T cells than in *LDHB* cKO cells. The same study showed that after activation, *LDHA* cKO and *LDHA/LDHB* DKO (double knockout) CD4⁺ T cells displayed more cell death, delayed cell cycle progression, decreased cell surface activation markers, reduced cell size and lower DNA/RNA protein content. This effect, however, was not observed in *LDHB* cKO T cells, indicating that *LDHB* is not essential in T cell activation (175).

3.9.13 LDHB is essential for lactate utilization and mitochondrial metabolism

Evidence suggests that LDHA and LDHB have differential roles in mitochondrial metabolism. In a mouse model of lung cancer, the abrogation of LDHA led to decreased glycolysis and enhanced mitochondrial metabolism, as determined by an increase in citric acid cycle metabolites and an increase in oxidative phosphorylation (177). However, the inhibition of LDH in isolated mitochondria, where LDHB is localized, led to decreased mitochondrial respiration when cells were cultured in lactate, the primary substrate for LDHB (178). Inhibition of LDHB resulted in a decreased lactate uptake from the serum and reduced the basal, ATP-linked, and maximal OCR (179). Human CD4⁺ T cells overexpressing LDHB displayed higher respiratory activity compared to control T cells. This effect was more pronounced in the presence of lactic acid, while lactic acid treatment reduced respiration in T cells with regular LDHB expression. The increase in mitochondrial respiration was not accompanied by higher mitochondrial mass, which implies that LDHB overexpression leads to higher mitochondrial activity. A co-culture experiment with colorectal carcinoma spheroids in the same study showed that LDHB-overexpressing T cells preferentially migrated into the spheroids and secreted more cytotoxic effector molecules. Again, this effect was more pronounced when the co-culture was treated with lactic acid (180). Overall, these results suggest that LDHB mediates lactate utilization, contributes to oxidative phosphorylation, heightens mitochondrial activity and improves anti-tumor response, especially in the presence of lactic acid, which is abundant in the tumor microenvironment.

3.9.14 LDHB regulates telomere length by interacting with telomerase

Evidence from a study in pancreatic cancer cells suggests that LDHB contributes to telomerase activity independently of its metabolism-regulating functions by interacting with TERT (telomerase reverse transcriptase) and regulating the interaction of TERT with TERC (telomerase RNA component). A knockdown of LDHB inhibited telomerase activity and reduced telomere lengths, whereas the knockdown of LDHA did not affect telomere lengths. The LDHB knockdown also increased senescence-associated- β -Galactosidase activity, while the LDHA knockdown did not induce features of cellular senescence. These results indicate

that LDHB activity might protect cells from telomere shortening and replicative senescence (181).

3.10 Prevalence of senescent T cells in solid tumors and hematological malignancies and their prognostic impact

Emerging evidence highlights the role of aging T cells in the inhibitory tumor microenvironment (TME). It was observed that tumor-infiltrating T cells (TILs) have senescent characteristics at tumor sites in non-small cell lung cancer (NSCLC), multiple myeloma, ovarian cancer, breast cancer, AML and follicular lymphoma (182-186). Senescent T cells regulate the TME through their SASP, which actively influences other immune cells in the TME. Some contents of the SASP, such as IL-6, IL-8 and TNF, can induce premature senescence in T cells, while the secretion of IL-10 and TGF- β suppresses effector immune cells. In addition to establishing a suppressive TME, the senescent T cells can directly influence tumor growth, invasion and metastasis via the SASP (91, 187). Conversely, in vitro experiments have shown that tumor cells and regulatory T cells present in the TME can induce T cell senescence (188-191).

Numerous studies have shown a correlation between the prevalence of CD28⁻ CD8⁺ T cells and CD57⁺ CD8⁺ T cells, notably prevalent in the peripheral blood and tumor microenvironment of patients with solid tumors and hematological malignancies. Patients with lung cancer exhibited a higher proportion of senescent-like CD28⁻CD57⁺ CD8⁺ T cells in their peripheral blood, particularly among those with stage IV lung cancer (192). A high percentage of CD57⁺ CD8⁺ T cells in the blood of patients with gastric cancer, melanoma and renal cell carcinoma is positively correlated with lower overall survival (193-195).

In newly diagnosed AML patients, the percentage of CD8⁺ T_{EMRA} cells (CCR7⁻CD45RA⁺) and CD57⁺ CD8⁺ T cells were increased compared to healthy controls. AML patients in complete remission (CR) displayed lower numbers of senescent T cells than untreated AML patients (196). It could also be demonstrated that a co-culture of healthy control CD8⁺ T cells with AML blasts induced CD57 expression in T cells (197). In untreated early-stage, chronic lymphocytic leukemia (CLL) patients with an inverted CD4:CD8 ratio showed increased CD57⁺CD28⁻CD27⁻ T cells. This was associated with a shorter time to first treatment and reduced progression-free survival (198).

Multiple myeloma is characterized by the accumulation of malignant plasma cells in the bone marrow, forming a specific TME. Multiple myeloma primarily affects elderly individuals. T cells obtained from the bone marrow of myeloma patients displayed a significant increase of CD57⁺CD28⁻ CD8⁺ T cells compared to healthy controls, indicating an accumulation of a senescent T cell population in the myeloma bone marrow. Following treatment with unspecified immunomodulatory drugs and dexamethasone, the percentage of CD57⁺CD28⁻ CD8⁺ T cells was lower in treated patients than in those who were chemo-naïve (184). Interestingly, a

different study identifying senescent CD8⁺ T cell clones (KLRG-1⁺/CD57⁺/CD160⁺/CD28⁻) in myeloma patients found normal-for-age telomere lengths in these cells, suggesting telomere-independent and potentially reversible senescence (199). In addition, an early relapse in myeloma patients who underwent autologous stem cell transplantation was associated with high numbers of CD28⁻CD57⁺PD-1⁺ T cells (200).

3.11 Influence of senescent T cells on cancer immunotherapy

Cancer immunotherapy has emerged in recent years as a novel and effective form of treatment, harnessing the inherent ability of the immune system to detect and eliminate cancer cells. These include immune checkpoint inhibitors (ICIs) and adoptive cellular therapies (ACTs). As cancer immunotherapy depends on the immune system of the patient, immune dysfunction such as T cell senescence limits the efficacy of this treatment. Current approved checkpoint inhibitors target CTLA-4 (e.g., Ipilimumab), PD-1 (e.g., Nivolumab, Pembrolizumab), PD-L1 (e.g., Atezolizumab) and LAG-3 (Relatlimab) (201, 202). The effect of checkpoint inhibitors is associated with T cell activation through CD28 expression. As the loss of CD28 is a characteristic feature of senescent T cells, this limits immune checkpoint blockade. It was observed in melanoma patients that the loss of CD27 and CD28 and the expression of TIM-3 and CD57 on T cells could be associated with resistance to ICIs (203). Furthermore, a high number of CD28⁻CD57⁺KLRG-1⁺ CD8⁺ T cells in the peripheral blood of patients with advanced NSCLC is associated with a poor clinical response to PD-1 inhibition (204).

CAR-T cell and TCR-T cell therapy relies on functionally active T cells isolated from patients, reconstructed and expanded ex vivo to recognize specific antigens. CARs are fusion proteins consisting of an antigen-recognition domain derived from a monoclonal antibody and an intracellular signaling and costimulatory domain. In first-generation CARs, the intracellular signaling domains only consisted of CD3 ξ . In second-generation CARs, an additional co-stimulatory signal transduction domain (CD28 or 4-1BB) was added, while the third-generation CARs combine the two costimulatory domains CD28 and 4-1BB (205). Fourth-generation CARs secrete an immune modulatory molecule or cytokine after antigen recognition. Currently, fifth-generation “universal” CARs are being developed (206). At the time of this publication, seven commercially available CAR-T products targeting CD19 or B-cell maturation antigen (BCMA) have been approved by the FDA for large B-cell lymphoma, follicular lymphoma, mantle cell lymphoma, B-cell precursor acute lymphoblastic leukemia, and multiple myeloma (207, 208).

T cells isolated from heavily pre-treated cancer patients more frequently show a senescent phenotype, as described above. The patient's T cells have been exposed to the TME and have progressed toward terminal differentiation before isolation. A memory and stemness

phenotype, sustained proliferation and cytokine production of CAR T cells have been correlated with a favorable clinical response (12). In CLL-targeted CAR T cell therapies, the upregulation of genes involved in effector differentiation, exhaustion, apoptosis and glycolysis was observed in non-responders (209). CAR T cells with a central memory phenotype (CD27⁺CCR7⁺) were more frequent in DLBCL patients who had achieved complete remission (210).

4 Material and Methods

4.1 Material

4.1.1 Devices

Table 1: Devices used for cell culture

Device	Model	Manufacturer
Biological Safety Cabinet	Herasafe™	Thermo Fisher Scientific, Waltham, USA
Centrifuge	Heraeus Megafuge 1.0 R	Heraeus, Hanau, Germany
Centrifuge	Heraeus Megafuge 3.0 R	Heraeus, Hanau, Germany
Centrifuge	Sigma 1-14	Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany
Centrifuge	Heraeus Megafuge 40 R	Thermo Fisher Scientific, Waltham, USA
Centrifuge	Heraeus Megafuge 16 R	Thermo Fisher Scientific, Waltham, USA
CO ₂ free Incubator	B15	Heraeus, Hanau, Germany
CO ₂ Incubator	HERAcell®	Heraeus, Hanau, Germany
CO ₂ Incubator	Hfunction line	Heraeus, Hanau, Germany
Cytocentrifuge	Shandon Cytospin 4	Thermo Fisher Scientific, Waltham, USA
EasySep™ Magnet „The Big Easy“	18001	Stemcell™ Technologies, Vancouver, Canada
Flow cytometer	BD FACS Canto™ II Cell analyser	BD Biosciences, Franklin Lakes, USA
Flow cytometer	BD FACS Symphony A1 Cell Analyzer	BD Biosciences, Franklin Lakes, USA
Flow cytometer, sorting	BD FACS Aria™ III Cell Sorter	BD Biosciences, Franklin Lakes, USA
Magnet	MidiMACS™ Separator	Milteny Biotec GmbH, Bergisch Gladbach, Germany
Magnet (Dynabeads), 15 mL	Dynal MPC™-L	Thermo Fisher Scientific, Waltham, USA
Magnet (Dynabeads), 50 mL	Dynal MPC™-50	Thermo Fisher Scientific, Waltham, USA
Micropipettes single-channel	1000 µL; 200 µL; 100 µL; 10 µL; 2.5 µL	BRAND GmbH & Co. KG, Wertheim, Germany
Microscope	Light microscope Axiostar	Zeiss, Jena, Germany

Microscope	Wilovert Standard PH20 Inverted microscope	Helmut Hund GmbH, Wetzlar, Germany
Microscope EVOS	M5000	Thermo Fisher Scientific, Waltham, USA
Pipette controller	PIPETBOY acu 2	INTEGRA Biosciences GmbH, Biebertal, Germany
Agilent Seahorse Extracellular Flux Analyzer	XFp Analyzer	Agilent Technologies, Santa Clara, USA
Vortex mixer	Vortex genie 2	Scientific Industries inc, Bohemia, USA
Water bath	1003	GFL GmbH, Burgwedel, Germany

4.1.2 Chemicals and reagents

Table 2: Chemicals and reagents used for cell culture

Chemicals	Article number	Manufacturer
2-NBDG	11046	Cayman Chemical Company, Ann Arbor, USA
Ampuwa 1000 mL Plastipur®	1088811	Fresenius Kabi Deutschland GmbH, Bad Homburg, Germany
Antimycin A	A8674-25MG	Sigma-Aldrich, St. Louis, USA
Bovine Serum Albumin (BSA)	Sc-2323	Santa Cruz Biotechnology, Dallas, USA
Brefeldin A	ab120299	Abcam, Cambridge, UK
Calcein AM, 1 mg/mL in DMSO	80011-2	Biotium, Fremont, USA
CD8 microbeads, human	130-045-201	Milteny Biotec GmbH, Bergisch Gladbach, Germany
Dimethylsulfoxid (DMSO)	A994.2	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Dubecco's Phosphate Buffered Saline (1x)	D8537	Sigma-Aldrich, St. Louis, USA
Dynabeads™ Human-T-activator CD3/CD28	11131D	Thermo Fisher Scientific, Waltham, USA
efluor™ 640	65-0840-85	Thermo Fisher Scientific, Waltham, USA
Ethylendiamin-Tetraazetat (EDTA)	8040.3	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Etoposide 1000 mg/50mL HEXAL™	PZN-01546149	Hexal AG, Holzkirchen, Germany
FcR Blocking Reagent	120-000-265	Milteny Biotec GmbH, Bergisch Gladbach, Germany
Giemsa stock solution	T862.2	Carl Roth GmbH & Co. KG, Karlsruhe, Germany

Histopaque® 1007 (Ficoll)	10771-500ML	Sigma-Aldrich, St. Louis, USA
Hydrogen peroxide 3% solution	1799.99.99	Aug. Hedinger GmbH, Stuttgart, Germany
Ionomycin	10004974	Cayman Chemical Company, Ann Arbor, USA
May-Grünwald's solution	T863.1	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Methanol	0082.3	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
MitoSOX™ Red Mitochondrial Superoxide Indicator	M36008	Thermo Fisher Scientific, Waltham, USA
MitoTracker™ Deep Red FM	M22426	Thermo Fisher Scientific, Waltham, USA
MitoTracker™ Green FM	9074	Cell Signaling Technology, Danvers, USA
Monensin 1000x solution	00-4505-51	Thermo Fisher Scientific, Waltham, USA
OKT-3, Stock: 1 mg/mL	Orthoclone Okt-3®	Janssen-Cilag GmbH, Frankfurt/Main, Germany
Paraformaldehyde (PFA)	P6148	Sigma-Aldrich, St. Louis, USA
Perm/Wash Buffer (5x)	51-9008102	BD Biosciences, Franklin Lakes, USA
Phorbol-12-myristat-13-acetat (PMA)	P1585	Sigma-Aldrich, St. Louis, USA
Poly-D-Lysine	3439-100-1	R&D Systems, Minneapolis, USA
Proleukin®S (IL-2)	PZN-16771811	Clinigen Healthcare B.V., Schiphol, Netherlands
Rotenone	R8875	Sigma-Aldrich, St. Louis, USA
Trypanblue solution 0.4%	T8154-20ML	Sigma-Aldrich, St. Louis, USA
VectaMount Permanent Mounting Medium	H-5000-60	Vector Laboratories , Inc, Burlingame, USA
Viability™ 405/520 Fixable Dye	130-130-421	Milteny Biotec GmbH, Bergisch Gladbach, Germany

4.1.3 Consumables, cell culture media and buffers

Table 3: Consumables

Item	Item number	Manufacturer
14 mL Polystyrene round bottom test tube	352057	BD Biosciences, Franklin Lakes, USA
24-well cell culture plate	662160	Greiner Bio-One, Frickenhausen, Germany
96-well micro plate, flat bottom	655180	Greiner Bio-One, Frickenhausen, Germany

96-well micro plate, round bottom	650180	Greiner Bio-One, Frickenhausen, Germany
Cell culture flasks, 250 mL, 75 cm ²	658175	Greiner Bio-One, Frickenhausen, Germany
Cell culture tubes 15 mL	188271	Greiner Bio-One, Frickenhausen, Germany
Cell culture tubes 50 mL	227261	Greiner Bio-One, Frickenhausen, Germany
Coverslips 18x18 mm	190001818	IDL GmbH & Ko. KG, Nidderau, Germany
Cryos, 1 mL, Conical Bottom, Starfoot Base	123279	Greiner Bio-One, Frickenhausen, Germany
Cytosep™ Cytology Funnel	22045303	Thermo Fisher Scientific, Waltham, USA
Filtropur S 0,2	83.1826.001	Sarstedt AG & Co. KG, Nürnberg, Germany
LS-columns	130-042-401	Milteny Biotec GmbH, Bergisch Gladbach, Germany
Mr. Frosty™ Freezing Container	10110051	Thermo Fisher Scientific, Waltham, USA
Neubauer improved counting chamber (Depth 0.1 mm, 0.0025 mm ²)	717805	BRAND GmbH & Co., Wertheim, Germany
Pasteur pipettes	612361	Greiner Bio-One, Frickenhausen, Germany
Pipette tips 1000 µL TipOne®	S1111-6001	Starlab GmbH, Hamburg, Germany
Pipette tips, 20 µL	70.30.21	Sarstedt AG & Co. KG, Nürnberg, Germany
Pipette tips, 20 µL	70.3021	Sarstedt AG & Co. KG, Nürnberg, Germany
Pipette tips, 200 µL	70.760.012	Sarstedt AG & Co. KG, Nürnberg, Germany
Pipettes Cellstar™, 10 mL	607160	Greiner Bio-One, Frickenhausen, Germany
Pipettes Cellstar™, 2 mL	710180	Greiner Bio-One, Frickenhausen, Germany
Pipettes Cellstar™, 25 mL	760180	Greiner Bio-One, Frickenhausen, Germany
Pipettes Cellstar™, 5 mL	606180	Greiner Bio-One, Frickenhausen, Germany
Pipettes Cellstar™, 50 mL	768180	Greiner Bio-One, Frickenhausen, Germany
Polystyrene round-bottom tubes (5 mL) non-sterile	352008	BD Biosciences, Franklin Lakes, USA
Polystyrene round-bottom tubes (5 mL) sterile	352058	BD Biosciences, Franklin Lakes, USA
Reaction tubes, 1.5 mL	616201	Greiner Bio-One, Frickenhausen, Germany

Reaction tubes, 5 mL	72.701	Sarstedt AG & Co. KG, Nürnberg, Germany
Shandon Cytoslide™ (coated)	5991056	Thermo Fisher Scientific, Waltham, USA
Shandon Filter Cards	5991022	Thermo Fisher Scientific, Waltham, USA
Syringe BD Discardit™, 20 mL	300296	BD Biosciences, Franklin Lakes, USA

Table 4: Media and supplements

Article	Article number	Manufacturer
D-Glucose	G7021	Sigma-Aldrich, St. Louis, USA
Dulbeccos' Modified Eagle Medium (DMEM) Gibco	11960044	Thermo Fisher Scientific, Waltham, USA
Fetal Calf Serum (FCS), heat inactivated	F7524	Sigma-Aldrich, St. Louis, USA
HEPES Buffer 1M	882120	Lonza, Cologne, Germany
Human serum (AB), heat inactivated	-	Blood Transfusion Center Universitätsmedizin Mainz
L-Glutamine 200 mM	G7513	Sigma-Aldrich, St. Louis, USA
RPMI 1640 (- L-Glutamine)	R0883	Sigma-Aldrich, St. Louis, USA
RPMI 1640 (+ L-Glutamine -D- Glucose)	11879020	Thermo Fisher Scientific, Waltham, USA
Seahorse XF RPMI medium, pH 7.4	103576-100	Agilent Technologies, Santa Clara, USA
Sodium Penicillin/Streptomycin	P4333	Sigma-Aldrich, St. Louis, USA
Sodium pyruvate	S8636	Sigma-Aldrich, St. Louis, USA

Table 5: Buffers and solutions

Solution/Buffer solution	Composition
0.1% Trypan blue solution	0.1% Trypan blue in PBS
1% PFA solution	1 g PFA 100 mL PBS
4% PFA solution	4 g PFA 100 mL PBS
FACS buffer	Dulbecco's PBS 0.5% BSA
Freezing medium	10% DMSO (filtered sterile) in heat inactivated FCS
MACS buffer	Dulbecco's PBS 2% FCS

Red Blood Cell Lysis Buffer	1 mM EDTA 174 mM NH ₄ Cl 10 mM KHCO ₃ 0.1 mM EDTA pH = 7.3, sterile filtered
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Table 6: Composition of cell culture media

Medium	Composition
DMEM 10% FCS	10% heat inactivated FCS 1% L-glutamine 1% penicillin-streptomycin 2% HEPES Add to 500 mL DMEM, filtered sterile
RPMI 1640 10% AB-Serum	10% heat inactivated AB-Serum 1% L-glutamine 1% penicillin-streptomycin 2% HEPES Add to 500 mL RPMI1640, filtered sterile
RPMI 1640 10% FCS	10% heat inactivated FCS 1% L-glutamine 1% penicillin-streptomycin 2% HEPES Add to 500 mL RPMI1640, filtered sterile
Seahorse assay medium	9.7 mL XF RPMI medium, pH=7.4 0.1 mL 1M D-Glucose 0.1 mL Sodium pyruvate 0.1 mL L-glutamine

4.1.4 Kits and antibodies

Table 7: Kits

Kit	Components	Article number	Manufacturer
BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit I	10x Annexin V Binding Buffer FITC Annexin V Propidium Iodide Staining Solution	556547	BD Biosciences, Franklin Lakes, USA
Cell Event™ Senescence Green Flow Cytometry Assay Kit	Cell Event™ Senescence Green Probe (1000x) Cell Event™ Senescence buffer	C10841	Thermo Fisher Scientific, Waltham, USA
EasySep™ Human CD8 ⁺ T Cell Enrichment Kit	Human T cell enrichment cocktail D Magnetic Particles	19051	Stemcell™ Technologies, Vancouver, Canada

IntraPrep Permeabilization Reagent	Fixation agent (Formaldehyde) Permeability agent (Saponine)	A07803	Beckmann Coulter, Brea, USA
Seahorse FluxPaks	XFp Sensor Cartridges XFp Cell Culture Miniplates XFp Calibrant Solution	103022-100	Agilent Technologies, Santa Clara, USA
Seahorse XF Cell Mito Stress Test Kit	Oligomycin (3.314 mg) FCCP (3.304 mg) Rotenone/Antimycin A (3.311 mg)	103010-100	Agilent Technologies, Santa Clara, USA
TMRE-Mitochondrial Membrane Potential Assay Kit	1 mM TMRE (in DMSO) 50 mM FCCP in (DMSO)	ab113852	Abcam, Cambridge, UK

Table 8: Antibodies for Flow Cytometry

Anti-human (unless otherwise specified)

Antibody	Fluorophore	Clone	Article number	Manufacturer	Dilution
CD3	PE	SP-342	552127	BD Biosciences, Franklin Lakes, USA	1:100
CD3	APC	HIT-3a	300312	BioLegend, San Diego, USA	1:100
CD3	BB700	OKT-3	566818	BD Biosciences, Franklin Lakes, USA	1:100
CD4	APC-R700	OKT-4	566808	BD Biosciences, Franklin Lakes, USA	1:100
CD8	FITC	RPA-T8	555366	BD Biosciences, Franklin Lakes, USA	1:100
CD8	PE-Cy7	RPA-T8	301012	BioLegend, San Diego, USA	1:100
CD8	APC	RPA-T8	555369	BD Biosciences, Franklin Lakes, USA	1:100
CD8	PE	B9.11	A07757	Beckmann Coulter, Brea, USA	1:100
CD8	PE	MEM-31	21620084	ImmunoTools, Friesoythe, Germany	1:100
CD8	APC-H7	SK1	560179	BD Biosciences, Franklin Lakes, USA	1:100

CD25	FITC	M-A251	356106	BioLegend, San Diego, USA	2:100
CD27	APC	M-T271	130-093-186	Milteny Biotec GmbH, Bergisch Gladbach, Germany	3:100
CD27	PE-Cy7	O323	302838	BioLegend, San Diego, USA	1:100
CD28	Bv421	CD28.2	302930	BioLegend, San Diego, USA	1:100
CD28	FITC	CD28.2	555728	BD Biosciences, Franklin Lakes, USA	1:100
CD28	PE	CD28.2	555729	BD Biosciences, Franklin Lakes, USA	1:100
CD38	APC	240742	FAB2404A	R&D Systems, Minneapolis, USA	4:100
CD45RA	PE	HI100	304108	BioLegend, San Diego, USA	1:100
CD45RA	APC	HI100	550855	BD Biosciences, Franklin Lakes, USA	1:100
CD45RA	APC-H7	5H9	561212	BD Biosciences, Franklin Lakes, USA	1:100
CD45RA	V450	5Hg	560363	BD Biosciences, Franklin Lakes, USA	1:100
CD57	APC	NK-1	560845	BD Biosciences, Franklin Lakes, USA	1:100
CD57	BV421	NK-1	563896	BD Biosciences, Franklin Lakes, USA	1:100
CD57	efluor	TB01	48-0577-42	Thermo Fisher Scientific, Waltham, USA	1:100
CD57	PE-Cy7	TB01	25-0577-42	Thermo Fisher Scientific, Waltham, USA	1:100
CD57	Pacific blue	NC1	A74779	Beckmann Coulter, Brea, USA	1:100
CD57	Bv711	QA17A04	393328	BioLegend, San Diego, USA	2:100

CD62L	PE-Cy5	DREG-56	555545	BD Biosciences, Franklin Lakes, USA	1:100
CD87 (uPAR)	FITC	REA892	130-114- 849	Milteny Biotec GmbH, Bergisch Gladbach, Germany	2:100
CD98	PE	REA387	130-120- 137	Milteny Biotec GmbH, Bergisch Gladbach, Germany	3:100
CD127	PE	REA614	130-113- 976	Milteny Biotec GmbH, Bergisch Gladbach, Germany	1:100
CD152 (CTLA- 4)	PE	L3D10	349906	BioLegend, San Diego, USA	1:100
CD158b	FITC	CH-L	559784	BD Biosciences, Franklin Lakes, USA	5:100
CD160	PE	BY55	562118	BD Biosciences, Franklin Lakes, USA	2:100
CD223 (LAG-3)	Bv421	T47-530	565720	BD Biosciences, Franklin Lakes, USA	1:100
CD279 (PD-1)	APC	EH12.2H7	329908	BioLegend, San Diego, USA	1:100
Anti-rabbit IgG	PE	Poly4064	406421	BioLegend, San Diego, USA	5:100
CCR7	FITC	3D12	560548	BD Biosciences, Franklin Lakes, USA	1:100
Glut-1	Alexa Fluor 647	202915	566580	BD Biosciences, Franklin Lakes, USA	2:100
Granzyme B	APC	REA226	130-120- 703	Milteny Biotec GmbH, Bergisch Gladbach, Germany	5:100
Human Lactate Dehydrogenase B/LDHB Antibody	unconjugated	2057D	MAB9205	R&D Systems, Minneapolis, USA	1:100
IL-6	PE	MQ2- 13A5	130-100- 237	Milteny Biotec GmbH, Bergisch Gladbach, Germany	5:100

KLRG1	PE	13F12F2	12-9488-42	Thermo Fisher Scientific, Waltham, USA	2:100
KLRG1	BV605	14C2A07	368625	BioLegend, San Diego, USA	1:100:100
Lactate Dehydrogenase A/LDHA Antibody	unconjugated	2066C	MAB9158	R&D Systems, Minneapolis, USA	3:100
Perforin	FITC	delta G9	130-096-668	Milteny Biotec GmbH, Bergisch Gladbach, Germany	5:100
TIGIT	PE	REA1004	130-116-814	Milteny Biotec GmbH, Bergisch Gladbach, Germany	1:100
TIM-3	Alexa Fluor 488	344823	FAB2365G-100	R&D Systems, Minneapolis, USA	1:100

4.1.5 Cell lines

Table 9: Cell lines

SAOS 2 143	SAOS2/143 cell line is derived from the p53null SAOS2 sarcoma cell line by transfection with the human p53 gene harboring the mutation V143A (211) .
HeLa	The HeLa cell line is derived from human cervical adenocarcinoma.
OCI-AML3	OCI-AML3 is a human acute myeloid lymphoma cell line carrying mutations in NPM1 and DNMT3A genes. The cell line was kindly provided Catherine Wölfel (Mainz)
DAUDI	The DAUDI cell line is derived from the Burkitt's lymphoma of a 16 year old boy in 1967

The SAOS 2 413, OCI-AML3 and DAUDI cell lines were cultured in RPMI 10% FCS medium, and the HeLa cell line was cultured in DMEM 10% FCS medium.

4.1.6 Softwares

Table 10: Softwares used for evaluation and statistical analysis

Program	Manufacturer	Use
Prism 9.0	GraphPad, Boston, USA	Creation of graphs, statistical evaluation
FlowJo™ 10.7.1	BD Biosciences, Franklin Lakes, USA	Analysis of FACS data
ImageJ	Open Source, Developer: Wayne Rasband	Normalization of Seahorse data, analysis of Cytospin data
Wave 2.0	Agilent Technologies, Santa Clara, USA	Analysis of Seahorse data
Endnote X9	EndNote™, Clarivate Analytics, Philadelphia, USA	Citation

4.1.7 Use of AI Tools

Table 11: Use of AI Tools

AI Tool	Use	Reason	Where
ChatGPT	Revision of text drafts, translation	Improved readability	Throughout the thesis

4.2 Methods

4.2.1 Isolation of peripheral blood mononuclear cells (PBMCs)

Buffy coats from healthy donors were provided by the Transfusion Center of the University Medical Center Mainz. All blood donors gave written informed consent in accordance with the Declaration of Helsinki. The PBMCs were isolated from the peripheral blood by density gradient centrifugation. The blood was diluted 1:1 in PBS. 30 mL of diluted blood was carefully layered on 15 mL of Histopaque®. After centrifugation at 2200 rpm ($\pm$ 700 g) for 10 minutes at room temperature without brake, the PBMCs were isolated from the interphase between the blood plasma and the Histopaque®. The PBMCs were then harvested and washed three times with PBS. For immediate use, the remaining erythrocytes were removed by incubating the PBMCs with 5 mL of erythrocyte lysis buffer at room temperature for 3 minutes and washing the cells with PBS. To freeze the PBMCs, the cells were resuspended in fetal calf serum (FCS) containing 10% Dimethyl sulfoxide (DMSO), aliquoted at 1 mL with a cell density of 5×10^7 cells/mL, and stored at -80 °C for at least 24 hours before being transferred to the liquid nitrogen storage.

4.2.2 Isolation of CD8⁺ T cells by MACS[®] separation

CD8⁺ T cells were isolated from PBMCs by using the MACS[®] separation technique. For 10⁷ cells, PBMCs were resuspended in 80 µL MACS solution and 20 µL anti-CD8 Microbeads were added, mixed and incubated at 4°C for 15 minutes. The labeled cells were washed with 1 mL MACS buffer and resuspended in 500 µL MACS buffer. An LS MACS column was placed on a MACS separator and rinsed with 3 mL of buffer solution. The cells were then placed on the LS column and washed using buffer three times. Unlabeled cells ran through, and labeled cells were retained within the column, washed out with 5 mL of buffer and collected in a separate tube.

4.2.3 Isolation of CD8⁺ T cells by Easy Sep[™] Human CD8⁺ T Cell Enrichment

CD3⁺CD8⁺ T cells were isolated with the Easy Sep[™] Human CD8⁺ T Cell Enrichment Kit. PBMCs were prepared for a working concentration of 5 x 10⁷ cells/mL and a volume range of 0.5-8.5 mL. The cells were then placed in a 14 mL polystyrene round-bottom tube. 50 µL/mL of the enrichment cocktail was added to the sample and incubated at room temperature for 10 minutes. 150 µL/mL of the magnetic particles were vortexed for 30 seconds, placed on the sample and incubated for 5 minutes at room temperature. The suspension was brought to a volume of 5 mL with MACS buffer. The falcon was placed on “The Big Easy” Magnet and incubated for 5 minutes. After incubation, the magnet was picked up and the enriched suspension was poured off continuously into a new tube.

4.2.4 Restimulation and culture of human T cells

PBMCs and isolated CD8⁺ T cells were cultured and expanded by stimulation (weekly or twice weekly) with anti-CD3/anti-CD28 microbeads and IL-2 or OKT-3 and IL-2. The cells were collected in conical 50 mL tubes. Before every restimulation, the microbeads were magnetically removed by placing the tube in a magnet for three minutes. The cells were then transferred to a new conical tube while the beads remained on the wall of the previous tube. After centrifugation at 1500 rpm ($\pm$ 300g) for 5 minutes, the medium was decanted and the cells were resuspended in fresh RPMI medium with 10% AB-serum at a density of 0.5 x 10⁶ cells/mL in a 24-well plate. T cells were stimulated by adding 2 µL anti-CD3/anti-CD28 Dynabeads per 1 x 10⁶ cells and 600 U/mL IL-2. For cell culture with OKT-3, the cells were equally prepared and stimulated with 30 ng/mL OKT-3 and 600 U/mL IL-2. The cells were then cultured in the incubator at 37°C and a CO₂ concentration of 5 %.

4.2.5 Flow cytometry – Surface marker staining

5 x 10⁵ – 1 x 10⁶ freshly isolated PBMCs were placed in non-sterile 5 mL polystyrene round-bottom tubes and washed with PBS twice. Cells were stained with fluorochrome-coupled antibodies (see Table 8) for 15 minutes at room temperature in the dark, washed once with PBS and resuspended in 200 µL 1% PFA solution or 200 µL PBS for immediate use.

4.2.6 Flow cytometry - Data acquisition and analysis

Data was acquired on the BD FACS Canto™ II Cell analyzer and the BD FACS Symphony™ Cell analyzer. Unstained controls were used to define non-specific background staining and gate the populations accordingly. Fluorescence spillover was minimized by compensation. The data was analyzed using the FlowJo™ software. All cells were gated sequentially for lymphocytes first, then for single cells, then for CD8⁺ cells, and lastly for CD28⁺CD57⁻, CD28⁻CD57⁻, CD28⁺CD57⁺ and CD28⁻CD57⁺ cells.

4.2.7 Cell sorting

Flow cytometry cell sorting allows for the separation of cell subsets within a heterogeneous population based on the expression of intra- or extracellular structures labeled with fluorescent markers. This method involves capturing individual cells in liquid droplets, providing them with an electric charge and sorting them into sterile receiving tubes for further analysis.

After PBMC isolation and MACS separation for CD8⁺ T cells, the cells were resuspended in 1×10^6 cells/mL RPMI 1640 medium with 10% AB serum and incubated at 37°C overnight. The next day, $2-3 \times 10^7$ cells were washed and placed into a sterile 15 mL cell culture tube. The cells were stained with anti-CD8 PE-Cy7, anti-CD28 PE, and anti-CD57 Pacific blue (7 μ L each) and incubated for 15 minutes at room temperature in the dark. The cells were washed once and resuspended in MACS buffer at 1×10^7 cells/mL. Single-stained and unstained controls were prepared in sterile 5 mL round-bottom tubes. Cell sorting was performed in the BD FACS Aria™ III Cell Sorter. The cells were gated for CD8⁺CD28⁺CD57⁻, CD8⁺CD28⁻CD57⁻, CD8⁺CD28⁺CD57⁺ and CD8⁺CD28⁻CD57⁺ and sorted into sterile 5 mL tubes containing 1 mL RPMI 1640 medium with 10% AB serum. After sorting, the cells were counted, washed and resuspended in the medium for further experiments.

4.2.8 Proliferation - Cell Counting and Population Doubling Levels

On the day of restimulation, the number of viable cells was estimated by trypan blue exclusion test. 50 μ L of the cell suspension was mixed with 50 μ L trypan blue to exclude non-viable cells. The cells were then transferred to the Neubauer counting chamber and counted under the microscope in each of the four quadrants.

The cell count was determined using the following formula:

$$\text{Cell concentration (1x10}^6\text{/mL)} = \frac{Q1 + Q2 + Q3 + Q4}{4 \times 100} \times \text{Dilution}$$

The Population doubling levels (PDLs) were calculated with the following formula:

$$\frac{\log_{10}(\text{number of cells after expansion}) - \log_{10}(\text{number of cells seeded})}{\log_{10}(2)}$$

The cumulative population doubling levels were obtained by adding the values from each round of stimulation.

4.2.9 Proliferation - efluor™670 Proliferation Assay

The cell proliferation dye efluor™ 670 was used to monitor cell division *in vitro*. This fluorescent dye can penetrate the cell membrane and bind to intracellular proteins containing primary amines. During cell division, the dye is evenly distributed to the daughter cells and the fluorescent signal decreases with each division.

A working solution for efluor™ 670 was prepared by diluting the stock solution (5 mM) to 10 μ M with PBS. Freshly isolated PBMCs were washed twice with PBS and resuspended in 450 μ L PBS. 50 μ L of the efluor™ 670 working solution was added to the PBMCs to a final concentration of 1 μ M. The cells were incubated for 5 minutes at room temperature in the dark and washed. The sample for Day 0 was stained for anti-CD8 FITC, anti-CD28 PE and anti-CD57 PE-Cy7 and analyzed via flow cytometry the same day. The remaining PBMCs were resuspended in RPMI 1640 + 10% AB-Serum at 1×10^6 cells/mL, stimulated with OKT-3 (30 ng/mL) and incubated at 37°C. After three and five days of stimulation, the PBMCs were harvested, surface staining was performed and the cells were analyzed using flow cytometry.

4.2.10 Measurement of early and late-stage apoptosis

The Annexin V/Propidium iodide protocol is widely used to discriminate between viable, early apoptotic and late apoptotic cells. Annexin V binds to phosphatidylserine expressed on the intracellular leaflet of the plasma membrane in healthy cells. During apoptosis, phosphatidylserine translocates to the outer leaflet and can be labeled by Annexin V to identify apoptotic cells. In late apoptotic and necrotic cells, the integrity of the plasma membrane decreases, which allows the fluorescent propidium iodide (PI) to cross the membrane and stain DNA in the nucleus. Therefore, double positive cells for Annexin V and PI are late apoptotic, whereas a single Annexin V positivity identifies early apoptotic cells.

Freshly isolated, unstimulated PBMCs were harvested and washed with PBS. Then, they were incubated with 1 μ L anti-CD8 APC, 1 μ L anti-CD28 BV421, 1 μ L anti-CD57 PE-Cy7, 5 μ L Annexin V, and 2 μ L Propidium iodide for 15 minutes at room temperature. Cells were washed and resuspended in 150 μ L binding buffer. The samples were analyzed by flow cytometry. The experiment was repeated after 5 days of stimulation with 30 ng/mL OKT-3 and 600 U/mL IL-2.

4.2.11 Measurement of Granzyme B and Perforin and IL-6

The serine protease Granzyme B and the glycoprotein Perforin play an essential role in the cytotoxicity of CD8⁺ T cells, are found in cytotoxic granules and are released together after an adequate stimulus. Perforin can polymerize and form pores in the membranes of target cells, disrupting cellular homeostasis and allowing Granzyme B to enter the target cell. IL-6

production in T-cells has been associated with chronic inflammation. After 48 hours in culture, 1×10^6 PBMCs for the measurement of Granzyme B, Perforin, and IL-6 each were harvested and resuspended in 400 μ L RPMI 1640 + 10% AB-Serum. The PBMCs were subsequently stimulated with 2 μ L anti-CD3/anti-CD28 beads. A positive control was stimulated with 50 ng/mL PMA and 1 μ g/mL Ionomycin. Brefeldin A (10 μ g/mL) was added to the samples and the cells were seeded in a round bottom 95-well plate and incubated at 37°C for 5 hours. After incubation, the plate was centrifuged for 5 minutes at 1500 rpm, and the supernatant was decanted. The cells were resuspended in 200 μ L FACS buffer (0.5% BSA in PBS), and antibodies for surface staining were added. After 30 minutes of incubation in the fridge, the plate was centrifuged and washed 1x with FACS buffer. 200 μ L of 4% PFA was added, and the plate was incubated for 15 minutes at room temperature. The plate was then centrifuged at 1000 rpm for 5 minutes. The PBMCs were washed twice with FACS buffer and resuspended in 400 μ L FACS buffer. The plate was covered in foil and stored in the fridge overnight. The next day, the plate was centrifuged at 1000 rpm, and the supernatant was decanted. The PBMCs were resuspended in 400 μ L Perm/Wash buffer and incubated for 15 minutes at room temperature. After centrifugation, 3 μ L anti-Granzyme B APC, 5 μ L anti-Perforin FITC, and 5 μ L anti-IL-6 PE were added to the respective samples and incubated for 30 minutes in the fridge. The cells were washed once with Perm/Wash and resuspended in 400 μ L PBS. The samples were immediately analyzed via flow cytometry.

4.2.12 Degranulation assay

CD107a (also known as LAMP-1) is a transmembrane protein present in lysosomes and is widely used as a marker of degranulation in lymphocytes. CD107a is found on the membrane of the vesicles containing highly cytotoxic granules such as Granzyme B and Perforin. During degranulation, the membranes of the vesicles merge with the membrane of the T cell, and CD107a is consequently exposed to the cell's surface.

After 48 hours of stimulation with anti-CD3/anti-CD28 beads and IL-2, 1×10^6 PBMCs were washed, resuspended in fresh RPMI 1640 10% AB-Serum medium (0.5×10^6 /mL) and stimulated with 4 μ L/ 1×10^6 cells anti-CD3/anti-CD28 beads. The control was supplemented with 50 ng/mL PMA and 1 μ g/mL Ionomycin. 5 μ L anti-CD107a PE-Cy5 was added to both conditions. The cells were incubated for 1 hour at 37°C and cultured for another 5 hours at 37°C in the presence of 1 μ L/mL Monensin. After incubation, the samples were stained with anti-CD8 PE-Cy7, anti-CD28 FITC, and anti-CD57 BV421. Cells were analyzed via flow cytometry.

4.2.13 Ex vivo measurement of Beta-Galactosidase activity

The activity of the Beta-Galactosidase enzyme was measured using the CellEvent™ Senescence Green Flow Cytometry Assay Kit. Beta-galactosidase, a hydrolase enzyme located in lysosomes, catalyzes the conversion of Beta-galactosides into monosaccharides. In this assay, a beta-galactosidase-specific substrate is used. After the enzyme hydrolyzed the substrate, the hydrolyzed substrate stays inside the cell and emits a fluorogenic signal, allowing for detection via flow cytometry.

For ex vivo measurement of Beta-Galactosidase activity, 1×10^6 freshly isolated PBMCs were stained for anti-CD3 PE, anti-CD8 APC, anti-CD28 BV421 and anti-CD57 PE-Cy7. Further 1×10^6 cells were stained for anti-CD3 APC, anti-CD8 APC-H7, anti-CD27 PE, and anti-CD45RA V450. After a 15-minute incubation at room temperature, the cells were washed with FACS buffer and resuspended in 100 μ L PFA 1%, followed by a 10-minute incubation at room temperature. The cells were then washed to remove residual PFA. The CellEvent™ Senescence Green Probe (1000x) was diluted to a 1:500 working solution in CellEvent™ Senescence buffer. The cells were resuspended in 100 μ L of the working solution and incubated for 90 minutes at 37°C, protected from light and without exposure to CO₂. After incubation, the cells were washed and resuspended in 200 μ L FACS buffer for subsequent flow cytometric analysis.

4.2.14 Induction of senescence in T cells and Beta-Galactosidase Assay

To evaluate the senescence-inducing potential of different agents, freshly isolated PBMCs were resuspended in RPMI medium supplemented with 10% AB serum at 1×10^6 cells/mL. The cells were stimulated with 30 ng/ml OKT-3 and 600 U/mL IL-2. Separate groups of cells were treated with the following agents individually: 1 μ M Rotenone/Antimycin A, 5 μ M Etoposide, and 20 μ M Etoposide. Additionally, further cells were cultured in tumor-conditioned media. The conditioned media was collected from SAOS2/143 cells (incubated in RPMI 10% AB serum for 5 days at 37°C, diluted 1:10) and OCI-AML3 cells (incubated in RPMI 10% AB serum for 2 days at 37°C diluted 1:5). Cells in the tumor conditioned media were also stimulated with OKT-3 and IL-2 as described above. A negative control, receiving only stimulation without additional treatment, was included. After 48 hours of incubation at 37°C, the media from all experimental conditions were removed by centrifugation. The cells from each condition were washed twice with PBS to remove residual agents. The cells were then resuspended in fresh RPMI 10% AB serum with 30 ng/mL OKT-3 and 600 U/mL IL-2 and incubated for 72 hours at 37°C. After this incubation period, the Beta-Galactosidase assay was performed as previously described.

4.2.15 Expression of the Urokinase plasminogen activator receptor (uPAR) in untreated and senescence-induced T cells

The Urokinase receptor (uPAR; CD87) was measured on the cell surface of freshly isolated PBMCs. For this surface staining, untreated cells were stained for CD8, CD28, CD57, CD45RA, and CD87 and analyzed by flow cytometry. PBMCs were cultured in RPMI medium supplemented with 10% AB serum at a concentration of 1×10^6 cells/mL to evaluate the effect of senescence-inducing agents. PBMCs were stimulated with 30 ng/ml OKT-3 and 600 U/mL IL-2. Cells were individually treated with 5 μ M Etoposide, 20 μ M Etoposide, supernatant of SAOS2/143 cells (incubated in RPMI 10% AB serum for 5 days, diluted 1:10) and supernatant of OCI-AML3 cells (incubated in RPMI 10% AB serum for 2 days diluted 1:5). The treated PBMCs underwent medium replacement with fresh RPMI 10% AB serum after 48 hours. Cells were restimulated and cultured for an additional 72 hours before repeating the staining.

4.2.16 2-NBDG uptake assay

The fluorescent D-Glucose analog 2-NBDG is used to quantify glucose uptake through glucose transporters (GLUTs) in living cells. For every measurement, 1×10^6 PBMCs stimulated with 30 ng/mL OKT-3 for 48 hours were transferred in a polystyrene round-bottom tube and washed two times with PBS. The cells were stained with anti-CD8 PE-Cy7, anti-CD28 PE and anti-CD57. After incubation for 15 minutes at room temperature, the cells were resuspended in 200 μ L RPMI 1640 without D-Glucose and 1 μ L of 2-NBDG, diluted 1:10 in DMSO, was added to the cells. The cells were incubated at 37°C for specific time points of 15, 30 and 45 minutes to monitor the kinetics of 2-NBDG uptake. After each time point, the cells were washed once with PBS to stop the reaction and remove excess 2-NBDG. The cells were resuspended in 200 μ L PBS and analyzed via flow cytometry.

4.2.17 Detection of mitochondrial ROS

The measurement of mitochondrial ROS was performed using the MitoSOX™ Red superoxide indicator. This indicator is a derivative of dihydroethidium, which can be oxidized by reactive species and produce a fluorescent signal. MitoSOX™ Red accumulates in mitochondria and can be used to measure ROS produce by mitochondria.

The assay was performed on the day of PBMC isolation and after 48 hours of stimulation with OKT-3 (30 ng/mL). A positive control was treated with 200 μ M H₂O₂ for 20 minutes. 1×10^6 PBMCs were washed, treated with 1 μ M MitoSOX™ Red and incubated at 37°C for 30 minutes. Cells were then stained with anti-CD8 APC, anti-CD28 FITC, and anti-CD57 PE-Cy7, incubated for 15 minutes in darkness, washed and resuspended in 200 μ L of PBS for analysis in the flow cytometer.

4.2.18 TMRE assay

TMRE is a fluorescent, positively charged dye accumulating in active mitochondria because of its negative charge. Therefore, it can be used to determine the membrane potential of mitochondria.

The assay was performed on the day of PBMC isolation and after 48 hours of stimulation with OKT-3 (30 ng/mL) and IL-2 (600 U/ml). Treatment with 20 μ M FCCP for 10 minutes at room temperature was used as a negative control, as FCCP is an uncoupler of oxidative phosphorylation and eliminates the mitochondrial membrane potential. 1×10^6 PBMCs were incubated with 50 nM TMRE and incubated at 37°C for 30 minutes. The cells were stained with anti-CD8 APC, anti-CD28 FITC, and anti-CD57 Bv421, resuspended in 200 μ L PBS and analyzed by flow cytometry.

4.2.19 Measurement of mitochondrial mass and membrane potential

The carbocyanine-based dyes MitoTracker™ Green and MitoTracker™ Deep Red are cell-permeant reagents that label mitochondria in live cells. This allows for an analysis of the mitochondrial characteristics of cells by flow cytometry. The intensity of MitoTracker™ Deep Red is dependent on the mitochondrial membrane potential. Therefore, we used the combined staining of cells with MitoTracker™ Deep Red and MitoTracker™ Green to determine depolarized mitochondria in T cells. For this measurement, RPMI 1640 medium was warmed to 37°C and used to prepare a working solution for a combination of 10 nM MitoTracker™ Deep Red and 50 nM MitoTracker™ Green. Freshly isolated, unstimulated PBMCs were washed with PBS, resuspended in the MitoTracker™ working solution and incubated at 37°C for 30 minutes. After incubation, ice-cold FACS buffer was added to the cells before washing them twice with PBS. The cells were labeled with the surface markers anti-CD8 APC-H7, anti-CD28 PE and anti-CD57 Pacific blue and incubated at 4°C for 20 minutes. After incubation, the cells were washed once, resuspended in 200 μ L PBS and immediately analyzed by flow cytometry.

4.2.20 Seahorse XF Cell Mito Stress Test

The Agilent Seahorse XF Cell Mito Stress Test was used to compare the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of CD8⁺ T cell subsets. During the assay, modulators of respiration were added to the samples to investigate important parameters of mitochondrial function. After measuring the basal respiration, Oligomycin, an inhibitor of the ATP-Synthase, was injected, reducing mitochondrial respiration. This decrease in OCR was used to calculate ATP production. The uncoupling agent FCCP was added, resulting in an uninhibited flow of electrons through the electron transport chain. The OCR reached its maximum and was used to measure the spare respiratory capacity, which signifies the cell's ability to respond to an increased energy demand. The last injection was composed of a mixture of Rotenone, a complex I inhibitor and Antimycin A, a complex III inhibitor. This

combination inhibits mitochondrial respiration and enables the calculation of non-mitochondrial respiration.

CD8⁺ T cells were sorted into the four subpopulations, taken in RPMI 1640 + 10% AB serum and stimulated with 30ng/mL OKT-3 and 600 U/mL IL-2 and incubated at 37°C for 48 hours. The day before the assay, the sensor cartridge was hydrated by filling each well with 200 µL XFp calibrant and each outside chamber with 400 µL XFp calibrant. The cartridge was placed in a 37°C non-CO₂ incubator overnight. On the day of the assay, an XFp Cell Culture Miniplate was coated by adding 50 µL Poly-D-Lysine to each well to allow T cell adherence. The coated plate was incubated for 1 hour at room temperature, the Poly-D-Lysine was then aspirated and the plate was washed with 200 µL of sterile water. The plate was left to dry under the hood for 30 minutes. 80 µL of Seahorse assay medium warmed to 37°C were added to each well and incubated for 1 hour in a non-CO₂ incubator. In the meantime, the CD8 T cell subsets were harvested and counted. 3×10^5 cells were centrifuged and washed once with a prewarmed assay medium. The assay medium was removed entirely and the cells were adjusted to a concentration of 3.75×10^6 cells/mL. The assay medium in the wells of the Cell Culture Miniplate was aspirated. 40 µL of the cell suspension were added each to the wells B-G. The wells A and H were filled with 40 µL assay medium. The plate was centrifuged for 1 minute at 200 g (~960 rpm). 140 µL assay medium was added to each well and 320 µL XFp Calibrant was added to the outside chamber. The plate was incubated at 37 °C in a non-CO₂ incubator for 1 hour.

Meanwhile, the working solutions for 1.5 µM Oligomycin, 2 µM FCCP, and 0.5 µM Rotenone/Antimycin A were prepared. The injection ports of the sensor cartridge were loaded with 20 µL Oligomycin, 22 µL FCCP and 25 µL Rotenone/Antimycin A. After calibrating the Seahorse XFp Analyzer with the sensor cartridge, the Cell Culture Miniplate was placed in the Analyzer and the assay was performed. Once the assay was completed, the cells were stained with 1 µL 1:10 diluted Calcein, incubated for 15 minutes in darkness and imaged at 4x magnification using the EVOS microscope. The ImageJ software counted cells and the result was used to normalize the cell numbers. The assay was analyzed with the Seahorse Wave 2.0 Desktop software.

4.2.21 Intracellular Measurement of Lactate Dehydrogenase A (LDHA) and Lactate Dehydrogenase B (LDHB) in T Cells by Flow Cytometry

The intracellular expression of Lactate Dehydrogenase A (LDHA) and Lactate Dehydrogenase B (LDHB) in T cells was assessed by flow cytometry using a two-step staining protocol with a monoclonal LDHA antibody, a monoclonal LDHB antibody and a PE-labeled secondary antibody. The DAUDI cell line was used as a positive control for LDHA expression. HeLa cells were used as a positive control for LDHB expression.

For the measurement of LDHA, 1×10^6 freshly isolated PBMCs were stained with surface markers for CD8, CD28, CD57, and CD45RA. For the measurement of LDHB, 1×10^6 freshly isolated PBMCs were stained with surface markers for CD3, CD4, CD8, CD28, CD57, CD27, CD45RA and KLRG1. Cells were incubated for 15 minutes at room temperature, followed by a wash with PBS. For fixation, 100 μ L of formaldehyde-containing Reagent 1 (IntraPrep Permeabilization Reagent, Beckman Coulter) was added and cells were incubated for 15 minutes. After a subsequent wash, 100 μ L of Reagent 2, a saponin-containing solution, was added to permeabilize the cells. Cells were incubated with Reagent 2 for 5 minutes without vortexing, allowing passive diffusion.

To block non-specific binding, 20 μ L of FcR blocking reagent was added to the permeabilized cells, followed by a 10-minute incubation. Without washing out Reagent 2 or the blocking reagent, 3 μ L of the unconjugated primary LDHA antibody and 1 μ L of the unconjugated primary LDHB antibody were added to separate tubes and the cells were incubated in darkness for 30 minutes to allow specific antibody binding.

After incubation, the cells were washed once with PBS. An additional 50 μ L of Reagent 2 was added to maintain permeabilization during the secondary antibody staining and the cells were incubated for 5 minutes. For the LDHA staining, 5 μ L of the PE-conjugated secondary antibody (anti-rabbit IgG PE) was added. For the LDHB staining, 0.5 μ L of the PE-conjugated secondary antibody (anti-rabbit IgG PE) was added. Subsequently, the cells were incubated in the dark for another 30 minutes. Finally, after washing to remove excess secondary antibody, the cells were resuspended in 200 μ L of PBS for flow cytometric analysis.

4.2.22 Cytospin and May-Grünwald staining

The morphology of CD8⁺ T cell subsets was analyzed by light microscopy following cytocentrifugation. After isolation of CD8⁺ T cells and cell sorting, 1×10^5 cells were resuspended in 100 μ L RPMI 1640 + 10% AB-Serum. Labeled slides, filter cards, and funnels were assembled on the slide clip and inserted in the Cytospin. 100 μ L of each sample was aliquoted into the appropriate funnel and centrifuged at 800 rpm for 5 minutes. Before continuing with the staining, the slides were air-dried for 10 minutes. Cells were fixed by rinsing the slides with 1 mL methanol and air-dried for 15 minutes. The slides were immersed in 100% May-Grünwald solution for 4 minutes and transferred directly to 4% Giemsa solution for 4 minutes. After the staining, the slides were briefly rinsed with water and left to air-dry for 10 minutes. The cells were then sealed, and coverslips were applied. The EVOS microscope was used to image the cells at 100 x oil immersion. The results were analyzed using ImageJ software by circling the circumference of the cells and their nuclei to determine the areas. The size of the cytoplasm was subsequently determined mathematically by subtracting the surface area of the nucleus from the surface area of the cell.

4.2.23 Software and programs

Flow cytometric data was analyzed with FlowJo 10.7.1. Metabolic flux experiments were designed and analyzed with the Seahorse Wave Desktop software, Windows Version V2.6.3.5 (Agilent). ImageJ was used to normalize the data from the metabolic flux experiments and analyze the data generated by light microscopy to evaluate cell size. Graphs and statistics were generated with GraphPad Prism version 9.

4.2.24 Statistical analysis

All statistical analyses were performed using GraphPad Prism version 9. To assess differences across multiple related groups, repeated measures (RM) one-way ANOVA with the Geisser-Greenhouse correction was used to account for potential violations of sphericity. When comparing multiple groups with unequal sample sizes or when some values were missing, a mixed-effects model was applied, followed by Dunnett's multiple comparisons test to compare each group to a control while adjusting for multiple testing. For pairwise comparisons between two related groups, a paired t-test was conducted. Correlation was determined using Spearman's rank correlation coefficient (Spearman's r). Significance levels were defined as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****)

For clarity, the following abbreviations are used throughout the text to describe CD8⁺ T cell subsets. CD8⁺CD28⁺CD57⁻ T cells are referred to as CD8⁺CD28 single-positive (**CD8⁺CD28SP**) cells, whereas CD8⁺CD28⁻CD57⁺ T cells are termed CD8⁺CD57 single-positive (**CD8⁺CD57SP**) cells. CD8⁺CD28⁻CD57⁻ T cells are designated as CD8⁺ double-negative (**CD8⁺DN**) cells and CD8⁺CD28⁺CD57⁺ T cells are referred to as CD8⁺ double-positive (**CD8⁺DP**) cells.

5 Results

5.1 CD8⁺CD57SP T cells accumulate with age

As a first step, we investigated the distribution of CD8⁺ T cell subsets in the peripheral blood of healthy donors in relation to age and sex. Using Pearson correlation (r), we observed a moderate positive correlation ($r = 0.49$, Figure 1a) between the frequencies of CD8⁺CD57SP T cells and age. Similarly, a moderate negative correlation ($r = -0.54$, Figure 1a) was observed between the percentage of CD8⁺CD28SP T cells and the age of the donors. No significant correlation was found between the prevalence of CD8⁺DN T cells and the increasing age of donors. However, a moderate positive correlation ($r = 0.46$, Figure 1b) was found between the prevalence of CD8⁺DP T cells and the age of the subjects. Furthermore, as shown in Figures 1c (CD8⁺CD28SP T cells) and 1d (CD8⁺CD57SP T cells), the correlation with age was independent of sex. Similarly, the correlation of CD8⁺DN and CD8⁺DP T cells with age was independent of sex (data not shown).

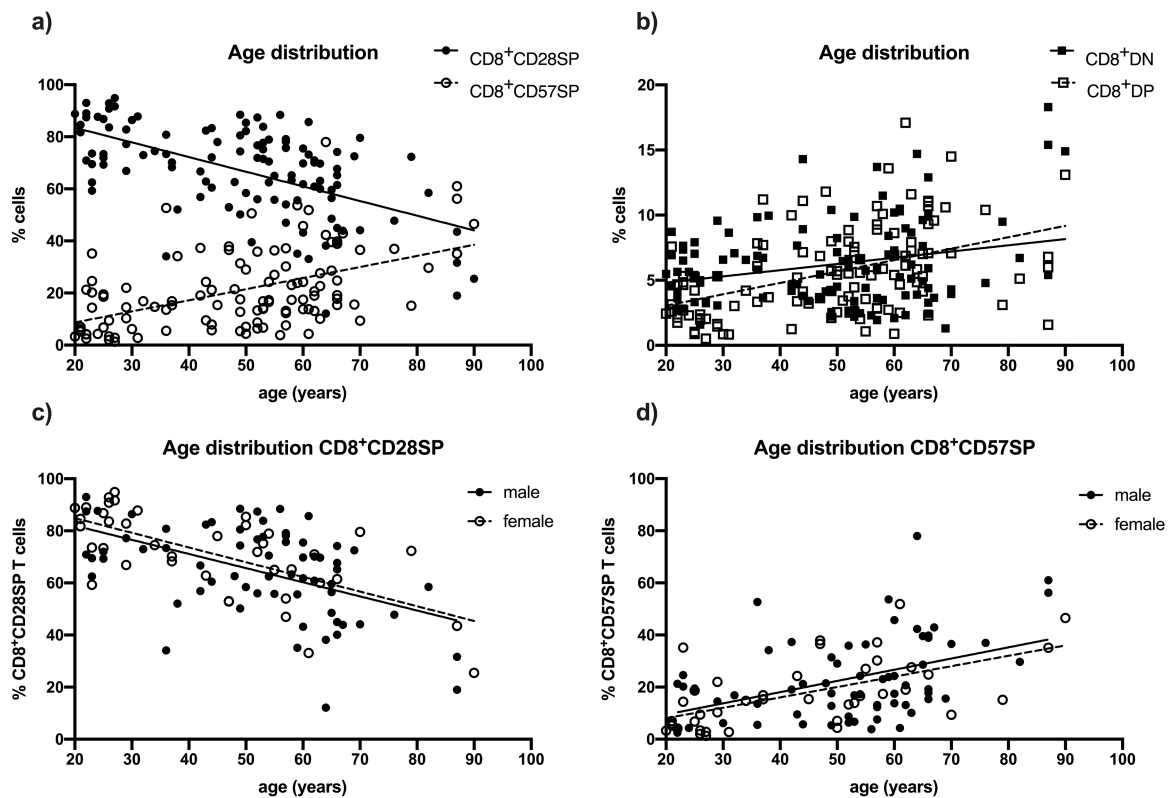


Figure 1: Age-related frequencies of CD8⁺ T cell subsets by CD28 and CD57 expression
Scatter dot plots demonstrating the correlation between age and frequency of indicated T cell subset in healthy donors. a) CD8⁺CD28SP $r = -0.54$ ($p < 0.0001$, 95% CI -0.67 to -0.39); CD8⁺CD57SP $r = 0.49$ ($p < 0.0001$, 95% CI 0.33 to 0.62); $n = 112$ b) CD8⁺DN $r = 0.12$ ($p = 0.214$, 95% CI -0.07 to 0.3); CD8⁺DP $r = 0.47$ ($p = 0.0001$, 95% CI 0.30 to 0.60), $n = 112$; c) male $r = -0.48$ ($p < 0.0001$, 95% CI -0.66 to -0.27), $n = 66$; female $r = -0.62$ ($p < 0.0001$, 95% CI -0.78 to -0.38; $n = 41$ d) male $r = 0.42$ ($p < 0.0002$, 95% CI 0.20 to 0.61); $n = 66$; female $r = 0.59$ ($p = 0.0001$, 95% CI 0.34 to 0.76); $n = 4$. 5 of the 112 donors were of unknown sex. The correlation was determined by Spearman's correlation coefficient, n =biological replicates.

5.2 Proliferation and apoptosis

5.2.1 The proliferative capacity of CD8⁺CD57SP T cells depends on stimulation conditions

The proliferative capacity of CD8⁺ T cell subsets was analyzed by two distinct approaches. The first method involved sorting CD8⁺ T cells into subsets based on their expression of CD28 and CD57, followed by stimulation through multiple rounds using anti-CD3/CD28 beads and IL-2. Cell proliferation was assessed through cell count and the Population Doubling Level (PDL) calculation. The naïve-like CD8⁺CD28SP T cell subset exhibited the highest proliferation. The CD8⁺DN and CD8⁺DP T cell subsets also demonstrated proliferative capacity, while the CD8⁺CD57SP T cells failed to proliferate and their numbers steadily declined over time (Figure 2a). Proliferation was further assessed by measuring eFluor 670 dye dilution. Here, bulk PBMCs were stimulated with OKT-3 and IL-2 and the reduction in mean fluorescence intensity (MFI) of CD8⁺ T cell subsets was measured after three and five days (Figure 2b). In this context, stimulation with anti-CD3/CD28 beads was intentionally excluded, as the CD8⁺CD57SP T cell subset, lacking CD28 expression, would not respond to anti-CD28 stimulation.

Significant differences were observed among the CD8⁺ T cell subsets in their proliferative potential. After three days, the CD8⁺CD57SP T cell subset showed significantly lower eFluor 670 reduction than the CD8⁺CD28SP cells ($p = 0.0009$), indicating a lower proliferation (Figure 2c). After five days, however, the CD8⁺CD57SP subset demonstrated a similar eFluor 670 reduction compared to the other T cell subsets (Figure 2d).

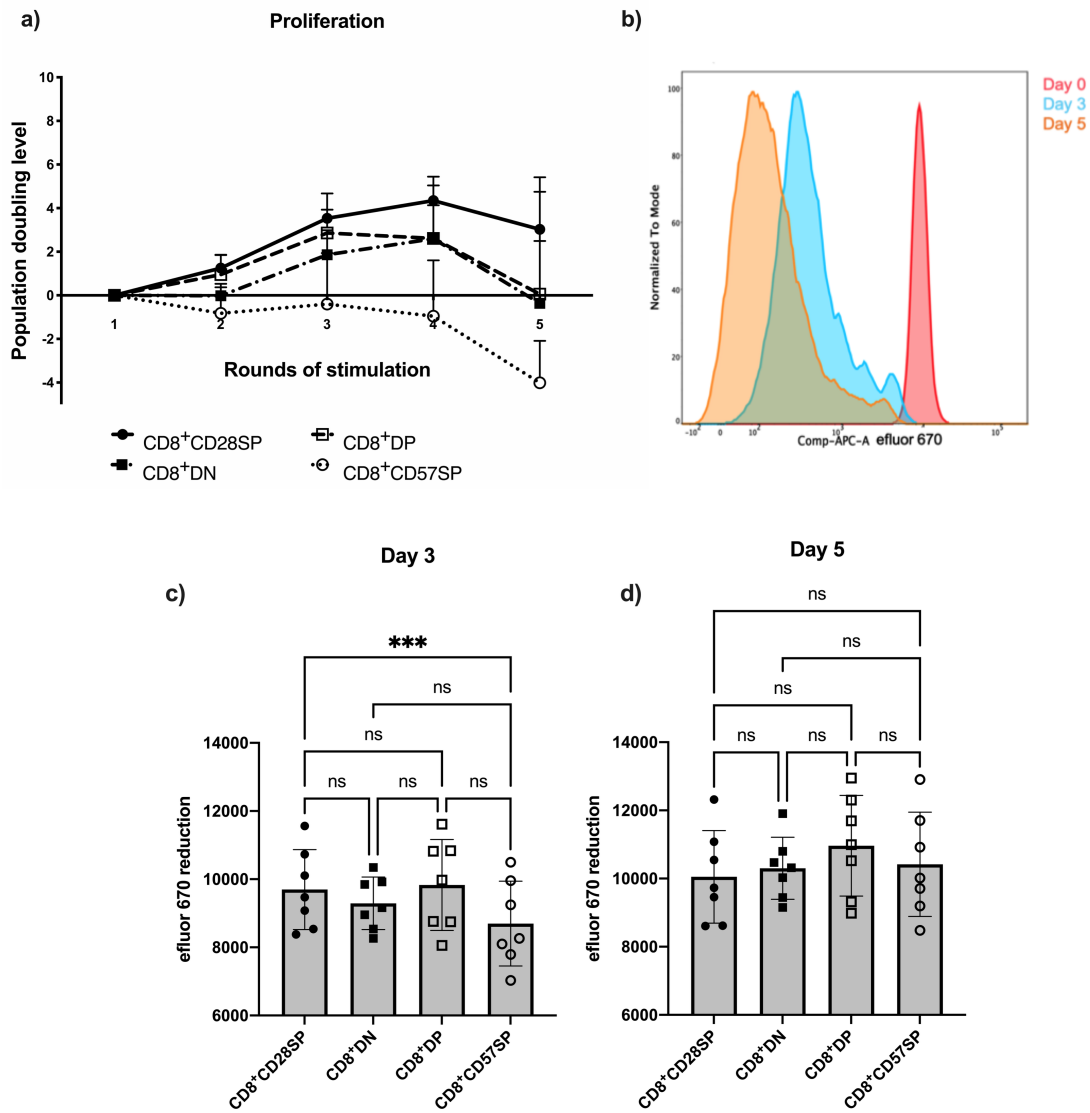


Figure 2: Proliferative capacity of CD8⁺CD57SP T cells Proliferation determined by cell count and eFluor 670 reduction via flow cytometry **a)** Population doubling level, CD8⁺ T cells were isolated from PBMCs, sorted into the subsets, and stimulated with 2 μ L anti-CD3/CD28 beads and 600 U/mL IL-2. Cells were counted under the microscope before restimulation after 5-7 days, n = 3 **b)** Representative image demonstrating the measurement of proliferation by eFluor 670 reduction in CD8⁺ T cells **c)** Scatter dot plot demonstrating eFluor 670 reduction after three days of stimulation with 30 ng/mL OKT-3 and 600 U/mL IL-2, n = 7 **d)** Scatter dot plot demonstrating eFluor 670 reduction after five days of stimulation with 30 ng/mL OKT-3 and 600 U/mL IL-2, n = 7 **e)** Scatter dot plot showing the fold increase of eFluor 670 reduction between day three and five of stimulation, n=7, Data are presented as mean $\pm$ SD. n = biological replicates, ns = not significant, p<0.05 (*), p<0.01(**), p<0.001 (***), p<0.0001 (****)

5.2.2 Apoptotic CD8⁺CD57SP T cells accumulate in cell culture

Apoptotic cells were identified by the combined staining of Annexin V and Propidium iodide (PI). In freshly isolated PBMCs (Figure 3a), few apoptotic cells (Annexin V⁺/PI⁺) were detected in the naïve-like CD8⁺CD28SP (mean = 0.55 %) and CD8⁺DN (mean = 0.7 %) T cell subset. Apoptotic cells (Annexin V⁺/PI⁺) were most abundant in the CD8⁺DP subset (mean = 9.7 %). CD8⁺CD57SP T cells showed more apoptotic cells (mean = 2.8%) than naïve-like CD8⁺CD28SP T cells ($p = 0,03$). However, their number was lower compared to the CD8⁺DP T cell subset ($p = 0,0059$). After a five-day stimulation with OKT-3 and IL-2 (Figure 3b), the number of apoptotic cells in naïve-like CD8⁺CD28SP and CD8⁺DN T cells remained very low. In sharp contrast, the percentage of apoptotic cells in CD8⁺DP and CD8⁺CD57SP T cells strongly increased after stimulation, resulting in 55.5% and 41.7% respectively.

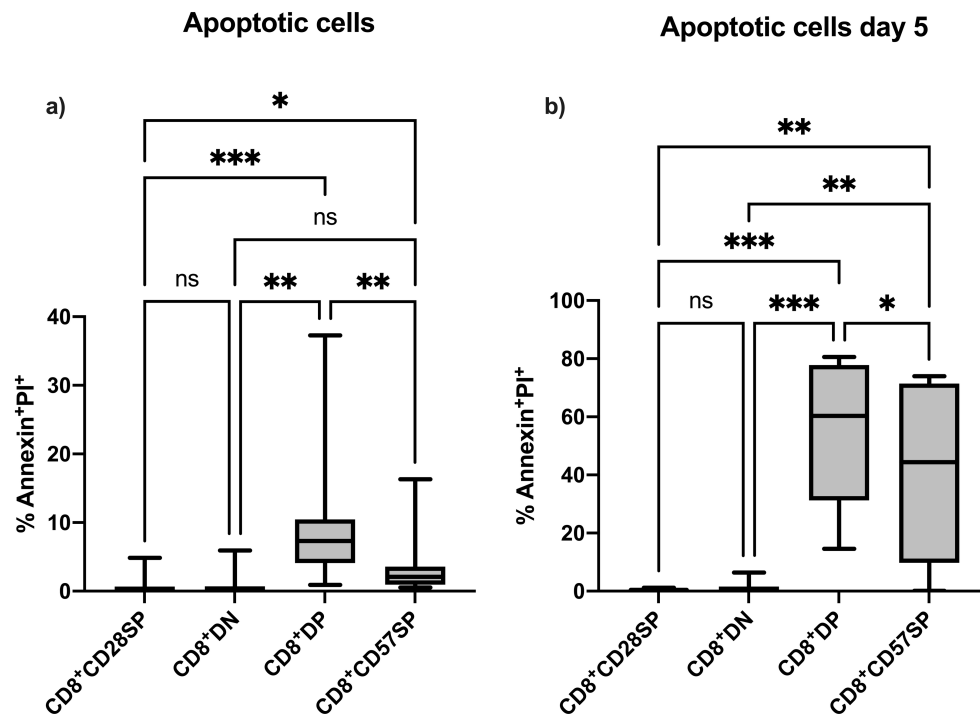


Figure 3: CD8⁺CD57SP T cells are more prone to apoptosis Identifying apoptotic CD8⁺ T cells by combined staining with Annexin V and Propidium iodide (PI) and measurement by flow cytometry; Data are presented as box plots with whiskers (min to max); **a)** Apoptotic cells on the day of PBMC isolation, n=22; **b)** Apoptotic cells after 5 days of stimulation with OKT-3 and IL-2, n=10; n=biological replicates, ns=not significant, $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***)

5.3 Characteristic surface marker profile of CD8⁺CD57SP T cells

5.3.1 High frequencies of T_{EMRA} cells are found within the CD8⁺CD57SP T cell subset

Based on the expression patterns of CD27 and CD45RA, the differentiation stages of CD8⁺ T cells were determined. Antigen “inexperienced”, naïve T cells express CD27 and CD45RA on their cell surface. CD27⁺CD45RA⁻ T cells represent central memory T cells and cells in the CD27⁻CD45RA⁻ subset are effector memory T cells. CD27⁻ T cells re-expressing CD45RA are terminally differentiated effector memory T cells (T_{EMRA}) (212). The co-staining for CD28, CD57, CD27, and CD45RA revealed distinct differentiation patterns. CD8⁺CD28SP T cells showed the highest expression of both CD27 and CD45RA on the cell surface (mean = 40.7%), followed by the CD8⁺DN T cell subset (mean = 31.3%), revealing a more dominant naïve phenotype (Figure 4a). The CD8⁺DP T cell subset included the most significant subset of CD27⁺CD45RA⁻ T cells (mean = 48.0%), indicating a predominance of central memory T cells (Figure 4b). Significantly more T_{EM} T cells were observed in the CD8⁺CD57SP T cell subset compared to the CD8⁺DN subset, however, no differences were observed between CD8⁺CD57SP T cells and CD8⁺CD28SP and CD8⁺DP subsets regarding the percentage of T_{EM} T cells. In contrast, the highest percentage of T_{EMRA} cells was observed in the CD8⁺CD57SP subset (mean = 58.5%; Figure 4d). Similarly, a high percentage of T_{EMRA} cells was represented in the CD8⁺DN subset (mean = 45.4%). A moderate positive correlation existed between the frequencies of CD27⁻CD45RA⁺ (T_{EMRA}) T cells and the increasing age of healthy donors ($r = 0.53$). Conversely, there was a strong negative correlation between the percentage of naïve (CD27⁺CD45RA⁺) T cells and age ($r = -0.82$; Figure 5a). Additionally, a moderate positive correlation was noted between the prevalence of effector memory (CD27⁻CD45RA⁻) T cells and increasing age ($r = 0.55$), while no significant correlation was observed between central memory (CD27⁺CD45RA⁻) T cells and age ($r = -0.13$; Figure 5b).

5.3.2 CD8⁺CD57SP T cells lose T cell homing markers

To further investigate the stages of differentiation between the four CD8⁺ T cell subsets, the expression of CCR7 and CD62L was analyzed by flow cytometry on freshly isolated, unstimulated T cells (Figure 6). The chemokine receptor CCR7 and the T cell homing marker CD62L showed the highest expression on naïve-like CD8⁺CD28SP T cells (mean_{CCR7} = 46%; mean_{CD62L} = 69%). The expression of both markers was reduced on CD8⁺DN and CD8⁺DP T cells. Interestingly, CCR7 was barely detected on CD8⁺CD57SP T cells (mean = 1.9%). Furthermore, the CD8⁺CD57SP T cell subset had a significantly lower expression of CD62L (mean = 29.34%) compared to CD8⁺CD28SP ($p < 0.0001$) and CD8⁺DN T cells ($p = 0.0005$).

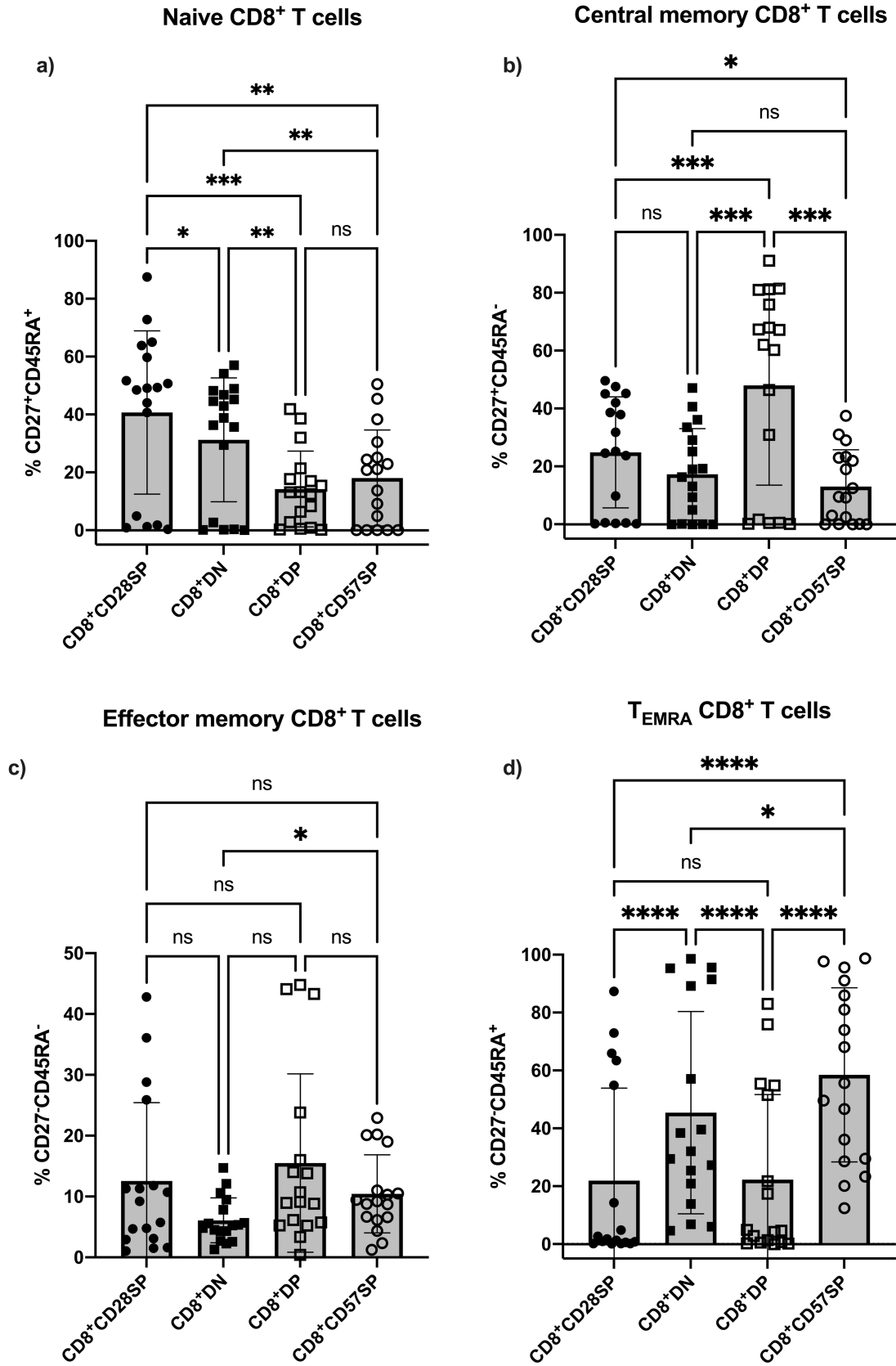


Figure 4: Markers of differentiation Scatter box plots demonstrating the frequencies of **a)** naïve CD27⁺CD45RA⁺; **b)** Central memory CD27⁺CD45RA⁻; **c)** Effector memory CD27⁻CD45RA⁻ and **d)** T_{EMRA} CD27⁻CD45RA⁺ subsets, gated on CD8⁺ T cells; n = 14; data are presented as mean ± SD, n = biological replicates, ns = not significant, p<0.05 (*), p<0.01 (**), p<0.001 (***), p<0.0001 (****)

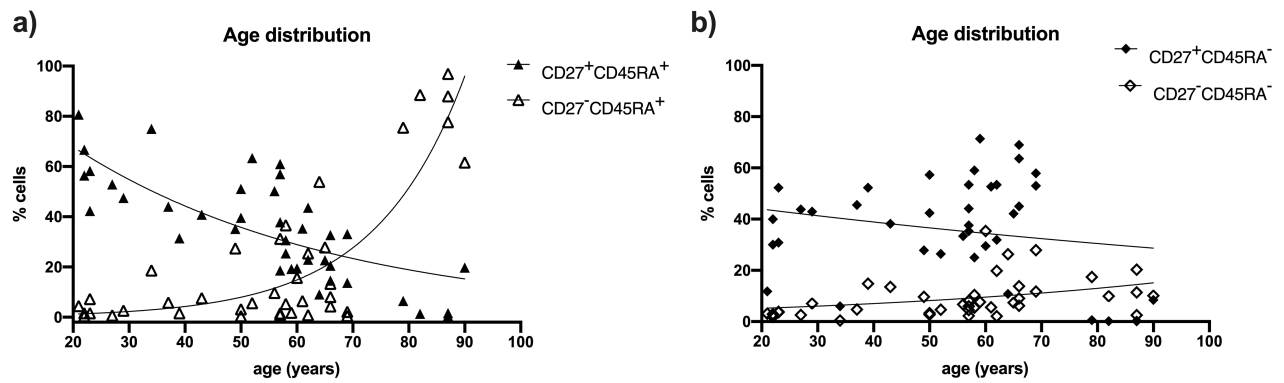


Figure 5: Figure 5: Age-related frequencies of CD8⁺ T cell subsets by CD27 and CD45RA expression Scatter dot plots demonstrating the correlation between age and frequency of indicated T cell subset in healthy donors. **a)** CD27⁺CD45RA⁺ $r = -0.82$ ($P < 0.0001$, 95% CI -0.90 to -0.68); CD27⁻CD45RA⁺ $r = 0.53$ ($p = 0.0004$, 95% CI 0.26 to 0.73); **b)** CD27⁺CD45RA⁻ $r = -0.13$ ($p = 0.41$, 95% CI -0.44 to 0.20); CD27⁻CD45RA⁻ $r = 0.55$ ($p = 0.0003$, 95% CI 0.28 to 0.74), $n = 40$, The correlation was determined by Spearman's correlation coefficient, n =biological replicates

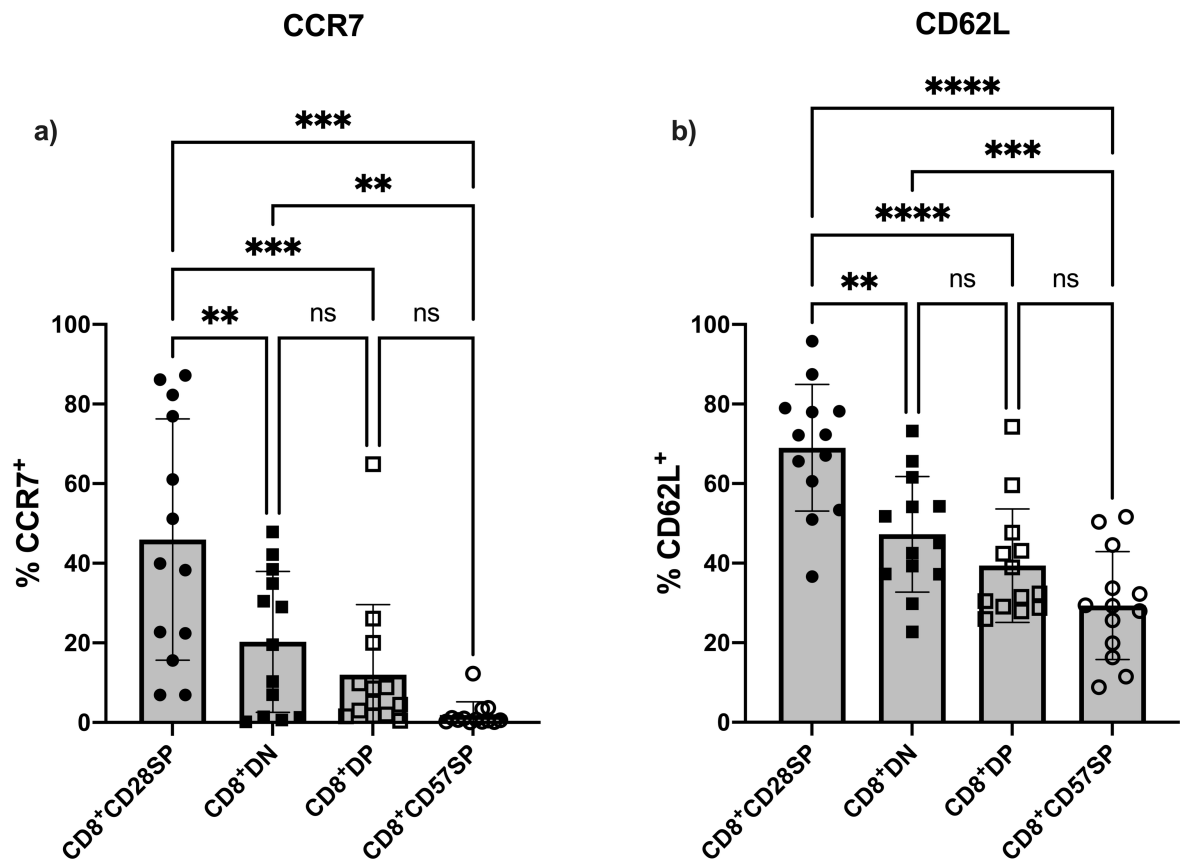


Figure 6: T cell homing markers CCR7 and CD62L Scatter box plots demonstrating the percentages of CCR7 and CD62L in freshly isolated CD8⁺ T cells, assessed by flow cytometry **a)** Expression of CCR7, $n = 13$; **b)** CD62L, $n = 13$. Data are presented as mean $\pm$ SD, n = biological replicates, ns = not significant, $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****)

5.3.3 IL-2 and IL-7 receptors are weakly expressed on CD8⁺CD57SP T cells “ex vivo” and upregulated following stimulation

The alpha chains of the IL-2 and IL-7 receptors were assessed by flow cytometry in unstimulated and stimulated CD8⁺ T cells. In unstimulated, freshly isolated CD8⁺ T cells, CD25 (IL-2R α) expression was low in all subsets (Figure 7a). The CD8⁺CD57SP T cell subset exhibited a significantly lower expression of CD25 compared to the naïve-like CD8⁺CD28SP subset (mean_{CD57SP} = 0.74%; $p = 0.033$). After 48 hours of stimulation (OKT-3 and IL-2), the expression of CD25 increased in all subsets (Figure 7b). The CD8⁺CD57SP T cell subset showed a marked increase in the expression of CD25 after stimulation (mean = 47.42%), with a mean fold increase of 78.3 compared to the day of isolation, which was significantly higher compared to the naïve-like CD8⁺CD28SP ($p = 0.049$) and CD8⁺DN ($p = 0.006$) subsets (Figure 7b). However, the expression of CD25 post stimulation in CD8⁺CD57SP T cells remained comparable to the other T cell subsets.

CD127 (IL-7R α) expression was measured on freshly isolated, unstimulated CD8⁺ T cells (Figure 7c). The expression was highest in the naïve-like CD8⁺CD28SP T cell subset (mean = 93.9%), followed by CD8⁺DP T cells (mean = 71.7%), and the CD8⁺DN T cells (mean = 34%). The weakest expression of CD127 was observed in the CD8⁺CD57SP T cell subset (mean = 12.3%). The expression of CD127 was also measured after 48 hours of stimulation (Figure 7d). After stimulation, the CD8⁺CD28SP and CD8⁺DP subsets maintained the highest percentage of CD127⁺ cells. However, compared to unstimulated cells, CD127 was downregulated in both the naïve-like CD8⁺CD28SP and the CD8⁺DP T cell subsets (mean fold increase <1), while it was upregulated on the CD8⁺CD57SP T cell subset (mean fold increase = 2.17). In contrast, stimulation did not affect the expression of CD127 in the CD8⁺DN T cell subset.

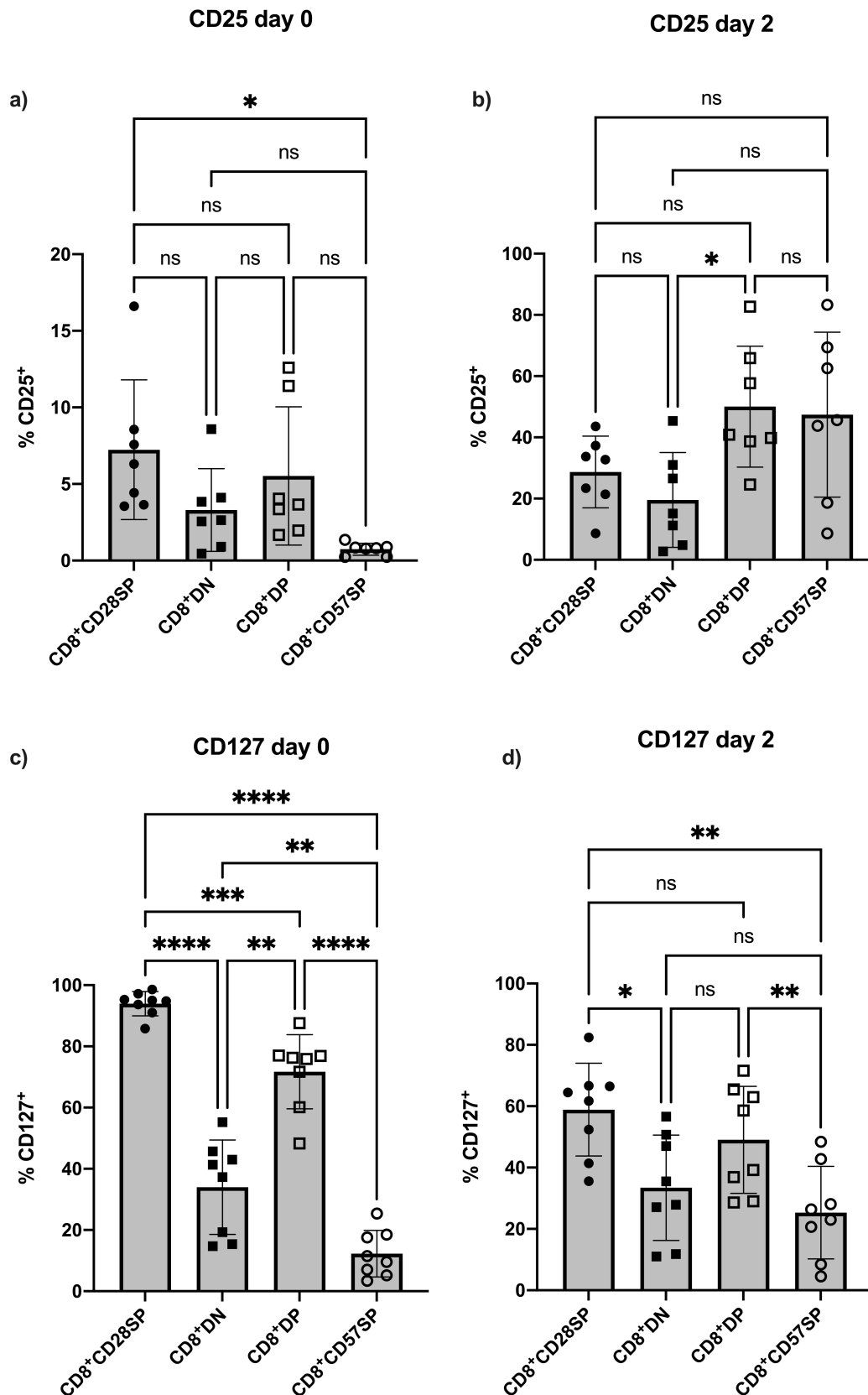


Figure 7: IL-2 and IL-7 receptor α -chain expression Scatter box plots showing the expression of CD25 and CD127 in freshly isolated and stimulated CD8⁺ T cells, assessed by flow cytometry. **a)** Expression of CD25, freshly isolated, $n = 7$; **b)** Expression of CD25 after 48 hours of stimulation with OKT-3 and IL-2, $n = 7$; **c)** Expression of CD127, freshly isolated, $n=8$; **d)** Expression of CD127 after 48 hours of stimulation with OKT-3 and IL-2, $n=8$. Data are presented as mean $\pm$ SD, n =biological replicates, ns=not significant, $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****)

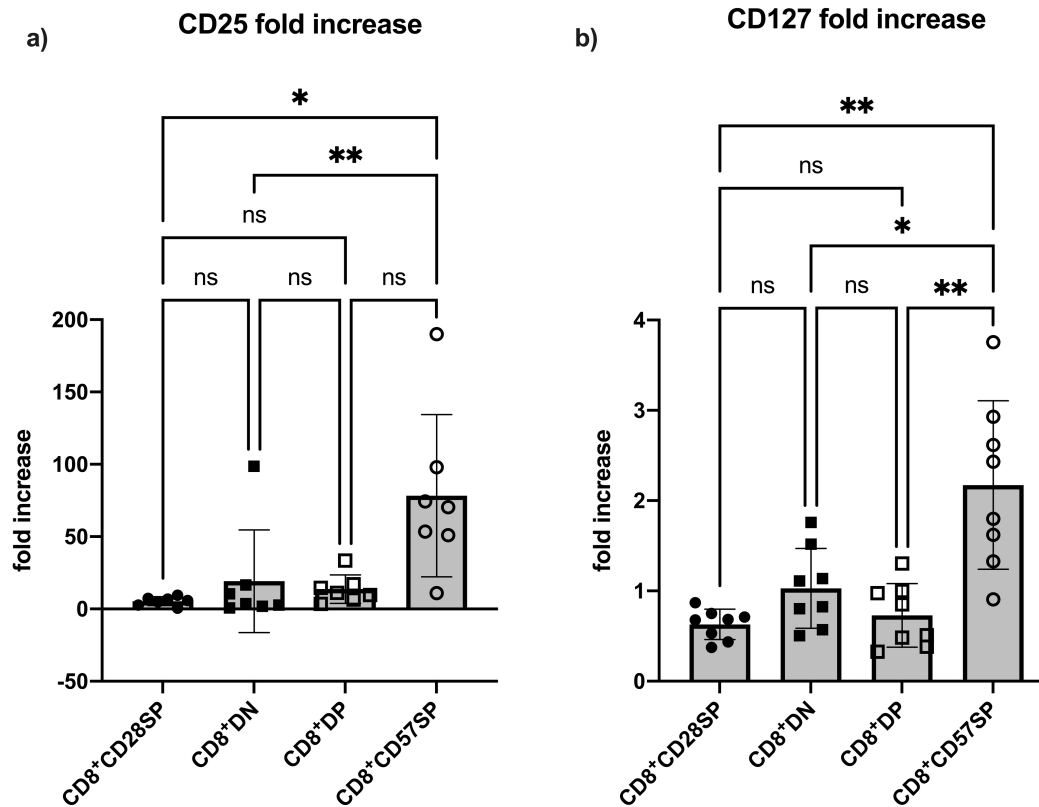


Figure 8: Fold increase of IL-2 receptor and IL-7 receptor α -chain expression following stimulation Scatter box plots showing the fold increase in the percentage of CD25 (IL-2 Receptor) expressing and CD127 (IL-7 receptor) expressing CD8⁺ T cells, following 48 hours of stimulation, assessed by flow cytometry **a)** Fold increase of CD25, $n = 7$; **b)** Fold increase of CD127, $n = 8$; Data are presented as mean $\pm$ SD, n = biological replicates, ns = not significant, $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****)

5.3.4 CD8⁺CD28⁻CD57⁺ T cells are distinct from exhausted T cells

The expression of several inhibitory receptors across different CD8⁺ T cell subsets revealed distinct patterns of marker distribution.

The highest frequency of PD-1⁺ T cells was observed in the CD8⁺DP T cell subset (mean = 73.9%). In contrast, CD8⁺DN T cells showed the lowest expression of PD-1 (mean = 35.56%). There was no difference in the percentage of PD-1⁺ cells between naïve-like CD8⁺CD28SP (mean = 42.9%) and CD8⁺CD57SP (mean = 45.9%) T cells. However, PD-1 expression levels were higher in CD8⁺CD57SP cells compared to CD8⁺DN cells (Figure 9a).

TIGIT expression was most pronounced on CD8⁺DP T cells (mean = 70.3%), while naïve-like CD8⁺CD28SP T cells displayed the lowest levels (mean = 24%). The expression of TIGIT was significantly higher on CD8⁺CD57SP cells compared to naïve-like CD8⁺CD28SP T cells ($p < 0.0001$), but no difference was observed between CD8⁺CD57SP and CD8⁺DN T cells (Figure 9b).

The lowest expression of KLRG-1 could be observed on naïve-like CD8⁺CD28SP T cells (mean = 36.2%). CD8⁺DN T cells expressed higher levels of KLRG-1 than the naïve-like CD8⁺CD28SP subset, while CD8⁺DP T cells had greater KLRG-1 expression than the CD8⁺DN subset. The highest expression of KLRG-1 was found on the CD8⁺CD57SP subset (mean = 92.3%), reflecting its association with advanced T-cell differentiation and senescence (Figure 9c).

The overall expression of CTLA-4 on freshly isolated, unstimulated T cells was very low (mean values ranging from 0.49% to 2.07%). Within this small subset of CTLA-4-expressing cells, CD8⁺CD57SP T cells exhibited higher CTLA-4 levels than the naïve-like CD8⁺CD28SP subset ($p = 0.0466$; Figure 9d).

The highest expression of TIM-3 was observed on CD8⁺DN (mean = 10.7%) and CD8⁺CD57SP T cells (mean = 8.5%), while the lowest expression was found on CD8⁺DP T cells (mean = 0.9%) (Figure 10a). The low expression of TIM-3 on CD8⁺DP T cells contrasts with the high expression of the other inhibitory receptors such as PD-1, TIGIT and KLRG-1 in this subset (Figures 9 a-c).

Interestingly, LAG-3 expression was very low on unstimulated T cells across all subsets (mean values ranging from 0.4% to 0.7%; Figure 10b).

5.3.5 NK-cell markers are expressed on CD8⁺CD57SP T cells

It was previously described that NK-cell markers are expressed on late differentiated T cells during aging (213, 214). Therefore, we measured the expression of the NK-cell-associated receptors CD160 and CD158b on freshly isolated, unstimulated CD8⁺ T cells. CD160 showed the lowest expression on the naïve-like CD8⁺CD28SP T cell subset (mean = 1.7%) compared to all the other T cell subsets (Figure 11a). Moreover, CD8⁺CD57SP, CD8⁺DN and CD8⁺DP subsets exhibited similar expression levels. The NK-cell receptor CD158b showed the highest expression on CD8⁺CD57SP (mean = 12.6%) and CD8⁺DN T cells (mean = 11.5%). Conversely, the naïve-like CD8⁺CD28SP (mean = 0.7%) and CD8⁺DP (mean = 2.3%) T cell subsets showed very low levels of CD158b expression (Figure 11b). Overall, the expression profile of CD160 and CD158b on the four analyzed CD8⁺ T cell subsets is rather similar, with CD8⁺DN and CD8⁺CD57SP T cells showing a higher expression of NK-cell markers than the naïve-like CD8⁺CD28SP and CD8⁺DP subsets.

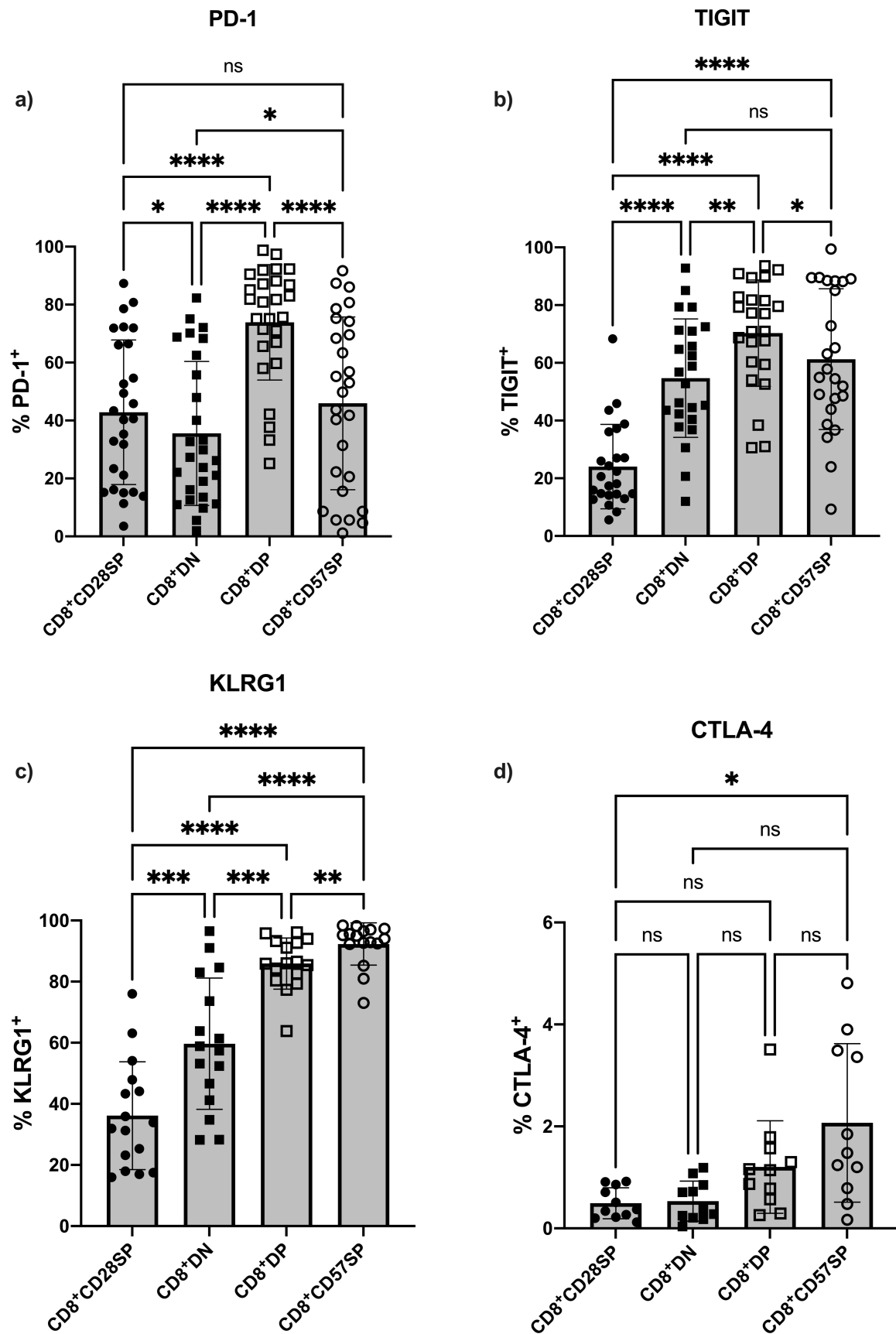


Figure 9: Expression of Inhibitory markers Scatter box plots showing the expression of inhibitory markers in freshly isolated CD8⁺ T cells, assessed by flow cytometry **a)** PD-1, n = 27; **b)** TIGIT, n = 24; **c)** KLRG1, n = 16; **d)** CTLA-4, n = 11. Data are presented as mean ± SD, n = biological replicates, ns = not significant, p < 0.05 (*), p < 0.01 (**), p < 0.001 (***), p < 0.0001 (****)

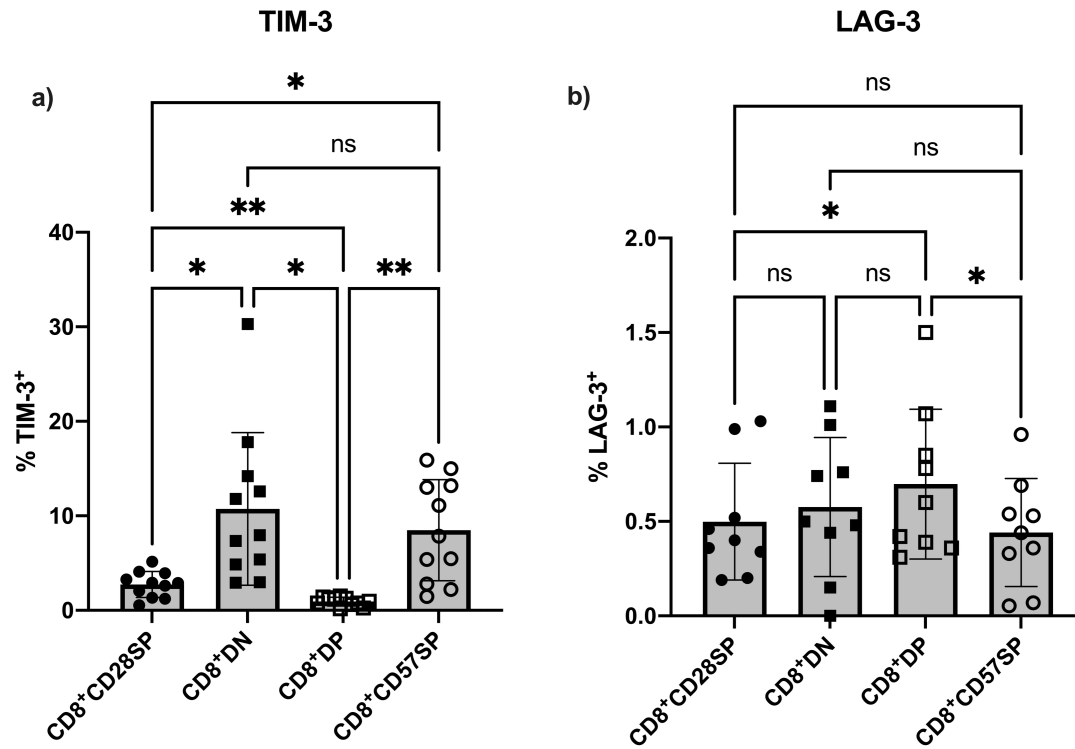


Figure 10: Expression of TIM-3 and LAG-3 Scatter box plots showing the expression of TIM-3 and LAG-3 in freshly isolated CD8⁺ T cells, assessed by flow cytometry **a)** TIM-3, n = 11; **b)** LAG-3, n = 9. Data are presented as mean $\pm$ SD, n = biological replicates, ns = not significant, $p < 0.05$ (*), $p < 0.01$ (**)

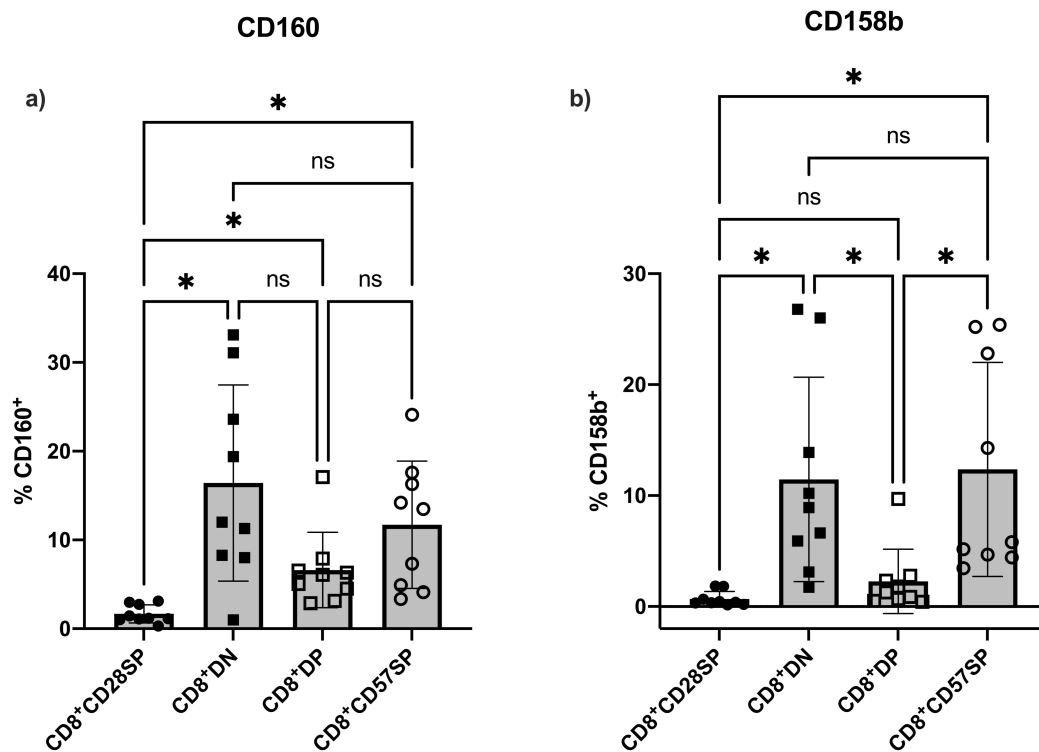


Figure 11: Expression of NK-cell receptors CD160 and CD158b Scatter box plots showing the expression of CD160 and CD158b in freshly isolated CD8⁺ T cells, assessed by flow cytometry **a)** CD160, n = 9; **b)** CD158b, n = 9. Data are presented as mean $\pm$ SD, n = biological replicates, ns = not significant, $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****)

5.4 Classic markers of senescence

5.4.1 Expression of Senescence-associated β -Galactosidase is not a characteristic feature of CD8⁺CD57SP T cells

Senescence-associated β -Galactosidase (SA- β -Gal) is a widely used marker of cellular senescence and an abundance of SA- β -Gal⁺ CD8⁺ T cells was previously observed in aging humans (65). We aimed to determine whether SA- β -Gal expression correlates with surface markers of T cell senescence and terminal differentiation in healthy donors.

SA- β -Gal levels were determined in CD8⁺ T cell subsets on the day of PBMC isolation and after a 5-day treatment with senescence-inducing agents. In unstimulated, untreated CD8⁺ T cell subsets on the day of PBMC isolation, levels of SA- β -Gal were low across all subsets (mean = 6.18%) (Figure 12 a/b). A strong positive correlation ($r = 0.64$) was observed between the percentage of CD8⁺ T cells expressing SA- β -Gal and the increasing age of healthy donors (Figure 12 c). Unexpectedly, in freshly isolated PBMCs no significant differences in SA- β -Gal expression were found between the CD27⁻CD45RA⁺ (T_{EMRA}) and CD27⁺CD45RA⁺ (T_{naïve}) cells. Overall, their SA- β -Gal expression was lower than in CD27⁺CD45RA⁻ (T_{CM}) and CD27⁻CD45RA⁻ (T_{EM}) T cells (Figure 12a). Moreover, unstimulated senescent-like CD8⁺CD57SP T cells showed a significantly lower SA- β -Gal expression than naïve-like CD8⁺CD28SP T cells (Figure 12b). CD8⁺ T cells stimulated with 30 ng/mL OKT-3 and 600 U/mL IL-2 and treated with 20 μ M of Etoposide led to significantly increased levels of SA- β -Gal compared to untreated cells (mean = 32.49 % vs 2.98%; $p = 0.0001$) (Figure 12d). Treating cells with inhibitors of cellular respiration, such as Rotenone and Antimycin A (which disrupt the electron transport chain of oxidative phosphorylation), also significantly increased SA- β -Gal levels (mean = 8.84 %; $p = 0.02$) (Figure 12 d). Similarly, culturing CD8⁺ T cells in the supernatant of the osteosarcoma cell line Saos2/143 and the leukemia cell line OCI-AML3 significantly increased SA- β -Gal to 15.39 % and 14.64 %, respectively (Figure 12d).

The analyzed CD8⁺ T cell subsets under senescence-inducing conditions revealed notable differences in SA- β -Gal expression. Senescent-like CD8⁺CD57SP T cells consistently exhibited low SA- β -Gal expression after stimulation and exposure to senescence-inducing conditions. In particular, SA- β -Gal expression in CD8⁺CD57SP T cells was significantly lower than in CD8⁺DP T cells following treatment with 1 μ M Rotenone/Antimycin A ($p = 0.004$), 5 μ M etoposide ($p = 0.03$), 20 μ M etoposide ($p = 0.004$), and culture in Saos2/143 supernatant ($p = 0.046$). Across most conditions, the CD8⁺DP T cell subset showed the highest levels of SA- β -Gal expression. Their expression was significantly elevated compared to naïve-like T cells (CD8⁺CD28SP) under untreated conditions ($p = 0.023$) and after treatment with 5 μ M etoposide ($p = 0.038$). Additionally, SA- β -Gal expression in CD8⁺DP T cells was significantly higher than

in CD8⁺DN T cells under both 5 μ M ($p = 0.019$) and 20 μ M ($p = 0.0001$) etoposide treatment (Figure 13).

When CD8⁺ T cell subsets were classified based on CD27 and CD45RA expression, a different pattern emerged. A trend toward increased SA- β -Gal expression was observed in T_{EMRA} (CD27⁻CD45RA⁺) CD8⁺ T cells following stimulation and culture under various senescence-inducing conditions. Notably, T_{EMRA} cells showed significantly higher SA- β -Gal expression compared to central memory T cells (T_{CM}; CD27⁺CD45RA⁻) after treatment with 5 μ M etoposide ($p = 0.019$) and in culture with Saos2/143 cell supernatant ($p = 0.048$). In all tested conditions except for treatment with 20 μ M etoposide, effector memory T cells (T_{EM}; CD27⁻CD45RA⁻) exhibited significantly higher SA- β -Gal expression compared to T_{CM} cells (Figure 14).

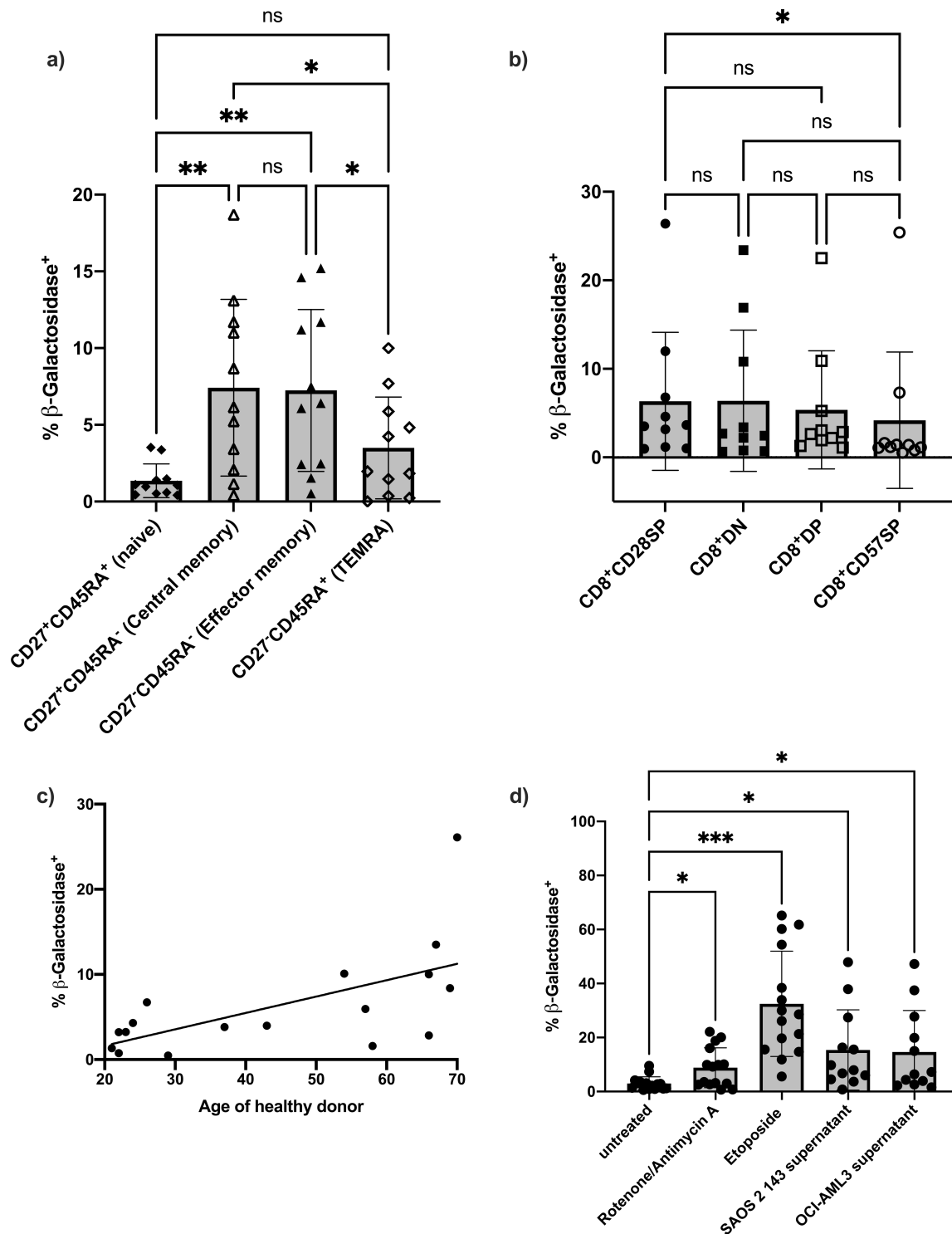


Figure 12: Expression of β -Galactosidase “ex vivo” and following treatment in senescence-inducing conditions Scatter box plots showing the expression of β -Galactosidase in peripheral blood CD8⁺ T cells, assessed by flow cytometry **a)** CD8⁺ T cell subsets by CD27 and CD45RA expression, freshly isolated $n=11$; **b)** CD8⁺ T cell subsets by CD28 and CD57 expression, freshly isolated, $n = 10$; **c)** Correlation of β -Galactosidase expression on CD8⁺ T cells and age of healthy donors, $r = 0,64$ ($P<0.0045$, 95% CI 0.23 to 0.86), $n = 18$ **d)** CD8⁺ T cells after 5 days with 30 ng/mL OKT-3 and 600 U/mL IL-2; untreated $n = 15$; 1 μ M Rotenone/Antimycin A, $n = 15$; 20 μ M Etoposide, $n = 15$; culture in SAOS 2 413 supernatant, $n = 12$; culture in OCI-AML3 supernatant, $n = 12$; Data are presented as mean $\pm$ SD, n = biological replicates, ns = not significant, $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***)

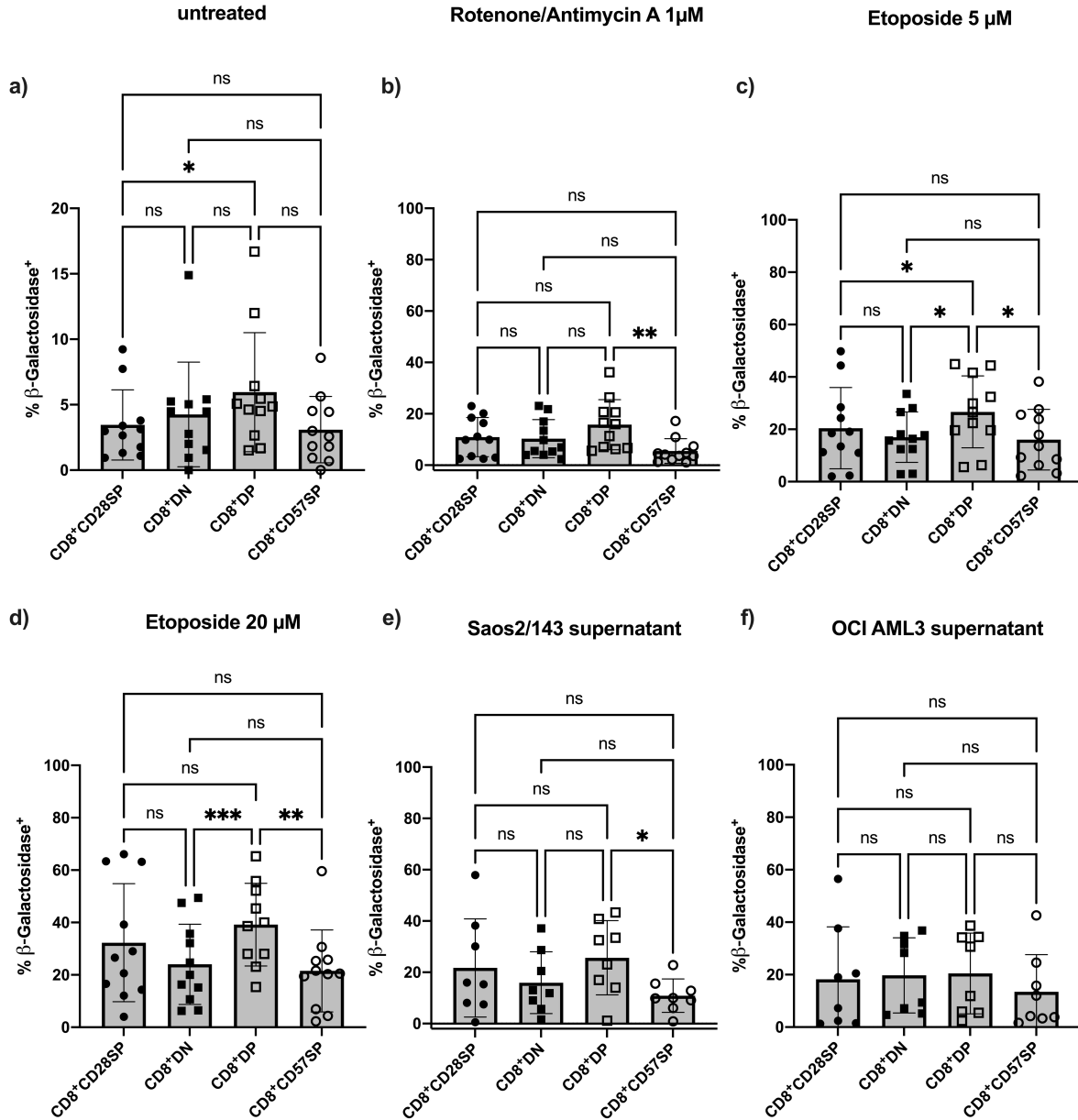


Figure 13: Expression of β -Galactosidase in CD8⁺ T cell subsets based on CD28 and CD57 expression after treatment in senescence-inducing conditions for 5 days Scatter box plots showing the expression of β -Galactosidase in peripheral blood CD8⁺ T cells, assessed by flow cytometry; stimulated with 30 ng/mL OKT-3 and 600 U/mL IL-2, assessed by flow cytometry **a)** untreated, n = 11 **b)** 1 μ M Rotenone/Antimycin A, n = 11 **c)** 5 μ M Etoposide, n = 11 **d)** 20 μ M Etoposide, n = 11; **e)** culture in Saos2/143 supernatant, n = 8; **f)** culture in OCI-AML3 supernatant, n = 8; Data are presented as mean $\pm$ SD, n=biological replicates, ns=not significant, $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***)

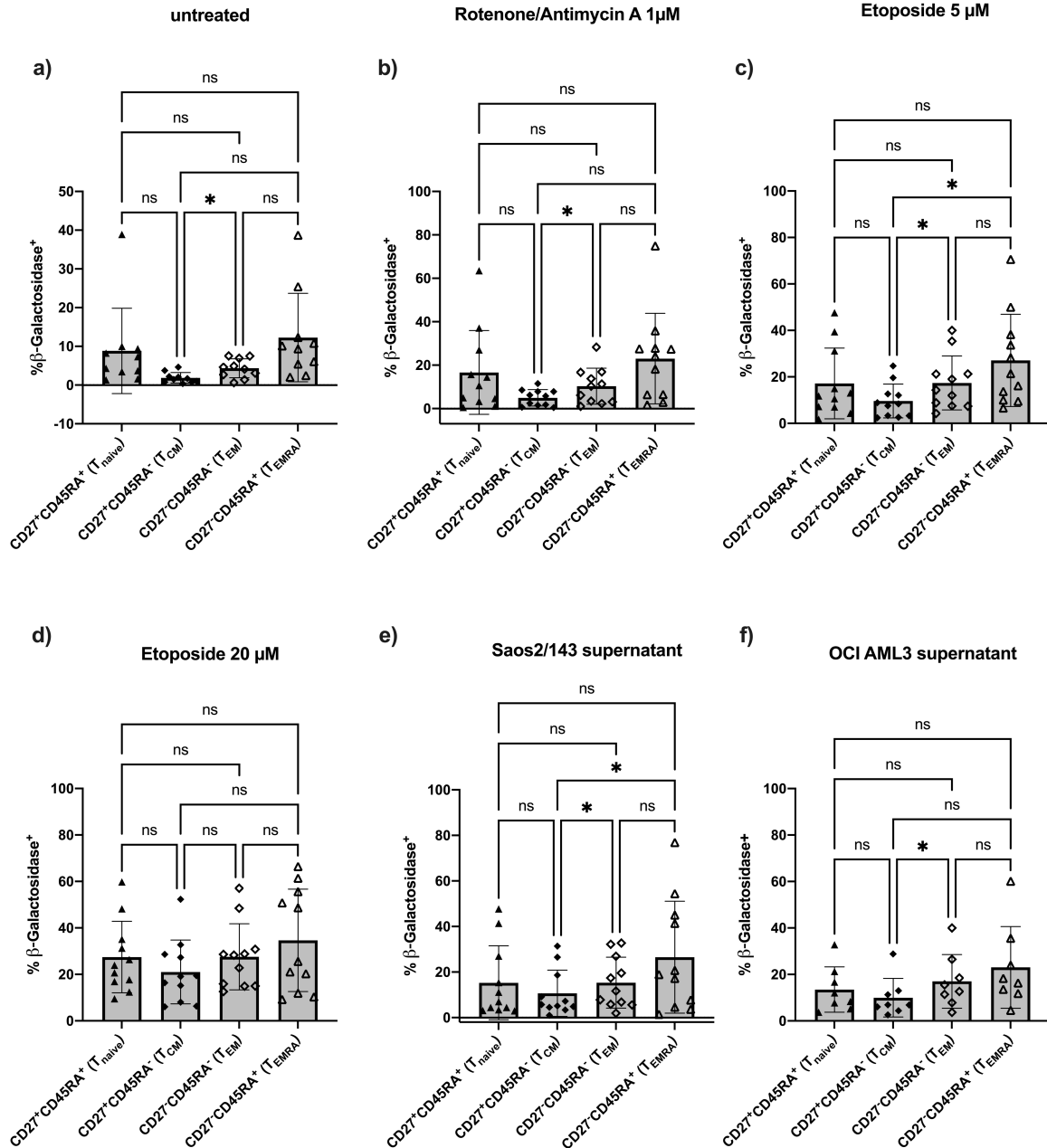


Figure 14: Expression of β -Galactosidase in CD8⁺ T cell subsets based on CD27 and CD45RA expression after treatment in senescence-inducing conditions for 5 days
 Scatter box plots showing the expression of β -Galactosidase in peripheral blood CD8⁺ T cells, assessed by flow cytometry; stimulated with 30 ng/mL OKT-3 and 600 U/mL IL-2, assessed by flow cytometry **a)** untreated $n = 10$ **b)** 1 μ M Rotenone/Antimycin A, $n = 11$ **c)** 5 μ M Etoposide, $n = 11$ **d)** 20 μ M Etoposide, $n = 11$; **e)** culture in Saos2/143 supernatant, $n = 11$; **f)** culture in OCI-AML3 supernatant, $n = 8$; Data are presented as mean $\pm$ SD, n =biological replicates, ns=not significant, $p < 0.05$ (*)

5.4.2 Expression of the Urokinase-type plasminogen activator Receptor (uPAR)

The Urokinase-type plasminogen activator receptor (uPAR) is a cell surface protein broadly induced during cellular senescence (66). In human T lymphocytes, uPAR has been previously described as an activation marker (67). We sought to determine if uPAR expression levels on CD8⁺ T cells correlate with surface markers indicating late-stage differentiation or T cell senescence.

Overall, the expression of uPAR was low on freshly isolated, resting CD8⁺ T cells of healthy donors (age range 21-69 years, mean age 46.25 years), with a mean uPAR expression of 2.54-5 % for most subsets, except the senescent-like CD8⁺CD57SP T cells which showed the highest percentage (mean = 7.8%, Figure 15a).

Following this observation, uPAR expression was evaluated after stimulation and treatment with senescence-inducing agents to determine whether uPAR, like SA- β -Gal, is upregulated on CD8⁺ T cells after senescence induction (Figure 15b). It was found that uPAR levels were significantly increased after treatment with Etoposide compared to the untreated condition. (mean = 36.69%, $p = 0.003$). Stimulation of CD8⁺ T cells in the supernatant of Saos2/143 tumor cells also showed a significant increase in uPAR (mean = 29.16%, $p = 0.01$). Likewise, culture in the supernatant of the OCI-AML3 cell line also significantly increased uPAR expression (mean = 19.84%, $p = 0.04$). The analysis of the expression of uPAR on the different CD8⁺ T cell subsets revealed that the senescent-like CD8⁺CD57SP T cell subset, although they slightly upregulate uPAR in response to Etoposide and to the Saos2/143 supernatant (Figure 16b/c) but not to OCI-AML3 supernatant (Figure 16d), their expression remained globally lower compared to the other T cell subsets after stimulation and senescence induction (Figures 16b-d).

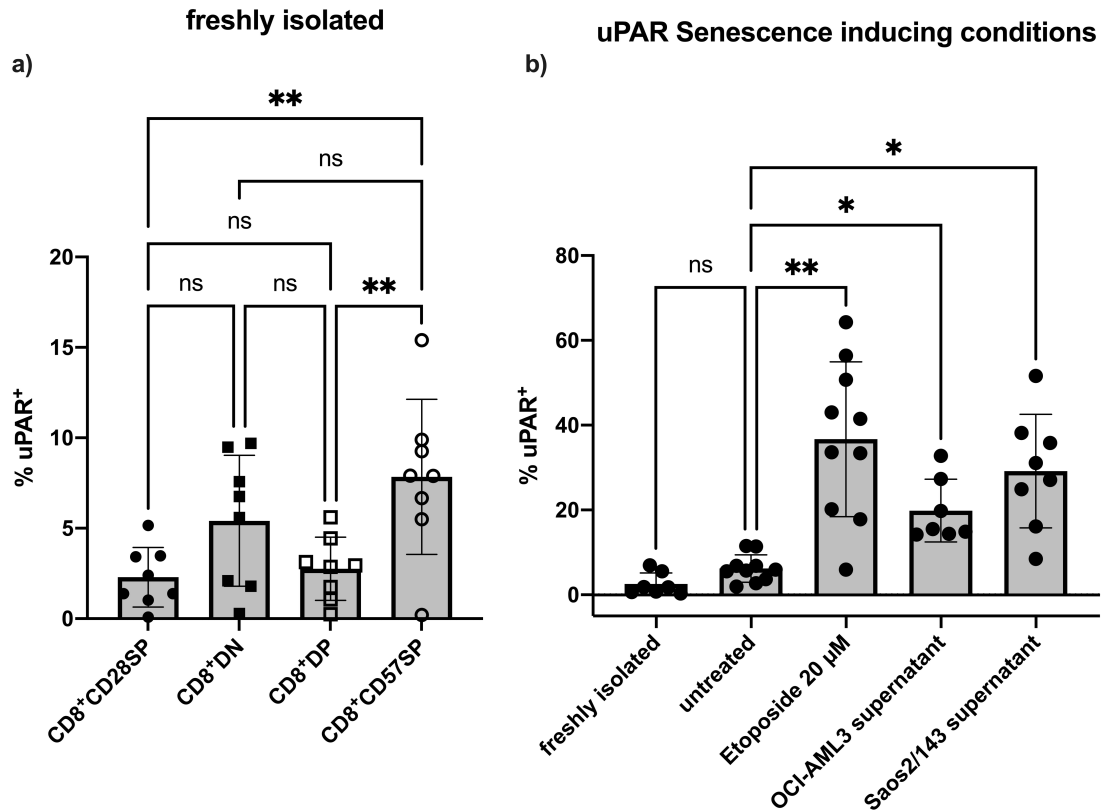


Figure 15: Expression of the Urokinase-type plasminogen activator Receptor (uPAR)

Scatter dot plots showing the expression of uPAR in peripheral blood CD8⁺ T cells, assessed by flow cytometry. **a)** in CD8⁺ T cell subsets from freshly isolated PBMCs, $n = 8$; **b)** in stimulated CD8⁺ T cells following treatment in senescence-inducing conditions ($n = 7$), without treatment ($n = 10$), treated with 20 μ M Etoposide ($n = 10$), cultured in the supernatant of OCI-AML3 cells ($n = 7$) or in the supernatant of Saos2/143 cells ($n = 8$). Data are presented as mean $\pm$ SD, n = biological replicates, ns = not significant, $p < 0.05$ (*), $p < 0.01$ (**)

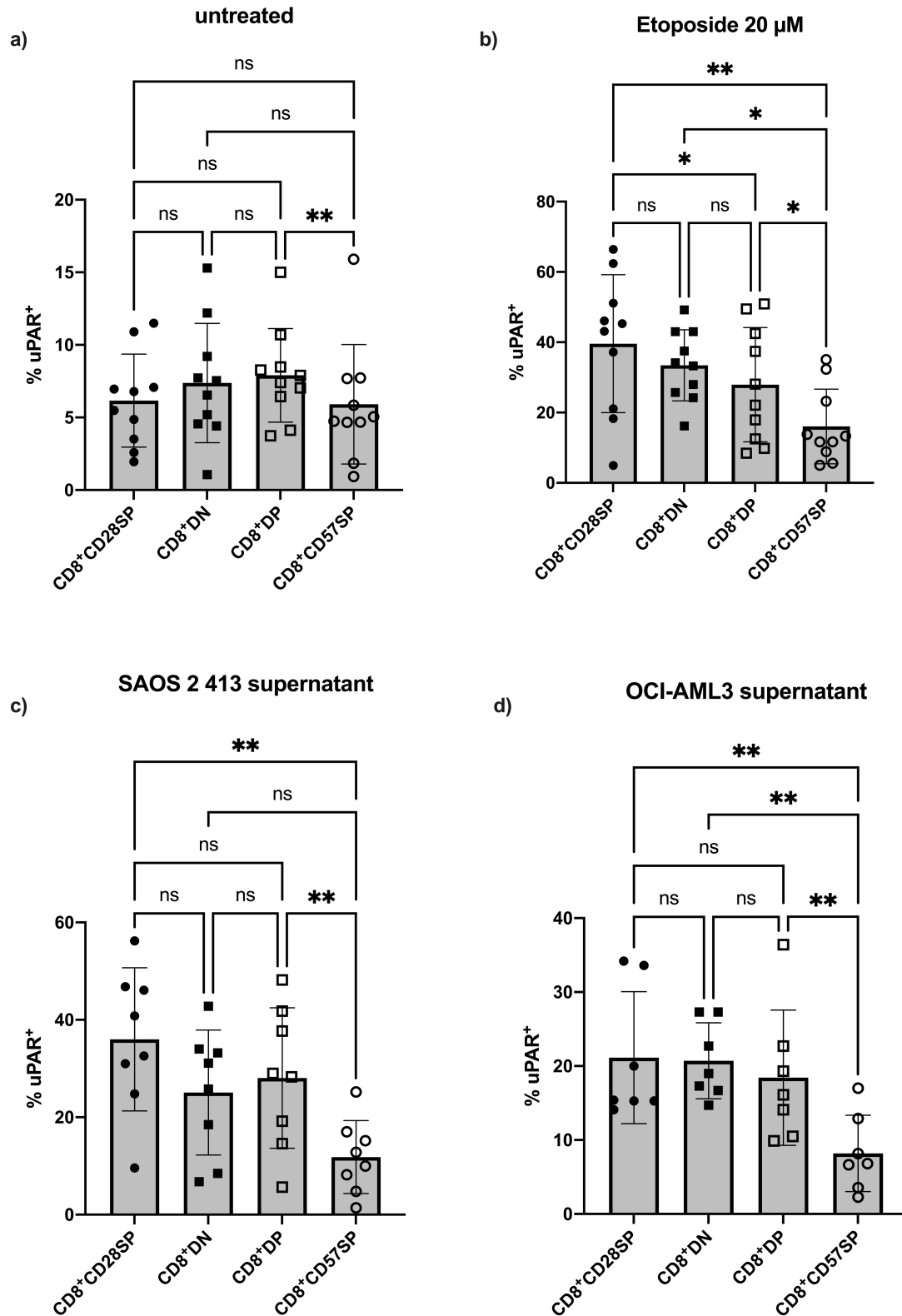


Figure 16: Expression of the Urokinase-type plasminogen activator receptor (uPAR) in stimulated CD8⁺ T cell subsets after treatment with senescence-inducing agents Scatter dot plots showing the expression of uPAR in peripheral blood CD8⁺ T cells, assessed by flow cytometry. **a)** untreated (n = 10); **b)** treated with 20 μM Etoposide (n = 10); **c)** cultured in the supernatant of Saos2/143 cells (n = 8); **d)** cultured in the supernatant of OCI-AML3 cells (n = 7). Data are presented as mean ± SD, n=biological replicates, ns=not significant, p<0.05 (*), p<0.01(**)

5.5 CD8⁺CD57SP T cells retain preserved effector functions

Degranulation, granzyme B, perforin, and IL-6 were measured in stimulated CD8⁺ T cell subsets to examine the cytolytic effector function and a component of the senescence-associated secretory phenotype (SASP) in senescent T cells.

Degranulation, an essential effector function of CD8⁺ T cells, was assessed by measuring the surface expression of CD107a (Figure 17a). Among all CD8⁺ T cell subsets analyzed, CD8⁺DP T cells exhibited the highest expression of CD107a (mean = 57%), highlighting a strong cytotoxic potential of this subset. The lowest expression of CD107a was observed in naïve-like CD8⁺CD28SP T cells (mean = 27.6%), reflecting their less differentiated state and limited cytotoxic capability. Notably, there were no significant differences between CD8⁺DN and CD8⁺CD57SP T cells.

CD8⁺CD57SP T cells demonstrated the highest percentage of **Granzyme B**-expressing T cells (mean = 46.5 %), identifying them as the subset with the greatest cytotoxic enzyme production. The lowest percentage of Granzyme B-expressing cells was observed in naïve-like CD8⁺CD28SP T cells (mean = 2.3%). CD8⁺DP cells exhibited the second-highest levels of Granzyme B expression (mean = 30%), suggesting that Granzyme B production increases with differentiation along the CD8⁺ T cell lineage (Figure 17b).

Perforin expression was higher in CD8⁺CD57SP T cells (mean = 44.9%), compared to all other subsets, emphasizing their potent cytotoxic potential. No significant differences in Perforin expression were observed among CD8⁺CD28SP, CD8⁺DN, and CD8⁺DP T cells (Figure 17c).

To evaluate a central component of the SASP, **IL-6** was measured in stimulated CD8⁺ T cells. Both CD8⁺DP and CD8⁺CD57SP T cells displayed the highest frequency of IL-6⁺ T cells among the CD8⁺ T cell subsets analyzed. Despite this, the overall levels of IL-6 production were low, with less than 10% of cells expressing IL-6 across all subsets.

Overall, CD8⁺CD57SP T cells demonstrated the highest Granzyme B and Perforin levels, underscoring their enhanced cytotoxic capabilities. Naïve-like CD8⁺CD28SP T cells consistently displayed the lowest cytotoxic and inflammatory potential across all markers. Furthermore, CD8⁺DP T cells emerged as a highly cytotoxic subset, as evidenced by their elevated CD107a expression and significant Granzyme B production.

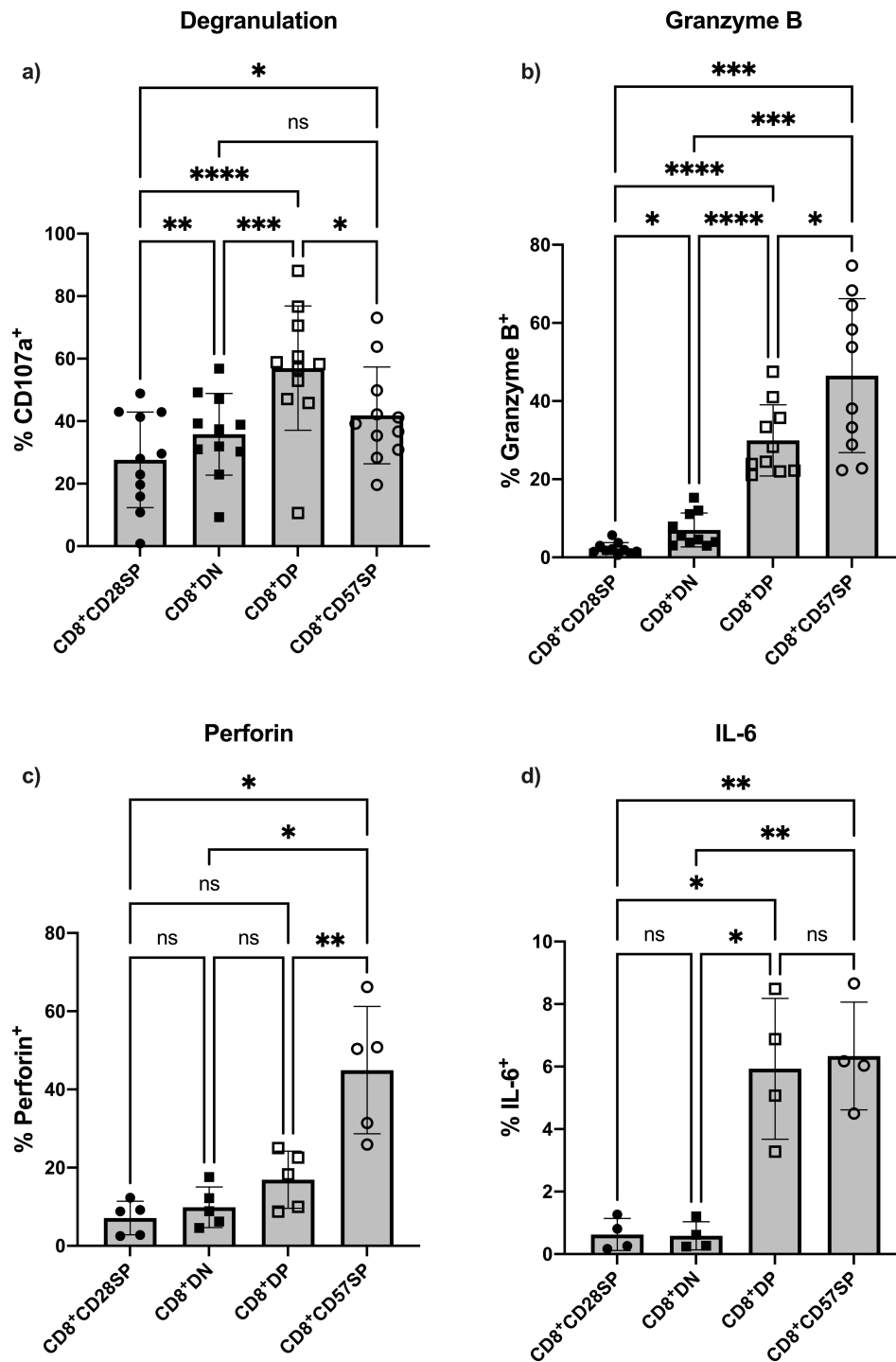


Figure 17: Functional characterization of T cell cytotoxicity and cytokine secretion

Scatter dot plots showing the formation of cytotoxic granules, production of cytotoxic molecules and IL-6 in stimulated peripheral blood CD8⁺ T cells, assessed by flow cytometry. **a)** Detection of CD107a surface expression on CD8⁺ T cells, $n = 11$; **b)** Intracellular detection of Granzyme B, $n = 10$; **c)** Intracellular detection of Perforin, $n = 5$; **d)** Intracellular detection of IL-6, $n = 4$; Data are presented as mean $\pm$ SD, n = biological replicates, ns = not significant, $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****)

5.6 Light microscopy reveals an altered cellular morphology of CD8⁺CD28⁻CD57⁺ T cells

The evaluation of cell size and cytoplasm size revealed that CD8⁺ T cells in the CD8⁺CD57SP subset were significantly larger than the other T cell subsets and showed an enlarged cytoplasm (Figures 18a, b). Cytotoxic granules were visible in some T cells of the CD8⁺CD57SP subset (Figure 19).

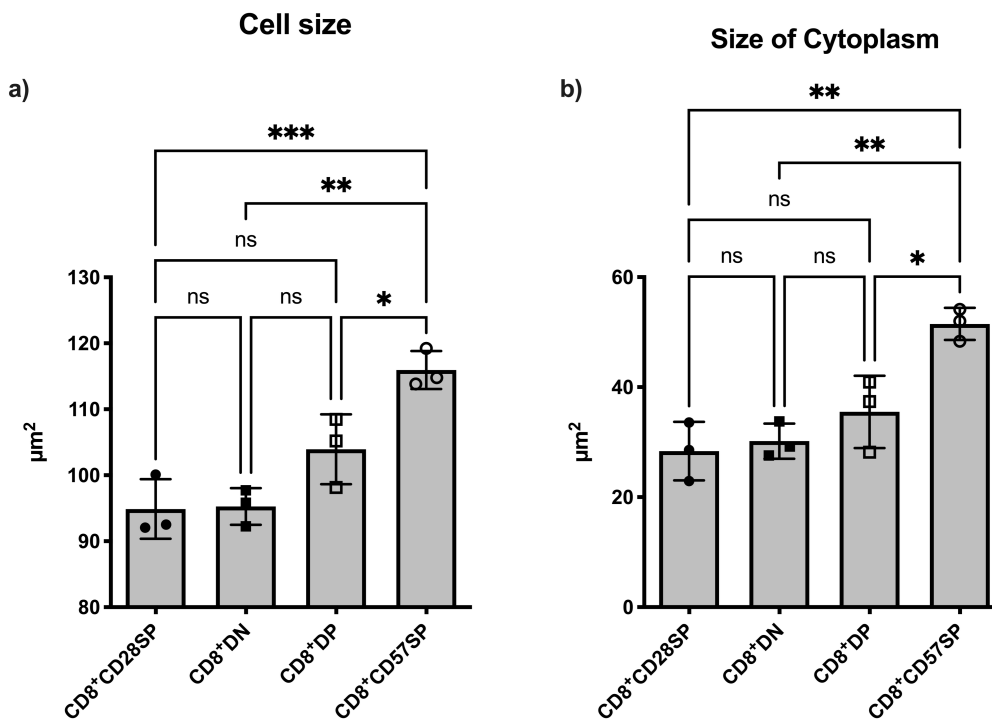


Figure 18: Analysis of cellular morphology by light microscopy Scatter dot plots showing mean cell size and mean cytoplasm size of sorted, unstimulated CD8⁺ T cell subsets. The cells were stained according to the May-Grünwald and Giemsa protocols. Imaging was performed at 100x oil immersion. The value for every biological replicate was calculated as the mean of 100 analyzed cells for each subset. **a)** Measurement of cell size, n = 3; **b)** Measurement of the size of the cytoplasm, n = 3. N = biological replicates, ns = not significant, p < 0.05 (*), p < 0.01 (**), p < 0.001 (***)

May-Grünwald staining

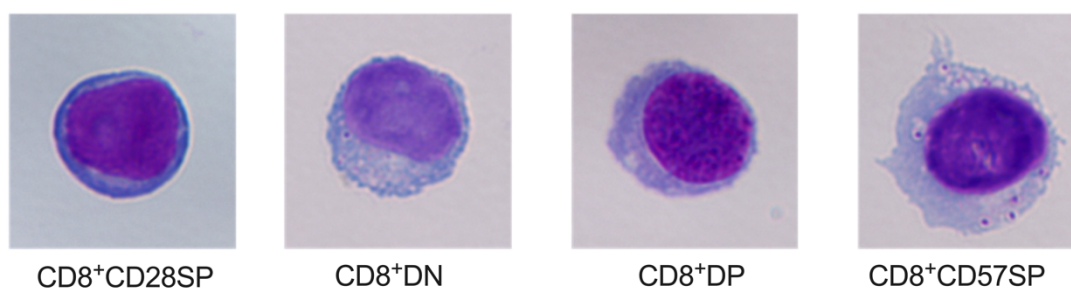


Figure 19: Representative images of sorted, unstimulated CD8⁺ T cell subsets after May-Grünwald staining Cytospin preparation, and imaging at 100 x oil immersion, representative images of 100 cells in each subset from three donors.

5.7 Analysis of mitochondrial function and glucose metabolism

5.7.1 Glucose uptake is impaired in CD8⁺CD57SP T cells

The cells' glucose uptake was assessed by flow cytometry using the fluorescent glucose analog 2-NBDG (2-(7-Nitro-2,1,3-benzoxadiazol-4-yl)-D-glucosamine). Three measurements over 45 minutes revealed that 2-NBDG uptake was significantly lower in the CD8⁺CD57SP subset compared to all the other subsets (Figures 20a-d).

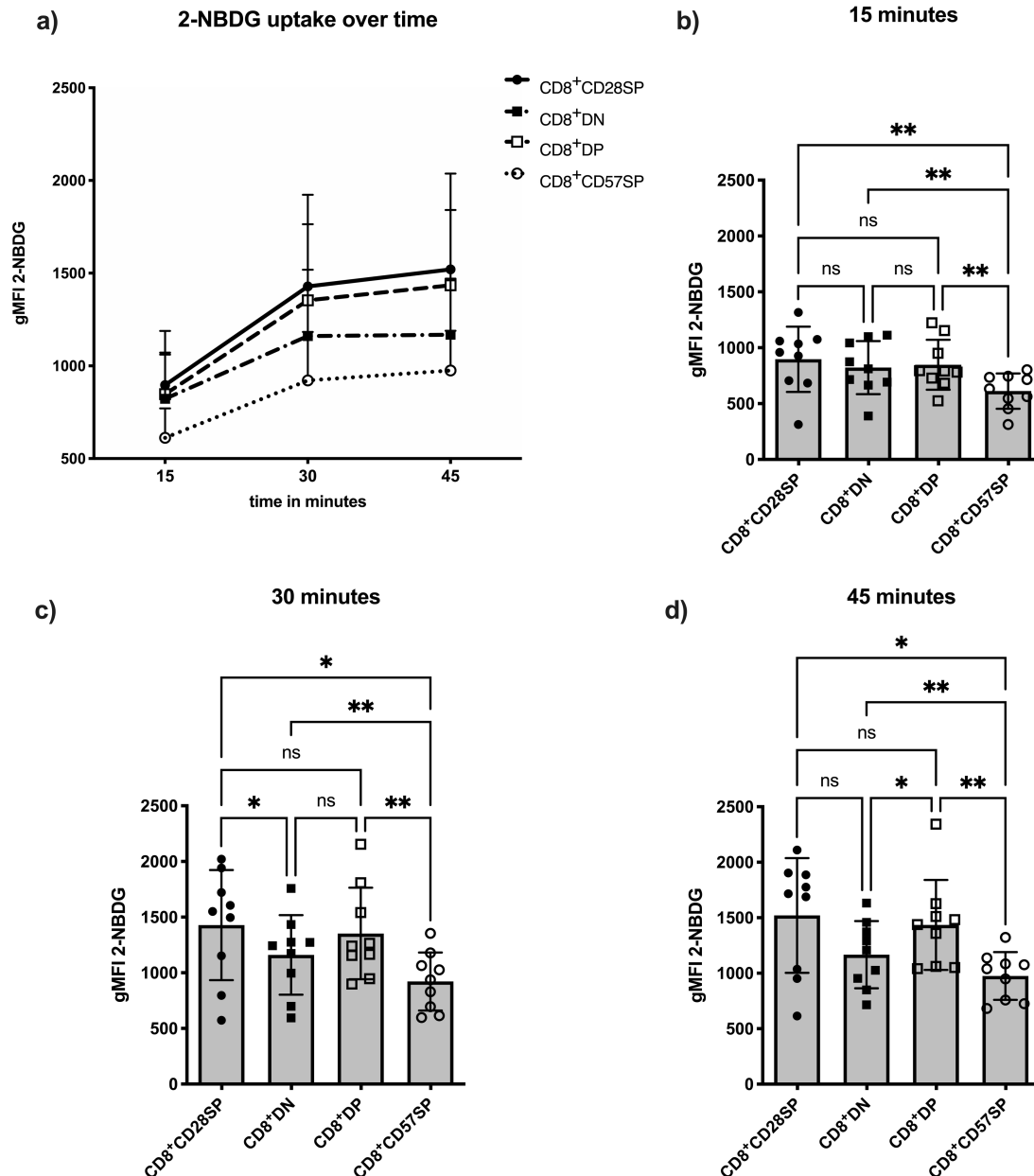


Figure 20: Glucose uptake by measurement of glucose analog 2-NBDG Scatter dot plots showing 2-NBDG fluorescence intensity in CD8⁺ T cell subsets. PBMCs were stimulated over 48 hours with 30ng/mL OKT-3. The uptake of 2-NBDG was assessed by gating on CD8⁺ T cell populations using flow cytometry. **a)** Fluorescence intensity of 2-NBDG over 45 minutes, n = 3 **b)** Fluorescence intensity of 2-NBDG at 15 minutes, n = 9 **c)** Fluorescence intensity of 2-NBDG at 30 minutes, n = 9 **d)** Fluorescence intensity of 2-NBDG at 45 minutes, n = 9; Data are presented as mean $\pm$ SD, n=biological replicates, ns=not significant, $p < 0.05$ (*), $p < 0.01$ (**)

5.7.2 Expression of the Glucose transporter GLUT-1 in CD8⁺CD57SP T cells

As previously described, the uptake of Glucose in senescent cells was low. Therefore, the Glucose transporter-1 (GLUT-1) expression was measured by flow cytometry. GLUT-1 expression was evaluated across various CD8⁺ T cell subsets on the day of PBMC isolation and after stimulation. Differences in GLUT-1 expression were assessed by comparison of geometric MFI, as >90% of T cells expressed GLUT-1 on the cell surface (data not shown). In freshly isolated, unstimulated CD8⁺ T cells, the highest levels of GLUT-1 expression among all T cell subsets were observed in the CD8⁺DP subset (Figure 21a). Interestingly, there were no significant changes in GLUT-1 expression levels after stimulation (OKT-3 and IL-2). Overall, no differences in the expression of GLUT-1 could be observed between the CD8⁺CD57SP T cell subset and the naïve-like CD8⁺CD28SP and CD8⁺DN subsets. In both unstimulated and stimulated conditions, GLUT-1 was significantly lower in CD8⁺CD57SP T cells compared to CD8⁺DP T cells.

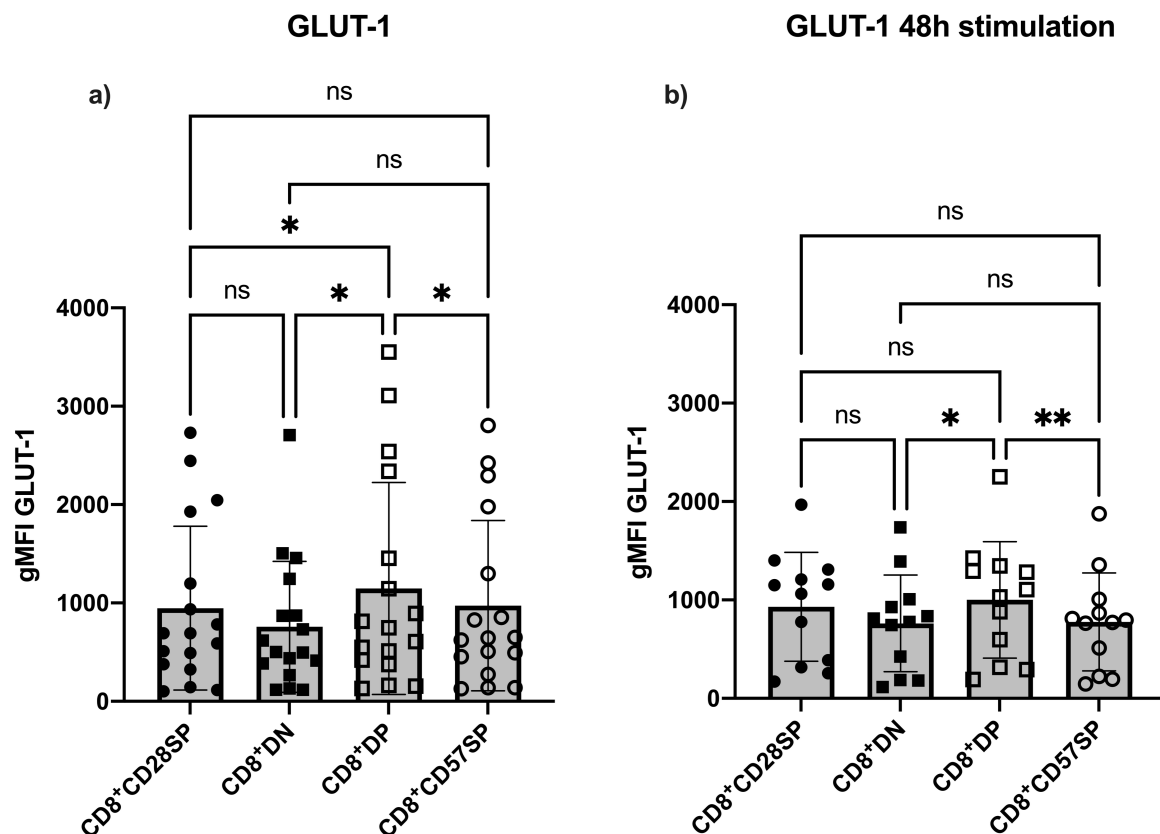


Figure 21: Expression of the glucose transporter GLUT-1 Scatter dot plots showing GLUT-1 expression in unstimulated and stimulated CD8⁺ T cells, assessed by flow cytometry; **a)** Expression of GLUT-1 on freshly isolated, unstimulated T cells, n = 17 **b)** Expression of GLUT-1 after 48 hours of stimulation with OKT-3 and IL-2, n = 12. Data are presented as mean $\pm$ SD, n = biological replicates, ns = not significant, $p < 0.05$ (*), $p < 0.01$ (**)

5.7.3 CD8⁺CD57SP T cells show an increased expression of the NAD⁺ depleting enzyme CD38

Maintaining a balanced ratio of NAD⁺/NADH, along with sufficient mitochondrial NAD⁺, is essential for optimal cellular function. Decreased NAD⁺ levels are significantly linked to a deterioration in both cellular and mitochondrial functions associated with aging. The expression of the NAD⁺-depleting enzyme CD38 was measured in freshly isolated, unstimulated CD8⁺ T cells to evaluate its role in modulating NAD⁺ levels across different T cell subsets (Figure 22). The expression of CD38 was found to be significantly elevated in the CD8⁺CD57SP subset (mean = 31.9%), as compared to the naïve-like CD8⁺CD28SP (mean = 19%) and CD8⁺DP (mean = 20.1%) subsets. An elevated expression of CD38 was also found on CD8⁺DN T cells (mean = 31.5%), which was comparable to the CD8⁺CD57SP subset. These findings highlight the role of CD38 in modulating NAD⁺ levels, with significantly elevated CD38 expression observed in CD8⁺CD57SP and CD8⁺DN T cell subsets compared to their naïve counterparts.

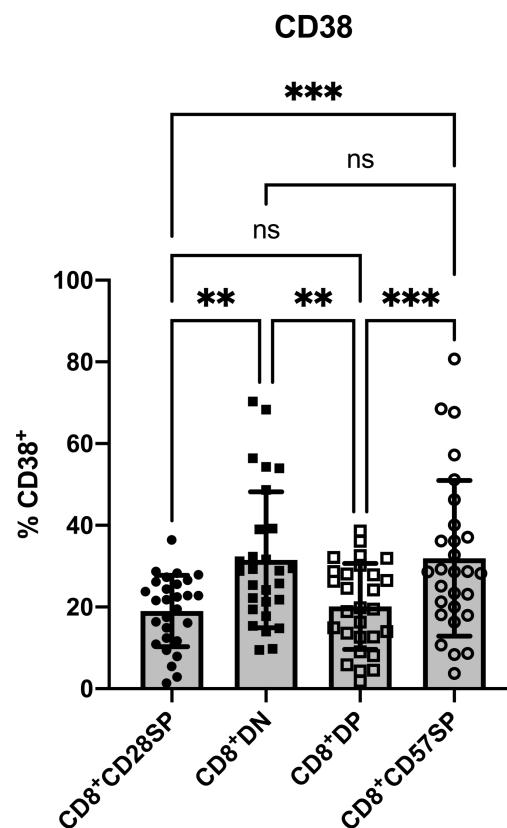


Figure 22: Expression of the NAD⁺ glycohydrolase CD38 Scatter dot plot showing the percentage of CD38 expression in freshly isolated CD8⁺ T cells, assessed by flow cytometry; $n = 28$; Data are presented as mean $\pm$ SD, $n =$ biological replicates, ns = not significant, $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***)

5.7.4 Mitochondrial mass and mitochondrial membrane potential

The combined use of MitoTracker Green (MG) and Deep Red (MDR) dyes was employed to simultaneously assess mitochondrial mass and membrane potential and identify depolarized mitochondria (Figure 23) within different subsets of unstimulated CD8⁺ T cells. MG binds to mitochondria independently of their membrane potential, allowing for the measurement of mitochondrial mass. In contrast, MDR depends on mitochondrial membrane potential and is used as a marker for mitochondrial function.

The CD8⁺DP T cell subset had the highest mitochondrial mass, as indicated by the increased mean fluorescence intensity of MG. Furthermore, this subset contained the highest percentage of cells with abundant mitochondrial content and high mitochondrial membrane potential (mean = 53.52%) (Figure 24a+b).

Senescent-like CD8⁺CD57SP T cells exhibited a higher mitochondrial mass and contained more cells with high mitochondrial mass and mitochondrial membrane potential than the CD8⁺DN subset, although no significant differences were observed compared to the naïve CD8⁺CD28SP cells (Figure 24a,b).

Cells with low mitochondrial mass and mitochondrial membrane potential were predominantly found in the CD8⁺DN subset. Senescent-like CD8⁺CD57SP T cells showed a higher number of cells containing mitochondria with low mitochondrial mass and mitochondrial membrane potential than CD8⁺DP cells. However, their numbers were comparable to naïve-like CD8⁺CD28SP T cells (Figure 24c). Depolarized mitochondria, defined as cells with a high mitochondrial mass but diminished membrane potential, were rare in unstimulated CD8⁺ T cells with mean values below 2% (Figure 24d).

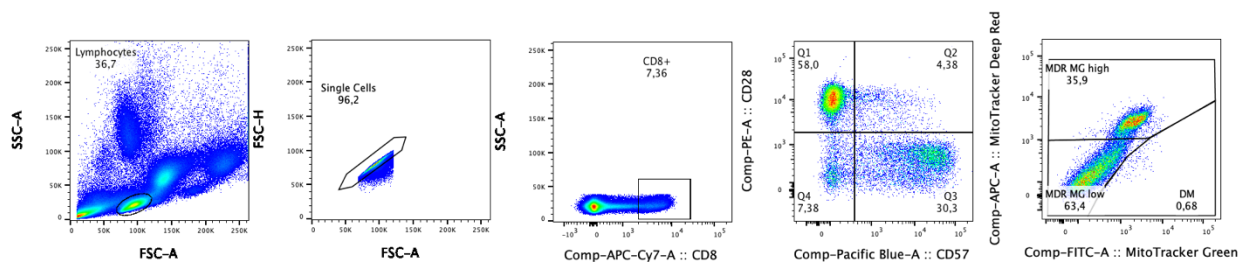


Figure 23: Gating strategy for MitoTracker Green and MitoTracker Deep Red staining
Representative gating strategy for assessing mitochondrial mass (MitoTracker Green) and membrane potential (MitoTracker Deep Red) to detect dysfunctional mitochondria (DM). Lymphocyte and single-cell populations were gated, followed by CD8⁺ cells. These were further divided into CD28/CD57 subsets and classified into three groups based on MitoTracker expression: high mitochondrial mass and potential (MDR, MG high), low mass and potential (MDR, MG low), and dysfunctional mitochondria (high mass, low potential or DM).

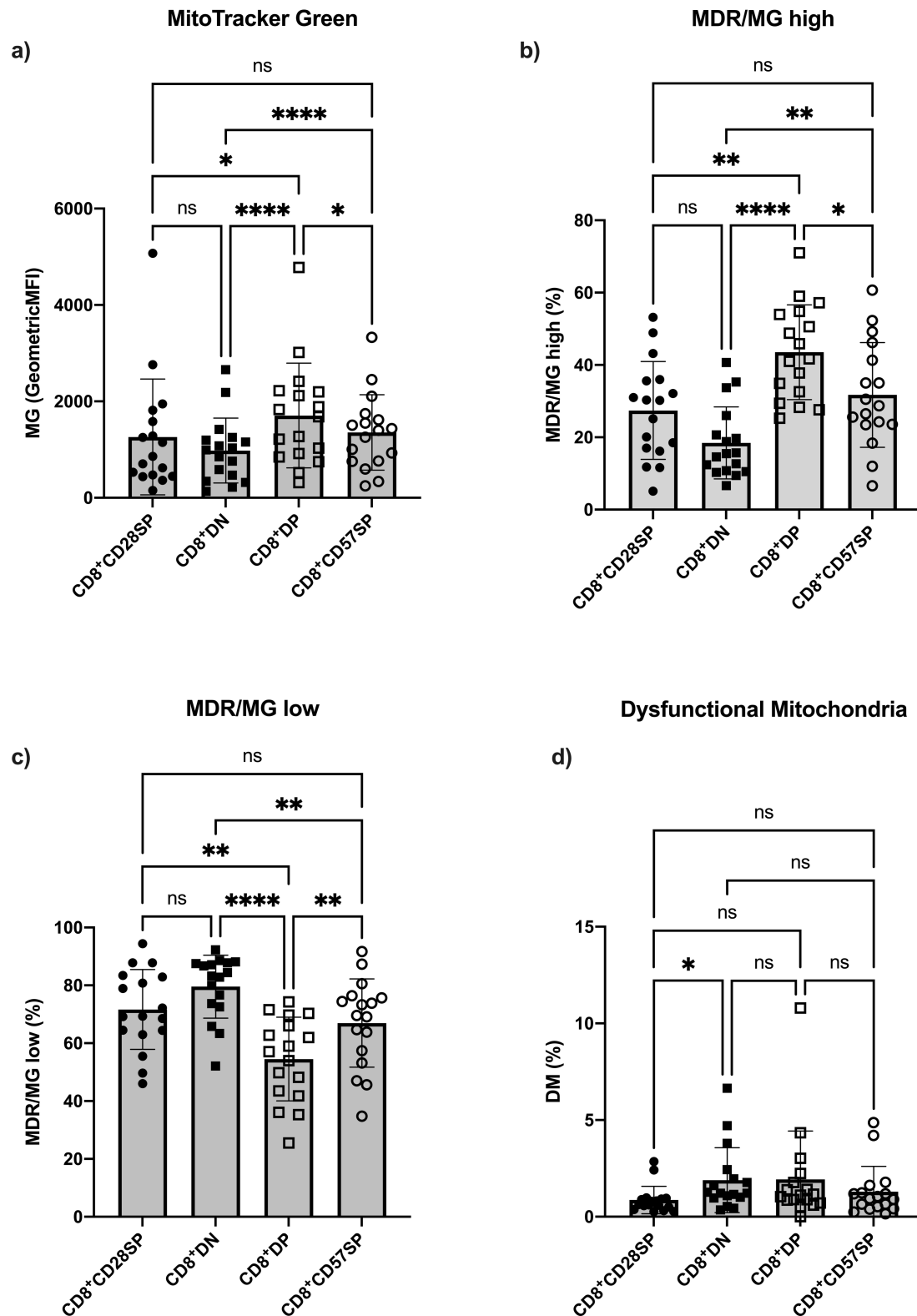


Figure 24: Analysis of mitochondrial mass and functional states of mitochondria Scatter dot plots showing the fluorescence intensity of MitoTracker Green and the percentages of mitochondrial subsets according to the differential intensity of MitoTracker Green and MitoTracker Deep Red in freshly isolated, unstimulated CD8⁺ T cells, assessed by flow cytometry. **a)** Mitochondrial mass indicated by gMFI of MitoTracker Green, $n = 17$. **b)** Mitochondria with high mitochondrial mass and membrane potential, $n = 17$ **c)** Mitochondria with low mitochondrial mass and membrane potential, $n = 17$ **d)** Depolarized mitochondria, $n = 17$. Data are presented as mean $\pm$ SD, n = biological replicates, ns = not significant, $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****)

5.7.5 CD8⁺CD57SP T cells show a high mitochondrial membrane potential

The geometric mean fluorescence intensity of TMRE was measured in freshly isolated, unstimulated CD8⁺ T cells to assess mitochondrial membrane potential. CD8⁺CD57SP T cells exhibited significantly higher TMRE intensity than naïve-like CD8⁺CD28SP ($p = 0.004$) and CD8⁺DN T cells ($p = 0.007$). Similarly, CD8⁺DP T cells displayed significantly higher TMRE intensity than naïve-like CD8⁺CD28SP ($p = 0.035$) and CD8⁺DN T cells ($p = 0.048$), but showed no significant difference compared to CD8⁺CD57SP T cells (Figure 25a).

MDR fluorescence intensity, used to assess mitochondrial membrane potential showed a similar trend. CD8⁺CD57SP T cells had significantly higher MDR intensity than CD8⁺DN T cells ($p = 0.0002$). CD8⁺DP T cells also exhibited higher MDR intensity compared to naïve-like CD8⁺CD28SP ($p = 0.0007$) and CD8⁺DN ($p = 0.0005$) subsets, while no significant differences were observed between CD8⁺CD57SP and CD8⁺DP T cells (Figure 25b).

After 48 hours of stimulation with OKT-3 and IL-2, TMRE fluorescence intensity increased across all T cell subsets, indicating an overall elevation in mitochondrial membrane potential (Figure 25c). Notably, CD8⁺CD57SP and CD8⁺DP T cells maintained the highest mitochondrial membrane potential after stimulation, while naïve-like CD8⁺CD28SP and CD8⁺DN subsets consistently exhibited lower mitochondrial membrane potential before and after stimulation (Figures 25a+c). Overall, CD8⁺CD57SP and CD8⁺DP T cells demonstrate significantly elevated mitochondrial membrane potential, as indicated by both TMRE and MDR intensities, compared to naïve-like CD8⁺CD28SP and CD8⁺DN subsets. This elevated mitochondrial membrane potential persists after stimulation.

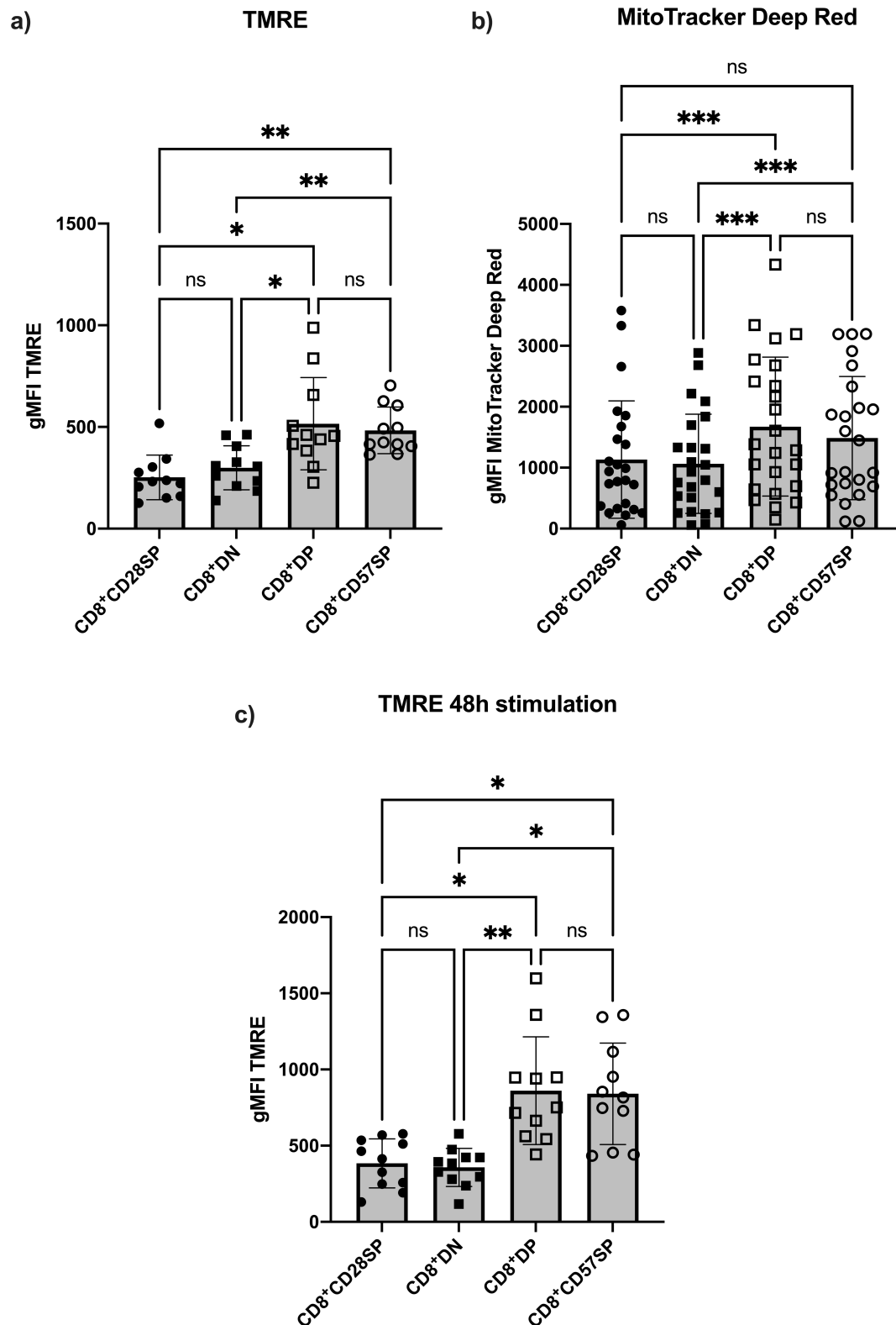


Figure 25: Mitochondrial membrane potential Scatter dot plots showing geometric mean fluorescence intensity of TMRE and MitoTracker Deep Red to determine mitochondrial membrane potential in CD8⁺ T cell subsets, assessed by flow cytometry; **a)** TMRE on the day of PBMC isolation, n = 11. **b)** MitoTracker Deep Red on the day of PBMC isolation, n = 24 **c)** TMRE staining after a 48-hour incubation period with OKT-3 and IL-2, n = 11. Data are presented as mean $\pm$ SD, n=biological replicates, ns=not significant, $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***)

5.7.6 Reactive oxygen species in T cell mitochondria

The buildup of reactive oxygen species (ROS) from mitochondria contributes to senescence and age-related diseases. We therefore measured mitochondria-derived ROS in CD8⁺ T cells before and after stimulation for 48 hours using the MitoSOX Red dye.

The highest percentage of cells expressing mitochondrial superoxide was found in the CD8⁺DP subset of freshly isolated CD8⁺ T cells (mean = 26.1%). The mitochondrial superoxide levels in CD8⁺CD57SP T cells were significantly higher than in naïve-like CD8⁺CD28SP T cells ($p = 0.026$) but not significantly different from the CD8⁺DN subset (Figure 26a). After stimulation, mitochondrial superoxide production increased in all subsets except the CD8⁺DN T cells (Figure 26b). The CD8⁺DP subset showed the highest levels of mitochondrial superoxide production after stimulation (mean = 80%), followed by the naïve-like CD8⁺CD28SP subset (mean = 63.5%). In contrast, CD8⁺DN (mean = 13.18%) and CD8⁺CD57SP T cells (mean = 34.46%) showed significantly lower levels. Treatment with 200 μ M H₂O₂ was performed as a positive control, and showed induction of MitoSOX⁺ CD8⁺ T cells ($p = 0.0045$) (Figure 26c).

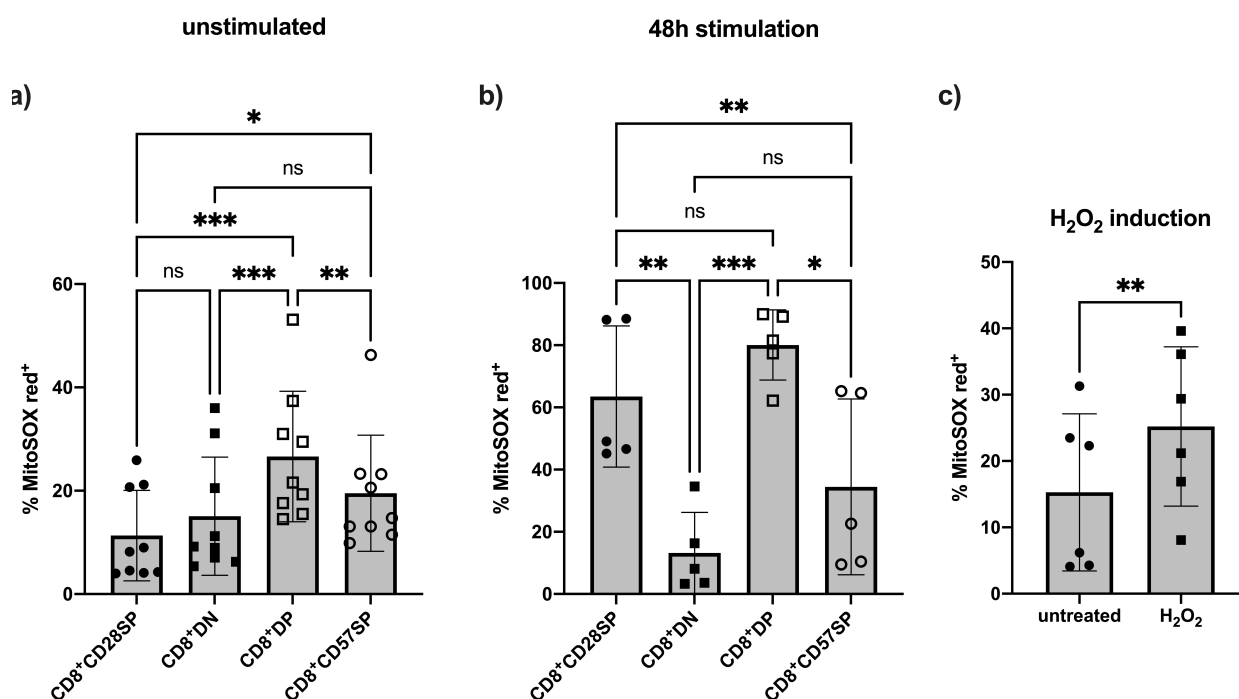


Figure 26: Mitochondrial superoxide Scatter dot plots showing the percentage of cells stained with MitoSOX in unstimulated and stimulated CD8⁺ T cell subsets **a)** Measurement on the day of PBMC isolation, $n = 9$. **b)** Measurement after a 48-hour incubation period with OKT-3, $n = 5$. **c)** Effect of treatment on bulk CD8⁺ T cells with 200 μ M H₂O₂ (used as an inducer of superoxide), $n = 6$; Data are presented as mean $\pm$ SD, n =biological replicates, ns=not significant, $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***)

5.7.7 CD8⁺CD57SP T cells show signs of mitochondrial dysfunction

Parameters of mitochondrial respiration were measured using the Agilent Seahorse XF Cell Mito Stress Test, during which the oxygen consumption rate (OCR) of cells and the extracellular acidification rate (ECAR), as a marker of lactate production, are continuously measured over time after the sequential injection of Oligomycin, FCCP, and Rotenone/Antimycin A (Figure 27a). The experiment was performed with CD8⁺ T cells, sorted into CD8⁺DN, CD8⁺DP and CD8⁺CD57SP subsets, and stimulated for 48 hours (OKT-3 and IL-2) prior to the assay. Figure 27b shows the result from a representative experiment, exhibiting the changes in OCR after the sequential injection of the different compounds as depicted in Figure 27a. It can be observed that the senescent-like CD8⁺CD57SP T cells subset had an overall low OCR. These cells also exhibited a low ECAR profile (Figure 27 c,d), although significance was not reached, most likely due to a small sample size. The low ECAR indicates low lactic acid levels in the cell's surroundings.

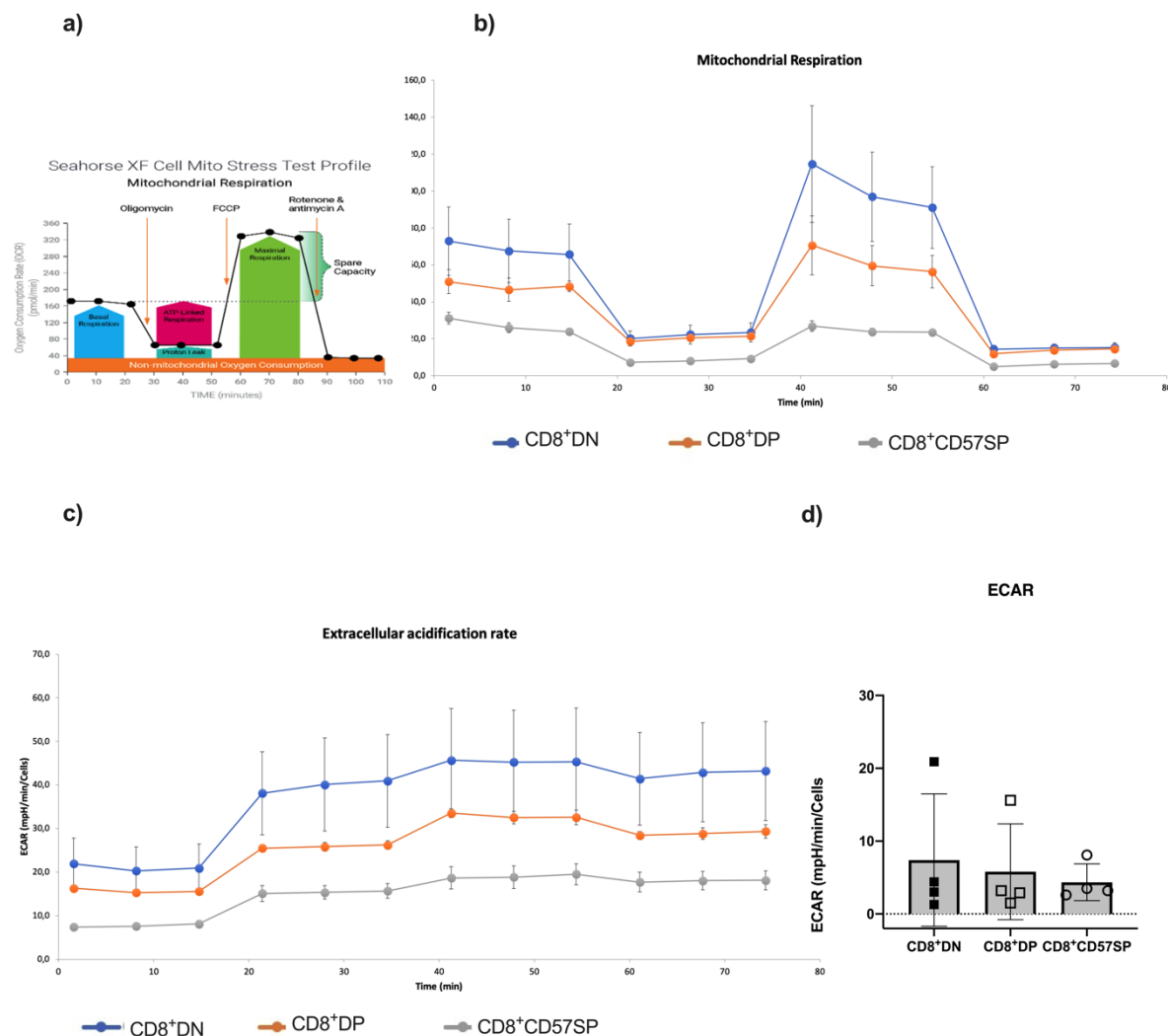


Figure 27: Analysis of mitochondrial respiration Experiments were performed using the Agilent Seahorse XF Cell Mito Stress Test **a)** Principle of the Seahorse XF Cell Mito Stress Test; image by Agilent Technologies 8 (Agilent Seahorse XF Cell Mito Stress Test Kit, User Guide Kit 103015-100) **b)** Measurement of the oxygen consumption rate (OCR) over time, result from a representative experiment **c)** Measurement of extracellular acidification rate (ECAR), result from a representative experiment **d)** ECAR (before injection of Oligomycin); $n = 4$; Data are presented as mean $\pm$ SD, $n =$ biological replicates

Further data analysis revealed a trend towards reduced metabolic activity in the CD8⁺CD57SP T cell subset. Specifically, this subset showed lower levels of basal respiration, maximal respiration, spare respiratory capacity, ATP production, and non-mitochondrial oxygen consumption compared to the other T cell subsets (Figure 28). These parameters of mitochondrial function are not directly measured but are calculated based on the OCR measurements after injection of the compounds. Likely due to the small sample size, these differences did not reach statistical significance.

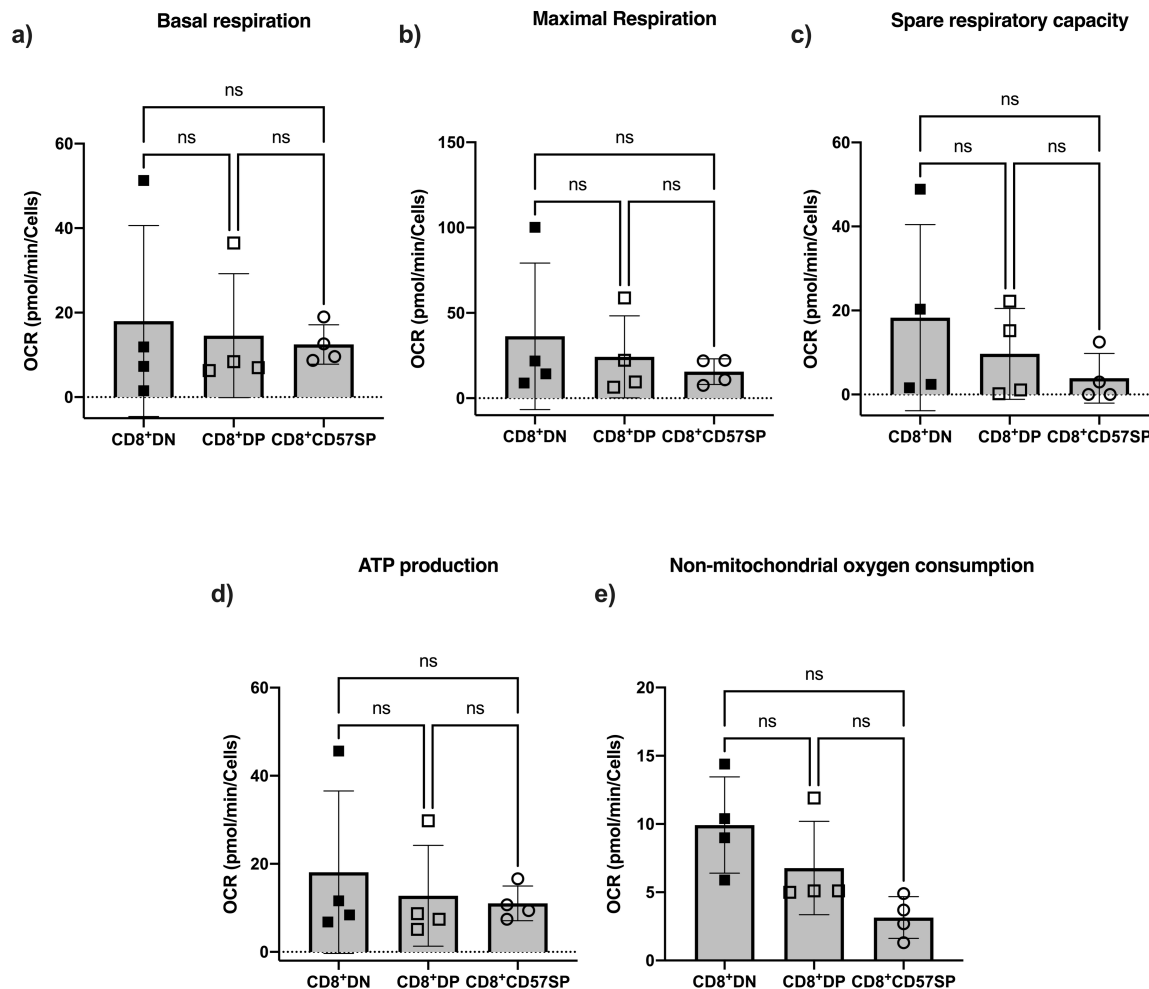


Figure 28: Key parameters of mitochondrial function Scatter dot plots showing key parameters of mitochondrial function based on OCR; experiments were performed using the Agilent Seahorse XF Cell Mito Stress Test **a)** Basal respiration; $n = 4$. **b)** Maximal respiration; $n = 4$. **c)** Spare respiratory capacity; $n = 4$; **d)** ATP production; $n = 4$; **e)** Non-mitochondrial oxygen consumption; $n = 4$. Data are presented as mean $\pm$ SD, $n =$ biological replicates, ns = not significant

5.8 Expression of Lactate Dehydrogenase subunits in CD8⁺ T cells

5.8.1 Establishment of the intracellular measurement of LDH subunits A and B

Lactate dehydrogenase (LDH) is a key metabolic enzyme that links glycolysis and oxidative phosphorylation (OXPHOS) by catalyzing the interconversion of pyruvate and lactate. LDHA and LDHB form distinct subunits of this enzyme, regulating the interconversion.

The DAUDI cell line, known for its high LDHA expression, was used as positive control to establish the intracellular measurement of LDHA. Flow cytometric analysis revealed an LDHA expression of 96.2% on DAUDI cells (Figure 29a). The HeLa cell line was used as positive control to establish the intracellular measurement of LDHB. Flow cytometric measurement revealed an LDHB expression of 99.8% on HeLa cells (Figure 29b). Following the successful measurement of LDHA subunits in established positive control cell lines, staining was performed on T cells, showing clear populations positive for LDHA in stimulated CD8⁺ T cells (45%) (Figure 29c) and positive for LDHB in unstimulated CD8⁺ T cells (97,2%) (Figure 29d).

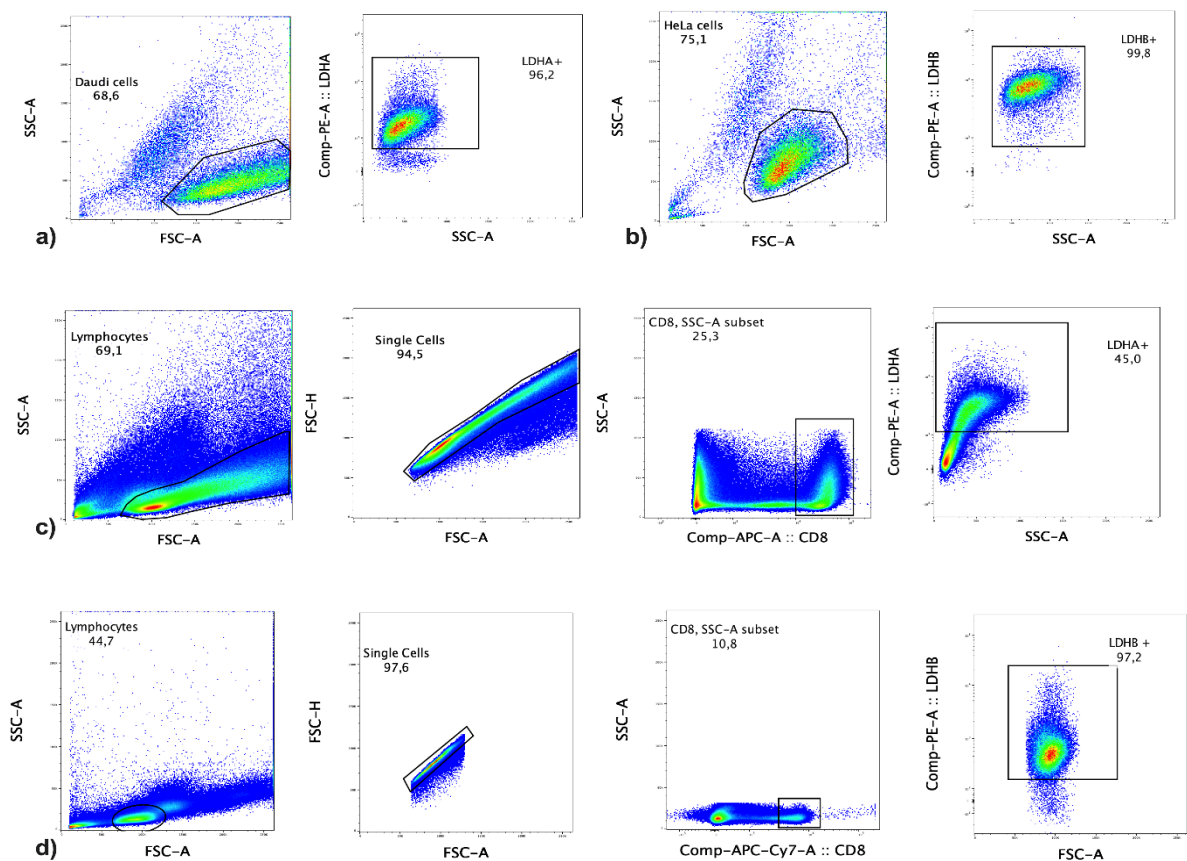


Figure 29: Establishment of intracellular LDHA and LDHB measurement by flow cytometry a) Gating strategy for LDHA in the DAUDI cell line b) Gating strategy for LDHB in the HeLa cell line c) Gating strategy for LDHA in PBMCs stimulated with 30ng/mL OKT-3 and 600 U/mL IL-2 for 48 hours d) Gating strategy for LDHB in PBMCs unstimulated, cultured for 48 hours; representative of three measurements

5.8.2 LDHA Expression in CD8⁺ T cells strongly increases after stimulation

LDHA expression was found to be low in freshly isolated CD8⁺ T cells, with a mean of 5.19%. This expression significantly decreased further after 48 hours of resting in culture without stimulation, reaching a mean of 2.38%. However, upon stimulation with OKT-3 and IL-2 for 48 hours, LDHA expression markedly increased, reaching a mean of 36.23% (Figure 30a). When gating by CD28 and CD57 expression, freshly isolated CD8⁺DP T cells exhibited significantly higher LDHA expression (as measured by gMFI) compared to all other subsets (Figure 30b). In 48-hour resting cells, senescent-like CD8⁺CD57SP T cells showed significantly lower LDHA levels compared to naïve-like CD8⁺CD28SP and CD8⁺DN subsets (Figure 30c). After 48 hours of stimulation, CD8⁺DP and naïve-like CD8⁺CD28SP T cells showed significantly higher LDHA expression than both CD8⁺DN and senescent-like CD8⁺CD57SP subsets, indicating a strong association between costimulatory marker expression and metabolic activation upon stimulation (Figure 30d).

5.8.3 LDHB Expression is low in senescent CD8⁺ T cell subsets

In contrast to LDHA, LDHB was expressed at high levels in CD8⁺ T cells in resting and stimulated conditions. In freshly isolated cells, LDHB expression was already high, with a mean of 74.14%, and increased to 96.88% after 48 hours of resting. After 48 hours of stimulation with OKT-3 and IL-2, expression levels rose slightly to 98.56%, with no significant difference between resting and stimulated conditions (Figure 31a). In freshly isolated T cells, LDHB expression (based on the gMFI) was highest in naïve-like CD8⁺CD28SP T cells, with lower levels observed in CD8⁺DP and senescent-like CD8⁺CD57SP subsets (Figure 31b). After 48 hours of resting, CD8⁺CD28SP and CD8⁺DN T cells displayed similarly high LDHB expression, while the senescent-like CD8⁺CD57SP and the CD8⁺DP subsets exhibited significantly lower levels. Notably, the lowest LDHB expression under this condition was observed in CD8⁺DP T cells (Figure 31c). Upon 48-hour stimulation, naïve-like CD8⁺CD28SP and CD8⁺DP T cells showed the highest LDHB expression, while senescent-like CD8⁺CD57SP (and CD8⁺DN) T cells maintained the lowest expression, consistent with a reduced metabolic profile in senescent-like subsets (Figure 31d). Further analysis based on CD27/CD45RA-defined subsets revealed that T_{EMRA} (CD27⁻CD45RA⁺) T cells exhibited the lowest LDHB expression compared to all other subsets, independently of the culture condition (freshly isolated, resting, or stimulated) (Figures 32a-c). In contrast, central memory (T_{CM}) and effector memory (T_{EM}) subsets showed significantly higher LDHB levels compared to naïve (CD27⁺CD45RA⁺) T cells following stimulation (Figure 32c).

When LDHB expression was evaluated based on KLRG-1 status, KLRG-1⁺ T cells showed significantly lower expression compared to KLRG-1⁻ T cells, further supporting the association between metabolic activity and differentiation or senescence status (Figure 32d).

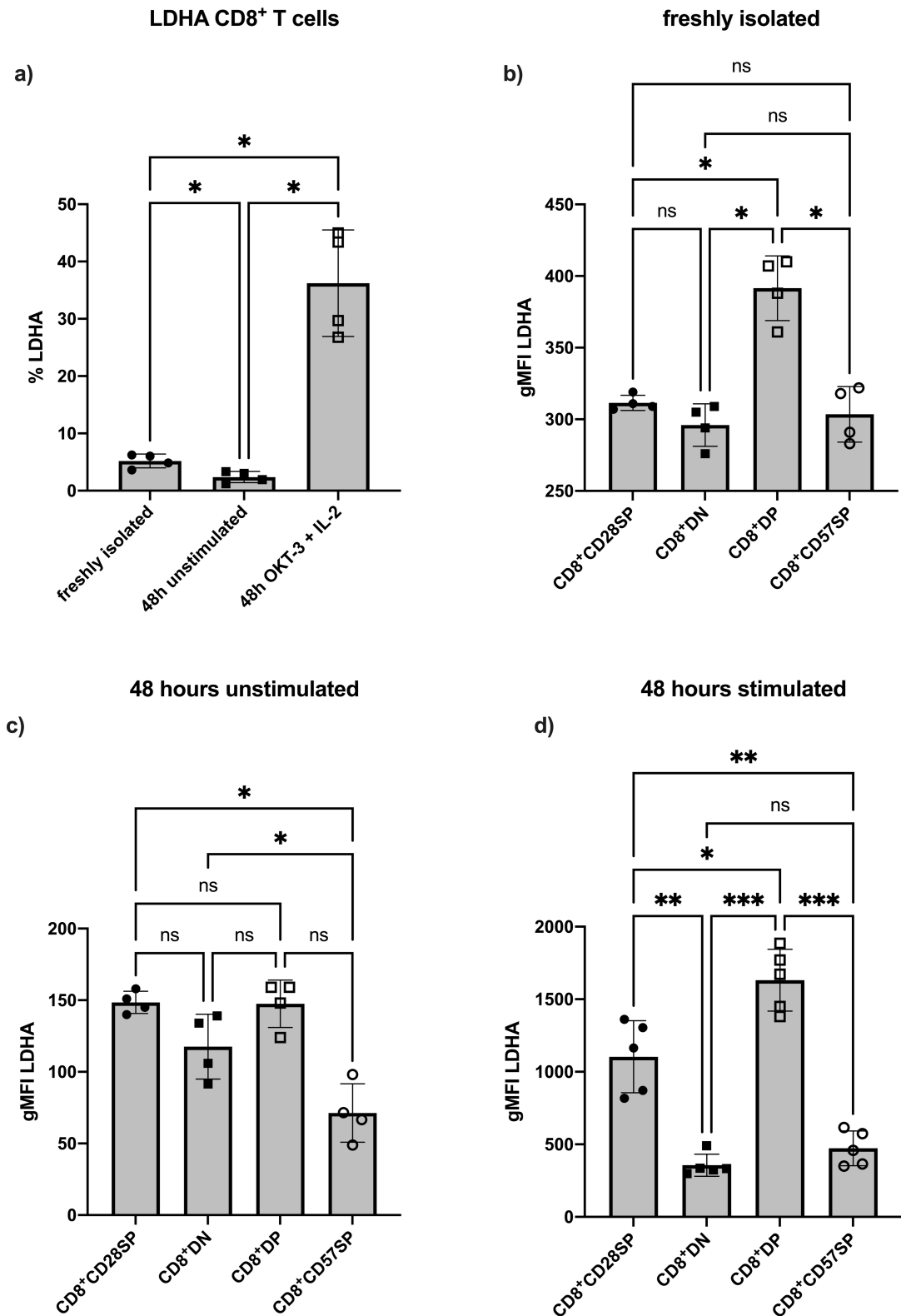


Figure 30: LDHA expression in CD8⁺ T cells Scatter dot plots showing the percentage and geometric mean fluorescence intensity (gMFI) of LDHA in CD8⁺ T cells **a)** Percentage of LDHA⁺ cells in freshly isolated, resting and stimulated CD8⁺ T cells, n = 4. **b)** gMFI of LDHA in CD8⁺ T cells subsets, freshly isolated, n = 4. **c)** gMFI of LDHA in CD8⁺ T cells subsets, 48 hours resting, n = 4 **d)** gMFI of LDHA in CD8⁺ T cells subsets, 48 hours after stimulation with OKT-3 and IL-2, n = 4; Data are presented as mean $\pm$ SD, n = biological replicates, ns = not significant, $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***)

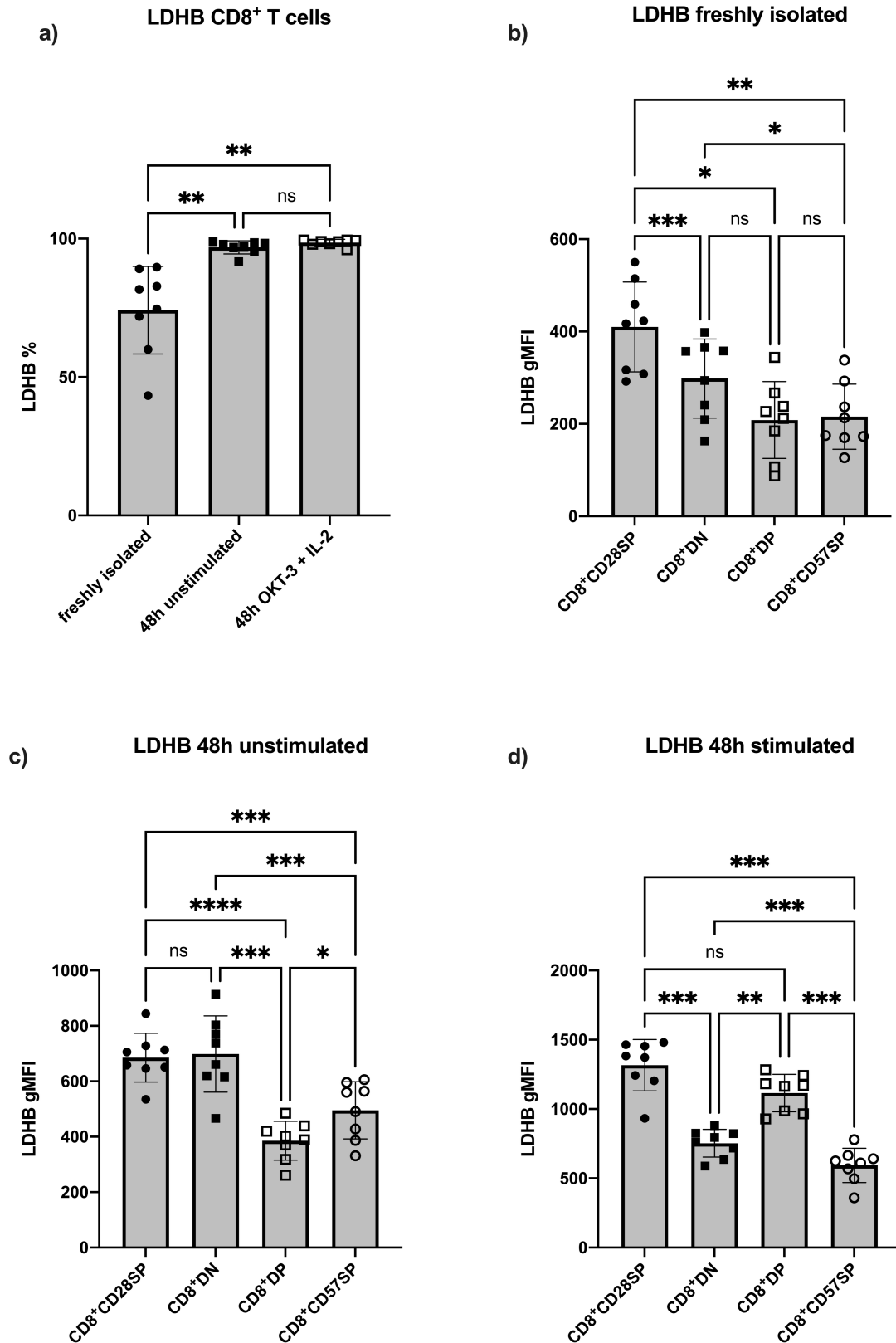


Figure 31: LDHB expression in CD8⁺ T cell subsets by CD28 and CD57 expression
 Scatter dot plots showing the percentage and geometric mean fluorescence intensity (gMFI) of LDHB in CD8⁺ T cells. **a)** Percentage of LDHB⁺ cells in freshly isolated, resting and stimulated CD8⁺ T cells, $n = 8$. **b)** gMFI of LDHB in freshly isolated T cell subsets, $n = 8$. **c)** gMFI of LDHB in 48 h resting T cell subsets, $n = 8$. **d)** gMFI of LDHB in the different T cell subsets, 48 hours after stimulation with OKT-3 and IL-2, $n = 8$; Data are presented as mean $\pm$ SD, n = biological replicates, ns = not significant, $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).

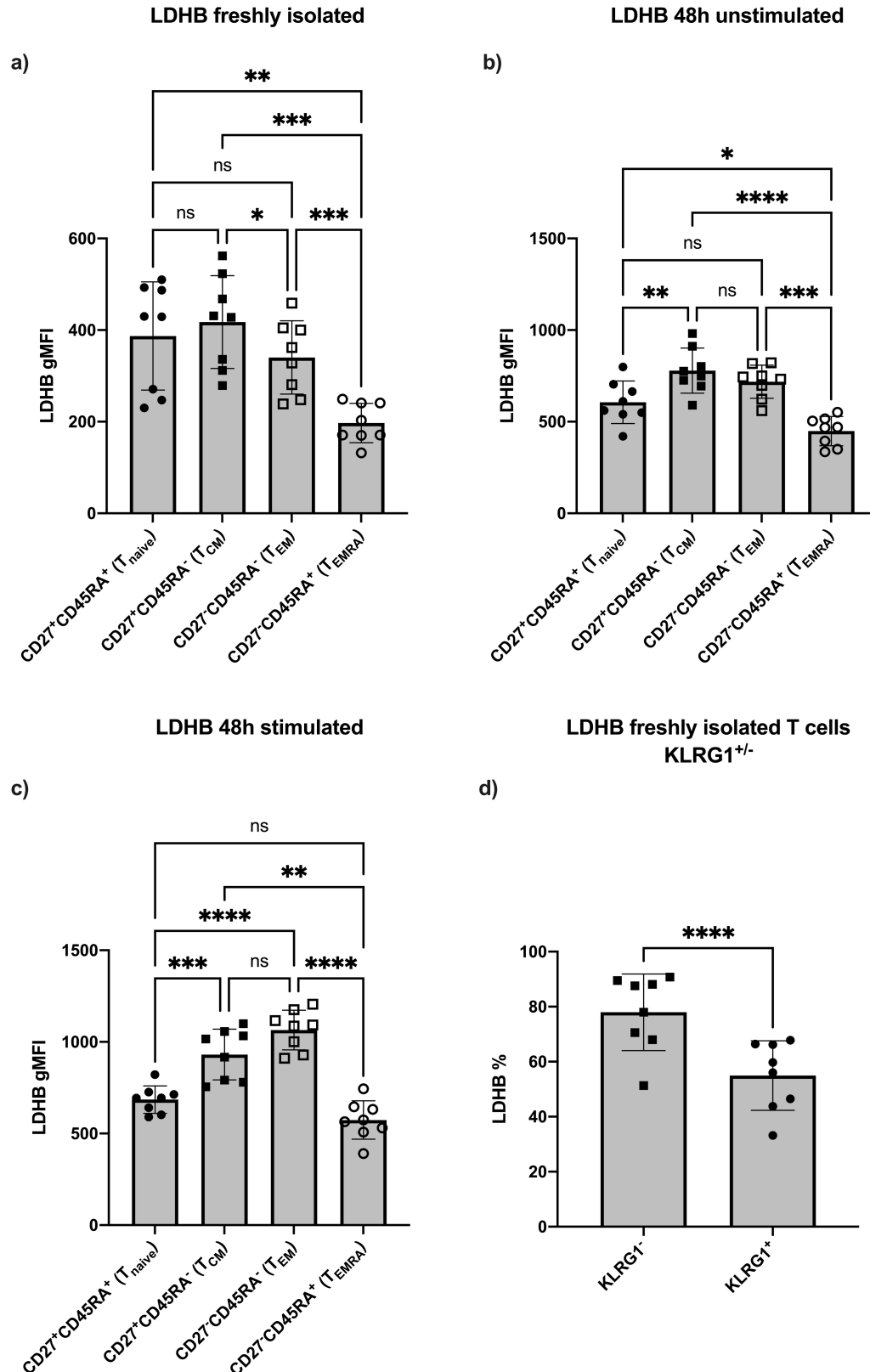


Figure 32: LDHB expression in CD8⁺ T cell subsets by CD27, CD45RA and KLRG-1 expression Scatter dot plots showing the percentage and geometric mean fluorescence intensity (gMFI) of LDHB in CD8⁺ T cells **a)** gMFI of LDHB, freshly isolated, n = 8. **b)** gMFI of LDHB, 48 h resting, n = 8 **c)** gMFI of LDHB, 48 hours OKT-3 and IL-2, n = 8 **d)** gMFI of LDHB in KLRG-1^{+/-} T cells, freshly isolated, n = 8; Data are presented as mean $\pm$ SD, n = biological replicates, ns = not significant, $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****)

6 Discussion

6.1 Identifying senescence-associated features in CD8⁺ T cells

Four distinct CD8⁺ T cell populations can be distinguished based on their differential expression of CD28 and CD57 markers.

In the first part of the study, we aimed to investigate the phenotype of CD8⁺CD57SP T cells, which were previously described as terminally differentiated and “senescent-like” (215). The four T cell populations were analyzed based on markers of differentiation and exhaustion and the T cell subsets were related to donor age. To further characterize CD8⁺CD57SP T cells, we evaluated their properties in cell culture, focusing on proliferation, apoptosis, morphology, cytotoxicity and the expression of classical senescence markers, such as SA-β-Gal and uPAR. Ultimately, this analysis aimed to determine whether the phenotype of CD8⁺CD57SP cells corresponds to previously established hallmarks of senescence, to offer a translational aspect of T cell senescence in cancer immunotherapy and to improve our understanding of their role in the aging process of the immune system.

We noted that the proportion of senescent-like CD8⁺CD57SP T cells in the peripheral blood of healthy individuals rises consistently with age, whereas the numbers of naïve CD8⁺CD28SP T cells decline as age increases. Additionally, the CD8⁺DP T cell subset demonstrated a moderate positive correlation with age. These findings are consistent with earlier research examining the distribution of CD8⁺ T cell subsets based on CD28 and CD57 expression as age increases (13, 15). Multiple studies have further distinguished these subsets based on CMV status. An earlier investigation revealed that the rise in CD8⁺CD57SP CD8⁺ T cells is influenced not only by age but also by CMV seropositivity. Conversely, the reduction of the CD8⁺CD28SP subset was found to be exclusively tied to advancing age (216). A study targeting the CD8⁺DP and CD8⁺DN subsets found that the frequencies of CD8⁺DP T cells only increased with age in CMV seronegative patients (13). These results might clarify why the proportions of the subsets, especially the CD8⁺DP subset, vary significantly among donors in our study. Therefore, future research on this T cell subset should take CMV status into account.

6.1.1 Impaired proliferative capacity of senescent CD8⁺ T cells

Several studies have shown that CD8⁺ T cells, which express the surface marker CD57 and have lost the co-stimulatory receptor CD28, do not proliferate and have reached a state of replicative senescence (89, 217-219). This concept was supported by the observation that CD8⁺CD28⁻ (CD8⁺CD57⁺) T cells have shortened telomeres and reduced telomerase activity (220-222).

This study evaluated the proliferation of CD8⁺ T cell subsets „in vitro“ using two different methods. The first method, which utilized population doubling levels (PDL) after stimulation

with anti-CD3/CD28 beads and IL-2, demonstrated that CD8⁺CD57^{SP} T cells failed to proliferate. At first, this implies that these cells display characteristics of senescence, as proliferative arrest is a hallmark of this state. Interestingly, the CD8⁺DP subset displayed intact proliferative capacity, suggesting that CD57 does not universally inhibit proliferation.

The second method, using eFluor 670 dye dilution and employing a different stimulation protocol by using OKT-3 and IL-2, revealed a lower proliferation in the CD8⁺CD57^{SP} subset than in naive CD8⁺CD28^{SP} cells after three days of PBMC stimulation. This result aligns with findings from a prior study that reported reduced proliferation in CD8⁺CD57^{SP} T cells after CFSE labeling and anti-CD3 stimulation for four days (13). After five days, however, no significant differences in efluor reduction between the groups were observed. This observation challenges the prevailing concept that CD8⁺CD57^{SP} T cells have entered a state of complete cell cycle arrest.

Several other studies have shown that this subset can proliferate under certain stimulation conditions. A previous study investigated CD8⁺ T cell subsets from the peripheral blood of head and neck cancer patients revealed similar levels of proliferation in CD27/28⁻CD57⁺ and CD27/28⁺ T cell populations. Furthermore, stimulation with CD2/3/28 beads and IL-2 revealed that CD27/28⁻CD57⁺ T cells were able to maintain proliferation, although slightly reduced compared to CD27/28⁺ T cells. Interestingly, the authors noted that the proliferation of CD27/28⁻CD57⁺ cells was improved with IL-15 stimulation (223).

The observed differences in proliferation between the two approaches may result from the different experimental conditions. Initially, our first method utilized anti-CD3/CD28 bead stimulation, potentially benefiting the CD28⁺ T cell subsets. Later experiments, however, switched to OKT-3, which solely targets CD3. Furthermore, it should be emphasized that in the first approach, sorted, “pure” T cell subsets were stimulated, while the protocol in the second approach involved stimulating bulk PBMCs and subsequent gating and analysis of the T cell subsets of interest. Our observation that the CD8⁺CD57^{SP} T cell subset does eventually proliferate after three days of stimulation is supported by findings from a previous study, which showed that the use of irradiated PBMCs provided several co-stimulatory ligands from B cells, dendritic cells, and macrophages. This led to a significant increase in telomerase activity and proliferation in CD8⁺CD28⁻ T cells (224).

A further limitation of this study is the reliance on IL-2 in our stimulation protocol, which may not be optimal for promoting proliferation in CD8⁺CD57^{SP} T cells. Previous findings suggest that IL-15 may improve proliferation in CD8⁺CD57^{SP} T cells. Therefore, future experiments should investigate this possibility (223). Moreover, incorporating additional cytokines like IL-7 or IL-21 into the stimulation protocol might improve the proliferative capacity of CD8⁺CD57^{SP} T cells (225). By optimizing the stimulation conditions through the use of alternative cytokines,

the technical challenges while performing cell culture experiments with senescent cells could be addressed. We currently face the issue that sorted CD8⁺CD57SP T cells are rather short-lived cells in vitro, limiting the range of experiments that can be performed. Overall, this study demonstrates that the proliferative potential of CD8⁺CD57SP cells depends on the stimulation conditions. While sorted CD8⁺CD57SP cells that received only anti-CD3/CD28 and IL-2 stimulation showed no proliferation, we found that this subset could proliferate after three days when cultured with bulk PBMCs and receiving OKT-3. Taken together with the results from previous studies, the notion that CD8⁺CD57SP cells are in a state of proliferative arrest needs further investigation to explore their behavior under varying cytokine conditions to determine optimal cell culture conditions.

6.1.2 Senescent T cells show increased apoptosis in cell culture

Besides irreversible cell cycle arrest, resistance to apoptosis is a feature of most, but not all, senescent cells (226). There exist conflicting reports regarding CD8⁺CD28⁻ (CD8⁺CD57⁺) T-cell sensitivity to apoptosis. Cell culture models of replicative senescence in CD8⁺ T cells, which have undergone multiple rounds of antigen-specific stimulation, have shown a loss of CD28 and a resistance to apoptosis (75). A different report showed that CD8⁺CD28⁻ T cells were less prone to apoptosis “ex vivo” and showed resistance to activation-induced apoptosis “in vitro” (227).

Several authors, however, have shown that CD8⁺CD28⁻ (CD8⁺CD57⁺) T-cells are susceptible to programmed cell death, as demonstrated by detection of apoptosis through assays such as annexin V binding, propidium iodide uptake and binding of activated caspase-3 (89, 228).

In freshly isolated PBMCs, apoptotic cells (Annexin V⁺/PI⁺) were most abundant in the CD8⁺DP and CD8⁺CD57SP subsets. Following a five-day stimulation with OKT-3 and IL-2, the percentage of apoptotic cells in these subsets increased significantly, culminating in apoptosis in about half of the CD8⁺DP and CD8⁺CD57SP populations. This indicates that while these subsets exhibit features of senescence, they are not apoptosis-resistant, as is commonly seen in other senescent cell types.

These findings align with prior studies examining the propensity of CD8⁺ T cell subsets to undergo apoptosis. For example, when PBMCs were treated with etoposide, an agent that induces DNA damage, the highest levels of apoptotic cells (7-AAD⁺/Annexin V⁺) were observed in the CD8⁺DP subset, followed by the CD8⁺CD57SP subset (13).

Furthermore, an earlier study investigated the expression of CD95 (Fas), a central apoptosis-related receptor in T cell subsets. The authors reported that CD8⁺DP cells showed the highest CD95 expression, followed by the CD8⁺CD57SP subset (15). The elevated CD95 expression

in the CD8⁺DP T cell subset may partially explain the increased apoptosis in response to stimulation, which we observed in our study using Annexin V and PI staining.

These results indicate that, unlike other senescent cell types resistant to apoptosis, CD8⁺ T cells with senescent characteristics from healthy individuals display a pronounced tendency toward apoptosis in cell culture.

6.1.3 Defining the surface marker signature of senescent T cells

By analyzing the CD8⁺CD57SP T cell subset, focusing on the expression of additional cell surface markers, we were able to further dissect the phenotype of senescent T cells.

The high frequencies of T_{EMRA} (CD27⁻CD45RA⁺) cells within this subset strongly suggest that these cells are terminally differentiated. The low percentage of central memory (CD27⁺CD45RA⁻) and naïve (CD27⁺CD45RA⁺) cells within this subset further supports the observation that CD8⁺CD57SP cells represent a stage of late T cell differentiation. In contrast, the CD8⁺DP subset is enriched in central memory cells, while the CD8⁺CD28SP subset contains the highest frequencies of naïve cells. Aging significantly influences the number of differentiated T cells, as seen by the increase in T_{EMRA} and effector memory (CD27⁻CD45RA⁻) T cells with age. This aligns with the accumulation of highly differentiated T cells, particularly within the CD8⁺CD57SP subset. The nearly complete loss of CCR7 and CD62L homing markers in senescent-like CD8⁺CD57SP cells stress their low migratory potential. This further supports the claim, that these cells are terminally differentiated effector cells with a diminished capacity for lymphoid tissue recirculation.

Our analyses showed that senescent-like CD8⁺CD57SP T cells exhibited a weak expression of CD25 (also known as IL-2 receptor α chain) and CD127 (IL-7 receptor α chain) under freshly isolated conditions. The low baseline expression of CD25 on senescent-like T cells ex vivo reflects their limited activation and reduced proliferative capacity in the early stages of activation.

It is well established that CD127 is highly expressed in naïve and memory T cells, which is consistent with our observation that a high percentage of naïve-like CD8⁺CD28SP and CD8⁺DP T cells, which share marker characteristics with central memory T cells, express CD127 ex vivo (229, 230). In comparison, senescent-like CD8⁺CD57SP T cells exhibited a very low CD127 expression, which is in line with their classification as late-differentiated effector cells.

Following stimulation with OKT-3 and IL-2, both CD25 and CD127 were upregulated in senescent-like CD8⁺CD57SP T cells, with CD25 exhibiting nearly an 80-fold increase in expression. This strong increase in CD25 aligns with the well-known role of T cell receptor activation in inducing the upregulation of CD25. Additionally, the abundance of IL-2 in the

stimulation protocol also likely contributed to a positive feedback loop, further enhancing CD25 expression. (231). Interestingly, the senescent-like CD8⁺CD57SP subset demonstrated a significantly stronger upregulation of CD25 compared to other T cell subsets, which could offer an explanation for their delayed but enhanced proliferative response observed after three days of stimulation.

The dynamics of CD127 expression showed a more nuanced pattern. Although a transient downregulation of CD127 is typical during T cell activation, potentially to reduce competition between IL-7 and IL-2 signaling, our findings revealed that senescent-like CD8⁺CD57SP T cells uniquely upregulated CD127 after 48 hours of stimulation, whereas the other subsets continued to downregulate CD127 (232). Despite this upregulation, CD127 expression remained lower in senescent-like CD8⁺CD57SP T cells compared to other subsets.

The upregulation of CD127 in senescent-like CD8⁺CD57SP T cells following stimulation might suggest a stronger reliance on IL-7 for their survival. It could also be a compensatory response due to their reduced baseline responsiveness to IL-2, which typically antagonizes CD127 expression (232). Taken together, the dynamic expression of CD127 and CD25 in this subset highlights their unique response to stimulation, characterized by a delayed activation profile.

KLRG-1 is a classical marker of terminal differentiation and senescence and is highly expressed on aged or senescent T cells (233). Our results show that KLRG-1 is highly expressed (>90%) on CD8⁺CD57SP T cells, while naïve-like CD8⁺CD28SP T cells show the lowest expression. This reinforces the classification of CD8⁺CD57SP T cells as terminally differentiated and senescent-like. PD-1 is a canonical exhaustion marker, upregulated under chronic antigen exposure and associated with impaired effector function in exhausted T cells (234). In our studies, the highest expression of PD-1 was observed on the CD8⁺DP T cell subset, suggesting that they may be functionally exhausted. This observation is further strengthened by the observation that TIGIT, another inhibitory receptor implicated in T cell exhaustion, is also strongly expressed on the CD8⁺DP T cell subset (235).

The co-inhibitory receptor TIM-3, though overall expressed at relatively low levels in a non-activated state, was higher on the CD28⁻ cells than on the CD28⁺ subsets. Intriguingly, despite strong exhaustion-associated markers (PD-1/TIGIT/KLRG-1), CD8⁺DP lack TIM-3, suggesting they may not be fully exhausted. Interestingly, CTLA-4 and LAG-3 expression levels were very low across all subsets, with no relevant differences observed.

Regarding NK-cell-associated markers, CD158b and CD160 were expressed in approximately 10–15% of CD8⁺CD57SP and CD8⁺DN T cells, while expression in the other two subsets did not exceed 5%. This modest expression suggests some overlap between NK and T cell phenotypes in these subsets, but the functional implications of this remain unclear.

6.1.4 SA- β -Gal expression increases during aging and induction of senescence, but is not a marker of senescent or terminally differentiated T cells

SA- β -Gal is one of the most widely used biomarkers to identify senescent cells. Its enzymatic activity can be detected at pH 6, which distinguishes it from lysosomal β -Galactosidase. SA- β -Gal serves as a surrogate marker for senescence in various cell types and is most commonly described in fibroblasts and epithelial cells (64). In CD8⁺ T cells, SA- β -Gal has also been suggested as a marker of cellular aging, as an accumulation of SA- β -Gal⁺ CD8⁺ T cells has been observed in older individuals (65). However, there exists only little data on the correlation between SA- β -Gal expression and the well-known markers of T cell senescence and terminal differentiation, such as CD28, CD57, CD27, and CD45RA. Additionally, there is currently no data on the effect of senescence-inducing conditions on SA- β -Gal expression in CD8⁺ T cell subsets. This study aimed to clarify the link between SA- β -Gal activity and CD8⁺ T cell subsets in healthy donors, focusing on senescent-like CD8⁺CD57SP T cells and T_{EMRA} (CD8⁺CD27⁻CD45RA⁺) cells, “ex vivo” and in senescence-inducing conditions.

In freshly isolated CD8⁺ T cells from healthy donors, SA- β -Gal expression was relatively low, with less than 10% of cells exhibiting detectable levels. Only two donors in our small cohort in their late 60s to early 70s could show a SA- β -Gal expression up to 30%. This stands in stark contrast with the high number of SA- β -Gal⁺ T cells previously reported in aging humans, where over 60% of CD8⁺ T cells expressed high levels of SA- β -Gal (65). Overall, our results indicate that baseline SA- β -Gal activity is low in circulating CD8⁺ T cells of healthy young to middle-aged ($\approx$ 20-60 years) donors in “ex vivo” conditions. However, despite the low overall SA- β -Gal levels, a correlation with increasing donor age was observed, once again supporting its role as an aging marker. This increase with age may point towards a gradual buildup of metabolic or lysosomal issues in aging CD8⁺ T cells.

Our results also highlight that CD8⁺ T cells show elevated SA- β -Gal levels under senescence-inducing conditions, even if they do not exhibit high basal levels “ex vivo”.

To mimic conditions of the tumor microenvironment, senescence was also induced in T cells using conditioned medium from tumor cell lines. While SA- β -Gal levels were higher in these conditions compared to stimulation alone, statistical significance was not reached. This variability was likely due to inter-individual differences in donor responses, which we found to be age-dependent. These findings suggest that the tumor microenvironment may contribute to the induction of SA- β -Gal in T cells but do not definitively establish it as a consistent driver of senescence.

When examining the different T cell subsets “ex vivo”, senescent-like CD8⁺CD57SP cells exhibited even lower SA- β -Gal levels than naïve-like CD8⁺CD28SP cells. No significant

differences in SA- β -Gal expression were observed between CD8⁺CD57SP and the other subsets (CD8⁺DP and CD8⁺DN). Similarly, when T cells were defined by CD27 and CD45RA, T_{EMRA} cells (CD27⁻CD45RA⁺) showed lower levels of SA- β -Gal compared to other subsets. The absence of elevated SA- β -Gal activity in these subsets challenges the assumption that these cells meet the criteria for cellular senescence previously established in other cell types, such as fibroblasts or epithelial cells.

Some of our findings regarding the distribution of SA- β -Gal⁺ T cells within different CD8⁺ T cell subsets also align with a previous study showing that they are detectable in all differentiation states, including in naïve T cells. The same study also demonstrated that CD57 and KLRG-1 were not exclusively expressed in SA- β -Gal⁺ T cells (65).

Intriguingly, even under senescence inducing conditions, senescent-like CD8⁺CD57SP T cells exhibited similar or even lower SA- β -Gal expression compared to less differentiated subsets. Similarly, the T_{EMRA} subset (CD27⁻CD45RA⁺) did not exhibit significantly higher SA- β -Gal expression compared to other CD8⁺ T cell subsets defined by CD27 and CD45RA. However, there was a tendency for T_{EMRA} cells to have higher SA- β -Gal levels, suggesting a possible association between terminal differentiation and increased SA- β -Gal activity after senescence induction. However, the variability in results across donors, precluded any statistical significance. Future studies with a larger cohort of donors will be necessary to determine whether T_{EMRA} cells reliably display increased SA- β -Gal.

Overall, these findings reinforce the notion that CD8⁺CD57SP and T_{EMRA} cells do not exhibit an elevated SA- β -Gal activity, either “ex vivo” or under the senescence-inducing conditions used in this study.

6.1.5 uPAR is upregulated in T cells after senescence induction, but is not sustained in senescent T cells

Despite its established role in cellular senescence and inflammation, the urokinase plasminogen activator receptor (uPAR) is not well characterized in T cells, and its function in T cell differentiation, aging, and senescence remains unclear (66, 236). Previous studies have primarily linked uPAR with T cell activation, as it is transiently upregulated on the surface of T cells following antigenic stimulation (67). This study investigated uPAR expression in CD8⁺ T cell subsets, with a particular focus on T cell senescence and differentiation. By evaluating its expression under baseline culture conditions and after senescence-inducing stimuli, we aimed to determine whether uPAR, like SA- β -Gal, is a “reliable” marker of T cell senescence or is restricted to specific differentiation stages.

In our study, freshly isolated, resting CD8⁺ T cells from healthy donors exhibited a low baseline expression of uPAR, with only 2.54 % of cells expressing the receptor. This finding is consistent

with previous research suggesting that uPAR is not constitutively expressed in unstimulated T cells of healthy donors (67). Notably, the CD8⁺CD57SP T cell subset displayed the highest percentage of uPAR⁺ cells among the freshly isolated T cell populations. However, the overall frequency of uPAR-expressing T cells remains relatively low in unstimulated senescent-like T cells, suggesting that uPAR is not an appropriate marker for senescence, at least in unstimulated T cells of healthy donors.

We therefore wanted to determine whether uPAR emerges as a suitable senescence marker in T cells after stimulation and the induction of senescence under conditions known to trigger T cell senescence, including etoposide treatment and exposure to senescence-associated secretory factors (i.e. tumor cell-derived conditioned medium). Both treatments significantly increased uPAR expression in CD8⁺ T cells, indicating that uPAR is upregulated in response to senescence-inducing stimuli, similar to SA-β-Gal.

Interestingly, the senescent-like CD8⁺CD57SP subset failed to upregulate uPAR upon senescence induction, despite initially showing the highest baseline levels. In contrast, the other CD8⁺ T cell subsets (CD8⁺CD28SP, CD8⁺DP and CD8⁺DN) exhibited significant uPAR upregulation in response to senescence-inducing conditions.

These findings suggest that uPAR expression could be associated with less-differentiated CD8⁺ T cells following stimulation and senescence induction rather than a definitive marker of senescent-like CD8⁺CD57SP T cells. The expression of uPAR may simply reflect a cellular stress or damage instead of directly indicating T cell senescence. The observation that uPAR is significantly upregulated after etoposide treatment, a well-known inducer of DNA damage, supports this idea, implying that its expression may primarily result from genotoxic stress rather than intrinsic senescence-related mechanisms.

Although the upregulation of uPAR is a common feature of many senescent cells, its role in senescent T cells appears distinct. In fibroblasts and epithelial cells, uPAR is often associated with the SASP and indicates permanent cell cycle arrest and inflammatory activity (237). Overall, these findings highlight the complexity of defining T cell senescence and emphasize the need to distinguish between markers of transient stress, terminal differentiation and true senescence in immune cells. While uPAR may be a useful indicator of cellular damage or activation in CD8⁺ T cells, it does not seem to fully represent the senescent-like state of CD8⁺CD57SP T cells, indicating that additional markers are necessary to characterize T cell senescence comprehensively.

6.1.6 Elevated intracellular levels of Granzyme B and Perforin indicate enhanced effector function in senescent T cells

We evaluated key parameters of cytotoxicity (degranulation, Granzyme B and Perforin production) to investigate the cytotoxic potential of senescent-like CD8⁺ T cells compared to less-differentiated T cell subsets. Our findings show that senescent-like CD8⁺CD57SP T cells have a higher degranulation potential compared to naive-like CD8⁺CD28SP T cells. Their degranulation capacity is comparable to CD8⁺DN cells but lower than CD8⁺DP cells. Intracellular staining for Granzyme B and Perforin revealed the highest levels in senescent-like CD8⁺CD57SP T cells.

In conclusion, the results indicate that despite exhibiting a senescent-like phenotype, this subset possesses significant cytotoxic potential. This aligns with previous research that emphasized the strong cytotoxic capabilities of highly differentiated T cells (13, 58).

The findings raise important questions regarding the therapeutic targeting of senescent-like T cells. While senescent cells are often considered dysfunctional and are often targeted for elimination, our data suggest that senescent-like CD8⁺ T cells retain a strong cytotoxic function (238). Thus reprogramming these cells to restore their proliferative ability while maintaining their cytotoxic potential may offer new options for cancer cellular therapy. Future studies should identify signals that can reinvigorate proliferation in senescent-like T cells without diminishing their effector functions.

6.1.7 Senescent-like T cells show increased cell and cytoplasmatic size

The morphological characteristics of CD8⁺ T cell subsets provide valuable insights into the phenotypic changes associated with cellular senescence. Our study revealed that senescent-like CD8⁺CD57SP T cells exhibited significantly larger cell sizes and an expanded cytoplasmic volume compared to the other subsets. This increase in size aligns with previous reports suggesting a link between cellular enlargement with senescence (239, 240).

The observation of cytotoxic granules in CD8⁺CD57SP T cells correlates with the established cytotoxic potential of this subset, as we have demonstrated that these cells express elevated levels of perforin and granzyme B. The presence of such granules suggests a functionally active cytotoxic capacity, despite the senescent-like phenotype. This contrasts with early-stage T cells, which typically exhibit smaller cell sizes and lack prominent granule formation.

6.2 The metabolic profile of senescent CD8⁺ T cells

Understanding the metabolic profile and the role of mitochondrial dysfunction in senescent CD8⁺ T cells is crucial for identifying potential metabolic targets to improve T cell fitness. The results from several metabolic assays provide a comprehensive overview of glucose

metabolism, mitochondrial function, oxidative stress, and metabolic enzyme expression in different CD8⁺ T cell subsets, with a focus on the senescent-like CD8⁺CD57SP subset.

6.2.1 Glucose Uptake and GLUT-1 Expression

Glucose uptake is critical for T cell activation, proliferation, and function. The results demonstrate that the senescent-like CD8⁺CD57SP T cell subset exhibited the lowest uptake of the fluorescent glucose analog 2-NBDG over time, indicating a potential impairment in glucose metabolism. In vitro experiments have shown that activated T cells switch from OXPHOS towards aerobic glycolysis, enhancing their glucose uptake in a CD28-dependent manner (241, 242). However, due to the absence of the co-stimulatory signal CD28, senescent-like T cells may not be able to increase glucose uptake after stimulation.

We then measured the expression of the glucose transporter GLUT-1 to determine whether the reduced glucose uptake is a result of a decreased GLUT-1 expression in senescent-like T cells. While over 90% of all CD8⁺ T cells expressed GLUT-1, its expression was highest in the CD8⁺DP subset, whereas the senescent-like CD8⁺CD57SP cells exhibited significantly lower levels than this subset. However, there were no significant differences in GLUT-1 expression between the senescent-like CD8⁺CD57SP T cells and naive-like CD8⁺CD28SP and CD8⁺DP T cells, in both resting and stimulation conditions. These results contrast with the significantly lower uptake of the glucose analog in senescent-like T cells. Several factors may account for this discrepancy: Even if GLUT-1 is present on the cell surface, its functionality might be compromised, hindering glucose uptake. Furthermore, while GLUT-1 is the most widely studied glucose transporter, T cells also express GLUT family members GLUT-3, GLUT-6 and GLUT-8 (243). Therefore, the reduced glucose uptake in the senescent-like T cell population could be due to altered expression or activity of other GLUT family members beyond GLUT-1. Additionally, senescent-like T cells may prioritize alternative fuels, such as fatty acids or amino acids, over glucose, leading to lower glucose uptake despite normal GLUT-1 expression. To clarify this discrepancy, future studies should investigate alternative glucose transporters, their activity, and the use of other energy sources, such as fatty acids and amino acids.

6.2.2 Expression of the NAD⁺ glycohydrolase CD38

Maintaining a balanced NAD⁺/NADH ratio and ensuring a sufficient supply of mitochondrial NAD⁺ are important for optimal cellular metabolism and mitochondrial function. CD38, a key NAD⁺-depleting enzyme, plays a central role in regulating NAD⁺ homeostasis by hydrolyzing NAD⁺ into ADP-ribose, cyclic ADP-ribose and nicotinamide (244, 245). Previous studies have demonstrated that CD38 expression increases with age, contributing to a progressive decline in NAD⁺ levels and mitochondrial dysfunction across multiple murine tissue types (246). Moreover, pharmacological inhibition of CD38 has been shown to restore NAD⁺ levels and

ameliorate age-related metabolic impairments, highlighting its potential as a therapeutic target in aging and metabolic disorders (247).

Given the importance of NAD⁺ metabolism in immune function and aging, we sought to investigate CD38 expression across different stages of human T cell differentiation as an indirect measure of intracellular NAD⁺ levels. Our analysis revealed that CD38 expression was significantly elevated in the senescent-like CD8⁺CD57SPT cell subset, as compared to the naïve-like CD8⁺CD28SP and transitional CD8⁺DP subsets. Interestingly, we also observed high CD38 expression in CD8⁺DN T cells.

These findings suggest that senescent-like T cells may experience a substantial decline in intracellular NAD⁺ levels, potentially impairing their metabolic and functional capacity. To validate this hypothesis, future research should focus on direct quantification of intracellular NAD⁺ and NADH levels in these subsets. Furthermore, investigating the effects of CD38 inhibition or NAD⁺ supplementation on T cell function may yield insights into strategies to reverse age-related immune dysfunction.

6.2.3 Mitochondrial mass and mitochondrial membrane potential

A key aspect of this study was to gain novel insights into the mitochondrial characteristics of the senescent CD8⁺ T-cell subset. Using an experimental approach combining MitoTrackers, this study determined mitochondrial mass and membrane potential ($\Delta\Psi_m$) as key indicators of mitochondrial activation and function.

Our data indicate that the senescent-like CD8⁺CD57SP T cell subset has a significantly higher mitochondrial mass than the CD8⁺DN subset, but not significantly different from naïve-like CD8⁺CD28SP T cells. Furthermore, both the senescent-like CD8⁺CD57SP and CD8⁺DP subsets displayed significantly higher mitochondrial membrane potential, compared to naïve-like and CD8⁺DN populations.

Our findings partially align with a previous study, which reported an age-associated increase in mitochondrial mass and mitochondrial membrane potential in CD8⁺ T cells (248). On the other hand, our results differ from those of an earlier study, which reported reduced mitochondrial content and decreased mitochondrial membrane potential in T_{EMRA} CD8⁺ T cells (CD27⁻CD45RA⁺) (123). This discrepancy may reflect the differences in the phenotypic and functional profiles of senescent-like and T_{EMRA} cells. T_{EMRA} cells (CD27⁻CD45RA⁺) and the CD8⁺CD57SP subset examined here may represent distinct stages of T cell differentiation and senescence, with potentially divergent patterns of mitochondrial dysfunction. Furthermore, it has to be taken into account, that the referenced study used the lipophilic cation JC-1 to determine membrane potential, while our study utilized TMRE and MitoTracker Deep Red markers (123).

The combination of MitoTracker Green and Deep Red enabled us to distinguish between intact and depolarized mitochondria at the single-cell level. Depolarized mitochondria, showing a high mitochondrial mass with low $\Delta\Psi_m$, were exceedingly rare (<2%) across all subsets in freshly isolated, unstimulated cells. The results also suggest that depolarization is not a hallmark of „ex vivo“ senescent-like CD8⁺CD57SP T cells per se and that they maintain mitochondrial integrity despite their senescent status.

Our results also demonstrate that following stimulation with OKT-3 and IL-2, mitochondrial membrane potential increased significantly across all subsets, with the greatest increase observed in CD8⁺DP and senescent-like CD8⁺CD57SP cells. This indicates that these cells retain a dynamic mitochondrial response capacity, despite exhibiting surface markers of senescence.

Taken together, our findings suggest that senescent-like CD8⁺ T cells, particularly the CD8⁺CD57SP subset, are not metabolically anergic but instead maintain, and in some cases enhance, mitochondrial content and membrane potential. This phenotype may support their strong capacity for cytokine secretion and cytotoxicity despite impaired proliferative ability.

In conclusion, the combined use of different mitochondria trackers reveals a more nuanced view of mitochondrial fitness in senescent-like CD8⁺CD57SP T cells. Rather than exhibiting uniform mitochondrial decline, the CD8⁺CD57SP subset retains high mitochondrial mass and membrane potential, possibly contributing to their strong cytotoxic potential.

6.2.4 Mitochondrial ROS production

Mitochondrial reactive oxygen species (ROS), particularly superoxide, are both a byproduct and a driver of cellular senescence and dysfunction in aging tissues, including the immune system (125). In CD8⁺ T cells, elevated mitochondrial ROS levels have been associated with impaired effector function, metabolic dysfunction and a pro-inflammatory phenotype (249). We used MitoSOX Red staining to assess mitochondrial superoxide levels in CD8⁺ T cell subsets before and after TCR stimulation, which revealed subset-specific patterns of ROS accumulation.

Our data show that senescent-like CD8⁺ T cells, specifically the CD8⁺CD57SP subset, exhibit only moderately elevated mitochondrial ROS levels at baseline, and respond with a limited increase in ROS production after stimulation. This contrasts with several studies reporting markedly elevated ROS in senescent or terminally differentiated T cells, such as the CD8⁺ T_{EMRA} (CD45RA⁺CD27⁻) subset, which have been shown to accumulate dysfunctional mitochondria and generate excessive ROS due to impaired mitophagy or altered metabolic flux (123, 250).

One possible explanation for this discrepancy is that the CD8⁺CD57SP phenotype may represent a metabolically distinct T cell subset from T_{EMRA} cells, despite overlapping senescence features. Our data indicate that while senescent-like CD8⁺CD57SP T cells exhibit a high mitochondrial mass and membrane potential, they do not produce correspondingly high levels of mitochondrial superoxide, suggesting a relatively preserved mitochondrial function and potentially an upregulation of antioxidant defenses. This stands in contrast to studies where T_{EMRA} CD8⁺ T cells display mitochondrial depolarization and ROS buildup, often alongside a reduced ATP production (145).

Furthermore, the moderate ROS production in response to stimulation in senescent-like CD8⁺CD57SP (but also CD8⁺DN) T cells may also indicate a decreased responsiveness to TCR signaling and cytokine co-stimulation, which is a well-known characteristic of highly differentiated CD8⁺ T cells. A prior study has shown that senescent-like T cells are hyporesponsive to TCR engagement and may exhibit impaired mitochondrial biogenesis or metabolic reprogramming upon activation (251). The limited ROS induction we observed after 48 hours of TCR stimulation might mirror this diminished activation potential.

Interestingly, the CD8⁺DP T cell subset in our study produced the highest levels of mitochondrial ROS, both at baseline and after stimulation. This subset may be more metabolically active or represent a transitional state between effector and senescent phenotypes, capable of stronger mitochondrial engagement after stimulation, which could possibly explain the elevated oxidative stress.

In summary, our findings partially align with previous observations, confirming increased mitochondrial ROS in highly differentiated CD8⁺ T cells. However, they differ in suggesting that not all senescent-like subsets accumulate ROS equally or respond strongly to activation. This underlines the need to distinguish between different senescent phenotypes (CD8⁺CD57SP vs. CD27⁻CD45RA⁺) when evaluating metabolic dysfunction and redox imbalance in aging T cells.

6.2.5 Reduced mitochondrial respiration of senescent T cells

Mitochondrial function is a key parameter when determining the metabolic fitness and functional capabilities of T cells. During activation and differentiation, T cells experience significant metabolic reprogramming, shifting between oxidative phosphorylation and glycolysis to meet energy demands and support effector functions (251). Dysregulation of these processes is increasingly recognized as a characteristic of T cell exhaustion and senescence.

Our Seahorse-based analysis of mitochondrial bioenergetics in live cells revealed that the senescent-like CD8⁺CD57SP T cell subset exhibits signs of impaired mitochondrial function. This is evident from the overall lower oxygen consumption rate (OCR) observed throughout

the assay, along with a reduction in key parameters such as basal respiration, maximal respiration, spare respiratory capacity, and ATP production. Meanwhile, the extracellular acidification rate (ECAR) was lower in the CD8⁺CD57SP subset compared to the CD8⁺DP and CD8⁺DN subsets, indicating lower glycolytic activity and lactate production in the senescent-like CD8⁺CD57SP subset. While these findings did not reach statistical significance, likely due to limited sample size, they nonetheless indicate a trend toward metabolic insufficiency and mitochondrial bioenergetic dysfunction in this senescent-like T cell population.

These results align with prior studies identifying mitochondrial impairment as a hallmark of aged, senescent and terminally differentiated T cells. It could be demonstrated that the basal and maximal oxygen consumption rate (OCR) was decreased in aged CD8⁺ T cells despite evidence of increased mitochondrial mass (252). Research on CD8⁺ T_{EMRA} (CD45RA⁺CD27⁻) cells has also shown lower basal OCR, reduced spare respiratory capacity, and hypopolarized mitochondria (123, 253).

It is also noteworthy to mention the low ECAR observed within the senescent-like CD8⁺CD57SP T cell population. Since many effector T cells rely on glycolysis during activation, the reduced ECAR in CD8⁺CD57SP T cells further supports the idea of metabolic exhaustion.

Collectively, these findings add to the growing body of evidence that CD8⁺CD57SP T cells are metabolically compromised, regarding both mitochondrial respiration and glycolytic flexibility.

6.2.6 Altered Lactate Metabolism in Senescent CD8⁺ T Cells: Evidence from LDHA and LDHB Expression Profiles

Lactate dehydrogenase (LDH) mediates the interconversion of pyruvate and lactate; LDHA favors the conversion of pyruvate to lactate, supporting glycolysis and LDHB catalyzes the reverse reaction. In our research, we further investigated the metabolic adaptations of CD8⁺ T cell subsets during activation and resting condition, focusing on the dynamic regulation of LDHA and LDHB in relation to costimulatory and senescence markers.

In freshly isolated CD8⁺ T cells, LDHA expression was low and decreased further after 48 hours of culture without stimulation. This reduction aligns with the quiescent metabolic state of unstimulated T cells, which rely primarily on OXPHOS rather than glycolysis (254). In contrast, LDHB expression was high at baseline and further increased after a resting period of 48 hours, underscoring the oxidative metabolism characteristic of non-activated T cells.

Upon stimulation, a robust increase in LDHA expression was observed, marking a substantial metabolic shift towards aerobic glycolysis. This confirms that TCR stimulation induces metabolic reprogramming favoring rapid ATP production and biosynthesis, consistent with the “Warburg-like” effect observed in activated T cells (255). The highest LDHA expression was

observed in CD8⁺DP and naïve-like CD8⁺CD28SP T cells, indicating that costimulatory signaling is tightly linked to an increase in glycolysis.

In contrast, senescent-like CD8⁺CD57SP and CD8⁺DPN subsets showed reduced LDHA induction, suggesting a diminished capacity for glycolysis. The reduced response of CD28⁻ subsets may stem from their lack of costimulatory signaling, which is essential for full metabolic activation. These findings reinforce the concept that CD28 expression correlates with the ability to mount a glycolytic response and generate effector function upon stimulation (256).

With regard to LDHB, while its expression remained high across conditions, important subset-specific differences emerged. Naïve-like CD8⁺CD28SP T cells consistently showed the highest LDHB levels, while senescent-like CD8⁺CD57SP cells had the lowest, both at rest and following stimulation. This pattern was also evident when analyzing differentiation states by CD27/CD45RA markers: T_{EMRA} (CD27⁻CD45RA⁺) T cells, which are closely aligned with senescence, exhibited the lowest LDHB expression across all timepoints. Additionally, KLRG-1⁺ T cells, another marker of senescence or terminal differentiation, also displayed significantly lower LDHB expression than their KLRG-1⁻ counterparts.

These observations suggest that senescent-like CD8⁺ T cells have impaired mitochondrial metabolism, as LDHB is critical for converting lactate to pyruvate to fuel the TCA cycle. Low LDHB may limit their ability to metabolize lactate, which is a disadvantage in nutrient-limited or lactate-rich environments such as the tumor microenvironment (257).

Taken together, these findings underscore that the metabolic phenotype is tightly coupled to differentiation status in CD8⁺ T cells. CD28⁺ subsets, especially CD8⁺DP T cells, show metabolic plasticity, increasing both LDHA and LDHB expression upon stimulation, positioning them as key responders in immune activation. In contrast, senescent-like CD8⁺CD57SP T cells exhibit a dual defect. First, a blunted LDHA induction and second, a persistently low LDHB, pointing to a metabolically exhausted state that may underlie their impaired immune function.

Future studies should aim to further clarify the role of LDHB in T cell senescence. One potential approach involves the overexpression of LDHB in senescent CD8⁺ T cells, to assess whether this intervention can reverse key features of the senescent phenotype, particularly the proliferative arrest characteristic of these cells. Additionally, such experiments could determine whether LDHB overexpression restores metabolic function, thereby enhancing mitochondrial activity and improving the overall energetic capacity of senescent T cells. It could already be shown in CD4⁺ T cells, that the overexpression of LDHB could increase cellular respiration, reduce the adverse effect of lactic acid and led to a preferential migration into tumor spheroids (180).

Conversely, LDHB knockdown or knockout in metabolically active subsets, such as naïve-like CD8⁺CD28^{SP} T cells, could reveal whether reduced LDHB contributes directly to features of senescence, including impaired cytokine production, proliferation, or survival. Such studies could also explore whether LDHB modulates susceptibility to activation-induced cell death or affects long-term memory potential. At the transcriptional level, it would be valuable to investigate how LDHB is regulated during T cell differentiation and senescence.

Together, these approaches would provide mechanistic insight into whether LDHB is merely a metabolic marker of senescence or an active driver of dysfunctional T cell states, with potential implications for therapeutic modulation in aging or cancer immunotherapy.

7 Conclusion

Given an increasing aging global population, the relationship between cancer and the aging immune system, known as immunosenescence, has become a significant new challenge in hematology and oncology. It is well established that the incidences of solid and hematologic cancers increase with age (1). Therefore, a better understanding of how senescent immune cells, particularly CD8⁺ T cells, influence anti-tumor immunity and responses to immunotherapy is needed. The results of this thesis add to this field of research by examining the phenotypic, functional and metabolic characteristics of senescent CD8⁺ T cells. A key discovery of this study is the detailed phenotypic analysis of senescent-like CD8⁺CD57^{SP} T cells. These cells accumulate with age and exhibit classic signs of senescence, such as reduced proliferative ability and changes in cell shape. However, they still maintain strong effector functions, including high levels of granzyme B and perforin. This paradox, a reduced replicative capacity but maintained cytotoxic activity, underscores the complex role these cells might have in cancer immunosurveillance. They could be beneficial in the short term but potentially harmful if dysregulated or unresponsive to additional stimulation.

From a translational perspective, an important aspect of this work is the identification and refinement of surface markers that characterize the senescent T cell phenotype. In addition to the established CD28⁻CD57⁺ markers, this thesis expands the senescence panel to include KLRG-1, re-expression of CD45RA, loss of CD27, and reduced expression of important homing and cytokine receptors. Importantly, classical exhaustion markers like PD-1 and TIM-3 were not strongly expressed on senescent cells, indicating that senescence and exhaustion, while both relate to T cell dysfunction, are distinct immunological states. This distinction has important implications for designing immune checkpoint therapies, which mainly target exhaustion pathways but may miss the role of senescent T cells in resistance to treatment or immune escape.

Investigating the metabolic dysfunction of senescent T cells is an important aspect of this work. This study demonstrates that senescent T cells exhibit reduced mitochondrial respiration and decreased glycolytic capacity, based on metabolic flux assay profiling and glucose uptake tests. Interestingly, despite these functional impairments, senescent T cells display a high mitochondrial mass and membrane potential *ex vivo*, indicating that mitochondrial structure does not necessarily equal metabolic functionality. Notably, these cells did not display significantly increased mitochondrial ROS, unlike some earlier studies (123, 250). A particularly novel and potentially significant finding relates to lactate metabolism. This study is among the first to show that senescent CD8⁺ T cells show a low expression of LDHB and do not strongly induce LDHA after activation. As many tumors create a lactate-rich, immunosuppressive environment, this inability to efficiently metabolize lactate may cause senescent T cells to be metabolically impaired within the tumor microenvironment. In cancer immunotherapy, where metabolic competition and nutrient starvation are known to decrease T cell effectiveness, this discovery suggests new research directions. Specifically, targeting metabolic pathways, such as LDH isoenzyme regulation, could help restore or boost senescent T cell function.

As T cell-based therapies such as immune checkpoint inhibitors, bispecific antibodies and CAR-T cells become more common in clinical practice, it is important to understand the functionality of a patient's T cells. In older individuals or in those who have undergone extensive treatment, the amount of senescent or metabolically compromised T cells could be an indicator of how effectively a future cellular-based treatment might succeed. Consequently, adding a senescence profile to immune diagnostics before the treatment could improve patient risk stratification and help to customize immunotherapy plans, especially in the era of precision medicine. Furthermore, the observation that tumor-derived factors can trigger senescence-like traits, such as increased SA- β -Gal and uPAR expression in T cells that are normally not senescent suggests that the tumor microenvironment might actively promote immune aging as a means of immune evasion. Strategies aiming to reverse or prevent therapy-induced senescence or to reprogram senescent T cells using metabolic interventions could effectively complement existing cellular or checkpoint therapies. In summary, this thesis provides a deep characterization of CD8⁺ T cell senescence, connecting surface phenotype, proliferation, effector function, and metabolism to the broader clinical issues of immune aging and cancer. As immunotherapy advances in oncology, current efforts focus on improving the expansion and "in vivo" persistence of T cells. An essential step towards achieving a stronger and more enduring effect of immunotherapy is addressing the metabolic dysfunction of senescent CD8⁺ T cells and, in the long run, restoring their ability to respond, especially for aging and cancer-prone individuals.

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Acknowledgement

I would like to express my sincere gratitude to my primary supervisors for giving me the opportunity to pursue my doctoral thesis within this exciting and stimulating research project. I am especially grateful to and for their dedicated supervision, insightful guidance, and continuous support throughout the course of this thesis.

I would also like to thank for her invaluable teaching, assistance, and patience. The stimulating discussions, collaborative spirit, and support provided by all members of AG are gratefully acknowledged.

Finally, I would like to express my deepest gratitude to my parents for their unwavering encouragement and constant support throughout my academic journey.

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