

PhD degree in Systems Medicine (curriculum in Molecular Oncology)

European School of Molecular Medicine (SEMM),

University of Milan and University of Naples “Federico II”

Settore disciplinare: MED/04

Linoleic Acid unleashes the anti-tumor potential of CD8+ T Cells by promoting Metabolic Reprogramming.

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Anno accademico 2022-2023

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Academic year: 2021-2022

To mom, dad, and Otto
“Love is not love
Which alters when its alteration finds,
Or bends with the remover to remove:
Oh no; it is an ever-fixed mark,
That looks on tempests, and is never shaken”

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LIST OF ABBREVIATIONS

4PBA	Phenylbutyric acid
AA	Arachidonic acid
ACT	Adoptive Cell Therapy
Ag	Antigen
Akt	Ak strain transforming
AMPK	AMP-activated protein kinase
ATP	Adenosine triphosphate
CAR	Chimeric Antigen Receptor
CDK	Cyclin-dependent kinases
CRAC	Calcium release-activated channels
CTL	Cytotoxic T Lymphocyte
CTLA4	Cytotoxic T-lymphocyte-associated antigen 4
CTR	Control
CXCL2	Chemokine (C-X-C motif) ligand 2
DAG	Diacylglycerol
DEG	Differentially expressed genes
DLN	Draining lymph node
DNA	Deoxyribonucleic acid
ECAR	Extra-Cellular Acidification Rate
EM	Electron microscopy
Eomes	Eomesodermin
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinases
ETC	Electron transport chain
Eto	Etomoxir
F16BP	Fructose-1,6-Bi-phosphate
FA	Fatty acid
FABP	Fatty acid-binding proteins
FAO	Fatty Acid Oxidation
FAS	Fatty Acid Synthesis
FCCP	Carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone
G6P	Glucose-6-Phosphate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLUT	Glucose transporter protein
GMP	Good Manufacturing Protocol
GPX4	Glutathione peroxidase 4
GRP	Glucose regulatory protein
GSH	Glutathione
GSSR	GSH cysteinylglycine disulfide
GTP	Guanosine triphosphate
GZMb	Granzyme beta
HDAC	Histone deacetylases
HIF-1 α	Hypoxia Inducible factor 1 alpha
HK	Hexokinase
ICB	Immune Checkpoint Blockade
IP3	Inositol 1,4,5-trisphosphate
IP3R	Inositol 1,4,5-trisphosphate receptor
LA	Linoleic Acid

Lag3	Lymphocyte Activating 3
LAL	Lysosomal acid lipase
LCFA	Long-chain Fatty Acid
LCMV	Lymphocytic choriomeningitis virus
LD	Lipid droplet
LDHA	Lactate dehydrogenase
LDLR	Low-density lipoprotein receptors
LPL	Lysophospholipid
mAb	Monoclonal antibody
MAM	Mitochondria Associated Membrane
MCU	Mitochondrial Ca ²⁺ Uniporter
ME	Module
MEK	Mitogen-activated protein kinase kinase
MERC	Mitochondria-ER Contact
MFI	Mean Fluorescent Intensity
MHC	Major histocompatibility complex
MPEC	Memory precursor effector cell
mTOR	Mammalian target of rapamycin
MUFA	Mono-saturated Fatty Acid
NADH	Nicotinamide adenine dinucleotide
NDLN	Non-draining lymph node
NFAT	Nuclear factor of activated T cells
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
Noco	Nocodazole
NSCLC	Non-small cell lung cancer
OA	Oleic Acid
OCR	Oxygen Consumption Rate
OLIGO	Oligomycin
OMM	Outer mitochondrial membrane
OVA	Ovalbumin
OXPHOS	Oxidative Phosphorylation
PC	Phosphatidylcholine
PD1	Programmed Death 1
PE	Phosphatidylethanolamine
PER	Proton Efflux Rate
PGC1	PPAR-gamma coactivator 1
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PPAR	Peroxisome proliferator-activated receptors
PPP	Pentose Phosphate Pathway
PRPS2	Phosphoribosyl Pyrophosphate Synthetase 2
PUFA	Poly-unsaturated Fatt
RANTES	Regulated upon activation, normal T cell, expressed and secreted
RNA	Ribonucleic acid
Rot./AA	Rotenone + Antimycin A
SCAP	SREBP cleavage activation protein
SCID	Severe combined immunodeficiency
SERCA	Sarco-endoplasmic reticulum Ca ²⁺ ATPase
SFA	Saturated Fatty Acid

SIRT6	Sirtuin 6
Slc1a5	Solute carrier transporter 1a5
SOCE	Store-operated Ca^{2+} entry
SRC	Spare Respiratory Capacity
SREBP	Sterol Regulatory Element-Binding Proteins
SSC	Side Scatter
STAT4	Signal Transducer And Activator Of Transcription 4
STIM1	Stromal interaction molecule 1
TAA	Tumor associated antigen
TAG	Triacylglycerol
T-ALL	T cell Acute Lymphoid Leukemia
Tbet	T-Box Transcription Factor 21
TCA	Tricarboxylic acid cycle
TCF1	T cell factor 1
T _{CM}	Central Memory T cell
TCR	T cell receptor
T _{EFF}	Effector T cell
T _{EM}	Effector-Memory T cell
TG	Thapsigargin
TIL	Tumor-Infiltrating Lymphocyte
Tim3	T-cell immunoglobulin and mucin-domain containing-3
T _M	Memory T cells
TME	Tumor Microenvironment
T _N	Naïve T cell
TOX	Thymocyte selection-associated HMG BOX
T _{PEX}	Precursor-exhausted T cell
T _{REG}	Regulatory T cell
TSA	Tumor-Specific Antigens
TSC1/2	Tuberous sclerosis complex 2
tSNE	t-distributed stochastic neighbor embedding
UPR	Unfolded protein response
VDAC	Voltage-dependent anion channels
WGCNA	Weighted gene co-expression network analysis
XBP-1	X-box binding protein 1
ZAP70	Zeta-chain-associated protein kinase 70

ABSTRACT

Adoptive T cell therapy (ACT) has achieved unprecedented clinical results in the treatment of cancer, but scarce intra-tumor infiltration, persistence and function of adoptively transferred T cells limit its efficacy, especially in solid tumors. Metabolic constraints imposed by the tumor microenvironment (TME) greatly influence the success of immune-based therapies. A common metabolic alteration in the TME is lipid accumulation, a feature often associated with defective anti-tumor responses. However, whether all lipids are detrimental to T cell functions and how they regulate different fate decisions remains poorly understood. Here, we identified Linoleic Acid (LA) as a major positive regulator of CTL activity. LA endows CTL with improved metabolic fitness and redirects them away from exhaustion and towards a memory-like phenotype with superior effector functions. Mechanistically, LA treatment fosters the formation of ER-mitochondria contacts (MERC) and mitochondrial-associated-membranes (MAMs), which in turn promotes calcium (Ca^{2+}) signaling, mitochondrial energetics, and CTL effector functions.

As a result, LA-instructed CD8⁺ T cells mediate superior control towards different tumor types both *in vitro* and *in vivo* following ACT on mouse models, overcoming the hustle of a highly immunosuppressive TME.

Our results pave the way for a new generation of adoptive T cell-based therapies, where LA can be used during *ex vivo* CAR- and TCR- T cell manufacturing as a novel approach to achieve metabolic reprogramming and long-term functionality, broadening the therapeutic efficacy of ACT to a wide range of malignancies.

INTRODUCTION

CD8+ T cells are protagonists of anti-tumor immune responses

The main roles of the adaptive immune system are (a) to elicit a highly specific cytotoxic response against the cognate antigen and (b) to form a long-lived immunological memory able to recall a strong immune response in case of a secondary antigen encounter.

T cells play a crucial role in immunity against pathogens and cancer. Antigen (Ag) encounters, costimulatory signals, and pro-inflammatory cytokines dictate naïve T cell (T_N) activation in secondary lymphoid organs. Once activated, antigen-specific T cells undergo clonal expansion and differentiate into effector cells, also known as Cytotoxic T Lymphocytes (CTL), many of which migrate to the site of infection through the blood ^{1,2}. CTL play a critical role in recognizing and destroying virally infected cells and cancer targets with remarkable efficiency and specificity. The increase in the effector population is typically correlated with antigen clearance and the subsequent contraction phase, after which the vast majority of CTL die *via* apoptosis while only 5-10% of antigen-specific memory CD8+ T cells can persist over time and ensure a rapid recall response upon further encounter with cognate antigen.

T cell activation and signaling pathways

The molecular and transcriptional programs involved in T cell activation and differentiation extend over a milieu of signaling pathways and intermediates that engage in an intricate crosstalk. Among these, the most relevant are calcium and PI3K/Akt/mTOR signaling.

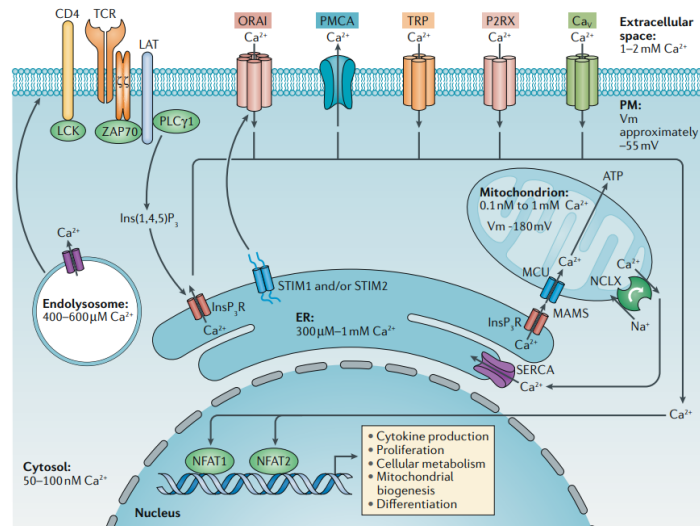
a. Calcium signaling.

In order to mount effective immune responses, T cells must transduce TCR signals from the surface of the plasma membrane, across the cytosol, to the nuclei. These signal transduction pathways require intracellular messenger molecules, the

function of which needs to be switched on and off with time. In this scenario, Ca^{2+} ions have been reported of paramount importance, since the on/off switch is tightly regulated by its concentration gradient across several cellular microdomains³. In fact, approximately 75% of genes controlling the production of cytokines and chemokines appear to be dependent on Ca^{2+} ⁴.

In the proximity of the cell surface, the Ca^{2+} signal transduction initiates with the TCR-mediated protein phosphorylation cascade leading to the activation of protein tyrosine kinases ζ -associated protein of 70 kDa (ZAP70) and phospholipase $\text{C}\gamma 1$ (PLC $\gamma 1$). In the cytosol, PLC $\gamma 1$ hydrolyzes phosphatidylinositol-4,5-bisphosphate (PI(4,5)P₂) to generate inositol-1,4,5-triphosphate (IP₃) and membrane-associated lipid, diacylglycerol (DAG)⁵. The DAG-mediated pathways are responsible for the activation of the Ras/Raf-1/MEK/ERK axis, and nuclear transport of nuclear factor kappa-light-chain-enhancer of activated B cells (NF κ B) to initiate the transcription of cytokine-related genes.

On the other hand, IP₃ carries the signal to the endoplasmic reticulum (ER), where it binds to its receptors and releases calcium into the cytosol, generating a calcium gradient that triggers the movement of the ER calcium sensor Stromal Interaction Molecule 1 (STIM1) towards ORAI channels on the plasma membrane. Upon translocation, STIM1 binds to and activates calcium release-activated (CRAC) channels creating a strong calcium influx from the extracellular milieu into the cytosol known as “store-operated Ca^{2+} entry” (SOCE). The significant increase in calcium levels inside the T cell leads to the activation of calmodulin, which undergoes a significant conformational change and allows for the dephosphorylation of multiple serine residues in the regulatory domain of the Nuclear Factor of Activated T cells (NFAT). Ultimately this process leads to NFAT translocation to the nucleus and initiates transcriptional activation of cytokine production genes^{3,4,6}.



Calcium signaling in T cell activation. (Taken from Trebak and Kinet. *Nature Reviews*. 2019)

The ability of T cells to modulate calcium current amplitude and frequency has proven to be essential for the maintenance of the activation signals in the nucleus. For instance, NF κ B can remain in the nucleus for a longer time; whilst NFAT is rapidly phosphorylated and inactivated if Ca²⁺ signals are not maintained, thus suggesting that calcium gradients not only provide an on/off switch but also a differential transcriptional program sustaining continuous stimulation versus acute activation and their resulting cytotoxic profiles⁷. Additionally, Faroudi and collaborators found that TCR signals triggered by low antigen concentrations are characterized by the low magnitude of Ca²⁺ oscillations, and ultimately lead to strong cytotoxicity, but weak IFN γ responses in human CTL. While on the other hand, high antigen concentrations effectively achieved both responses by promoting increased and sustained Ca²⁺ oscillations⁸. These discoveries further confirmed the existence of a calcium-dependent 'dual activation threshold' for controlling the extent of T cell activation and highlighted the importance of calcium signaling in regulating T cell activation and functionality.

In the clinic, several studies have extensively characterized the importance of calcium signals in the correct development of immune responses. For instance, T cells from patients suffering from severe combined immunodeficiency (SCID) show

abrogation of CRAC currents⁹. Further studies later proved that the mechanism behind these forms of primary SCID involved alterations or mutations in either *Oral* or *STIM1* genes that lead to impaired proliferation in response to mitogens⁴. Similarly, studies performed in non-small cell lung carcinoma (NSCLC), melanoma, and renal cell carcinoma (RCC) have shown that Ca^{2+} response is significantly impaired in Tumor Infiltrating Lymphocytes (TIL) with respect to peripheral blood T cells¹⁰. Moreover, calcium responses were reduced in peripheral blood T cells when transfected with Programmed Death 1 (PD1) suggesting a link between the checkpoint inhibitor and the strength of Ca^{2+} signaling.

b. PI3K/Akt/mTOR signaling axe.

The mammalian target of rapamycin (mTOR) is a highly conserved serine/threonine kinase that serves as an environmental sensor integrating extracellular cues into the signaling program that ultimately determines the fate of newly activated T cells¹¹.

Given the wide range of extracellular signals sensed by mTOR, there are various mechanisms leading to its activation, including cytokines (IL1, IL2, IL4, IL7, IL12, type I and II interferons), chemokines (GM-CSF, RANTES, CXCL12), metabolic cues (low levels of glucose, oxygen or amino acids), engagement of surface activation markers (CTLA4, PD1, OX40, ICOS), and ligation of pattern recognition and antigen-specific receptors (TCR and CD28)¹¹. In particular, TCR engagement leads to mTOR activation through phosphatidyl Inositol 3-kinase (PI3K)-driven phosphorylation of Akt and the subsequent inhibition of Tuberous Sclerosis Complex (TSC) 1/2. This results in the activation of the small GTPase Ras Homolog Enriched in Brain (Rheb), which activates mTOR Complex 1 (mTORC1) and the downstream phosphorylation of S6-kinase and 4E-BP1¹².

The role of mTOR in modulating the transcriptional program of T cells and its implications in T cell differentiation has been of paramount importance in the last few years. Strong evidence shows that mTOR activity regulates CD8⁺ differentiation programs by modulating T-box transcription factor TBX21 (T-bet) and

Eomesodermin (Eomes) expression in a TCR-dependent manner¹². While rapamycin treatment promotes Eomes and long-lived memory formation; induction of mTOR activity via PI3K and STAT4 pathways increases the expression of Tbet and promotes the formation of short-lived effector cells^{12–14}.

Additionally, mTOR plays a pivotal role as a metabolic sensor in T cells. The signaling cascade initiating from PI3K and leading to S6 phosphorylation is known to promote the translation of metabolic enzymes such as the nucleotide biosynthesis enzyme phosphoribosyl-pyrophosphate synthetase 2 (PRPS2)¹³, mitochondrial proteins involved in mitochondrial homeostasis and electron transport (TFAM, Complex I, and V components)¹⁴, and transcription factors such as Myc and Hypoxia Inducible Factor 1 alpha (HIF-1 α) that are essential for T cell metabolic reprogramming¹⁵. More recently, strong evidence has directly correlated mTOR activation and the development of metabolic programs in T cells. Deletion of Rheb - directly upstream of mTOR- reduced glycolysis levels, increased Fatty Acid Oxidation (FAO), and promoted a memory phenotype in CD8+ T cells. In contrast, the deletion of TSC2 led to increased glycolysis, diminished FAO, and expansion of the effector population¹⁶. These data, not only prove the direct involvement of mTOR in shaping of the metabolic landscape of CD8+ T cells but also emphasizes the strong connection between T cell metabolism and their differentiation status.

The conjunction of these signaling pathways and transcriptional programs has an enormous impact on T cell anti-tumor activity. Particularly, CTL depend deeply on signal transduction to initiate their cytotoxic program. For instance, a recent study performed on a murine melanoma model proved that perturbation of CRAC channels on CTL hampered their serial killing capacities by reducing degranulation in an NFAT-mediated manner¹⁷. Similarly, Hu and collaborators showed that inhibition of acylglycerol kinase -an upstream modulator of PI3K-mTOR- reduced CD8+ T cell proliferation capacities and dampened CTL antitumor responses¹⁸. Not surprisingly, CD8+ T cell functional impairment was accompanied by substantial metabolic defects including the reduced glycolytic rate in both basal and stressed

conditions¹⁸, further strengthening the theory that metabolism is at the core of T cell effector functions.

In summary, T cell activation is a multifaceted process characterized by the orchestrated activation of signaling pathways and intermediates that promote the translation and activity of transcription factors and other molecules involved in the establishment of a metabolic program. While calcium oscillations are responsible for signal transduction to the nucleus; the PI3K/Akt/mTOR signaling pathway is the main coordinator of the increased uptake of glucose that feeds the synthesis of biomolecules such as lipids or proteins to support proliferation and effector function.

T cell activation and differentiation require profound organelle remodeling

The increased energetic and transcriptional demands resulting from T cell activation pose an important challenge to its cellular machinery. In this scenario, organelle's morphology and activity play a crucial role in fueling the activation and differentiation processes. Here, we review some of the morphological and functional mechanisms necessary to sustain T cell activation and functions:

a. ER dynamics during T cell activation

Since T cell activation is known to induce physiological and fluctuating levels of ER stress, these data become of great importance to understanding the role of the mitochondria-ER network on T cell biology.

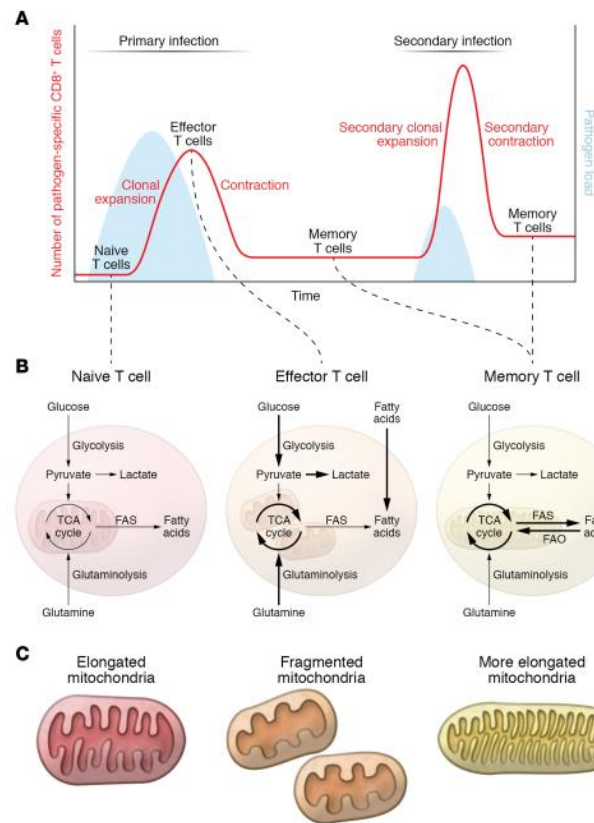
T cells require a significant increase in the number of proteins that are synthesized and folded in the ER to sustain clonal expansion, steady-state turnover, and cytokine production¹⁹. Under physiological conditions, these processes occur rapidly after antigen recognition and are reported to build the resilience and adaptability of T cells²⁰. In order to sustain ER homeostasis, the Un-folded Protein Response (UPR) is activated and metabolic rewiring is initiated by the concerted modulation of the T cell transcriptional programs. In particular, XBP-1 is known to increase the

expression of lipid-related genes (DGAT2 and ACC2), and up-regulate mitochondrial FAO and ketogenesis²¹; thus, linking ER stress and lipid metabolism.

b. Mitochondrial dynamics during T cell differentiation

Because newly activated T cells rely mostly on aerobic glycolysis to obtain ATP and fulfill the increasing metabolic demands of the activation process, the role of mitochondria in T cell activation was historically overlooked. However, mitochondria should not be only conceived as the cell's powerhouse. Besides energy production, mitochondria play an important role in several biosynthetic processes including calcium homeostasis, lipid synthesis, apoptosis, signaling, and cell cycle progression²². In fact, one of the first morphological changes observed in T cells upon activation is the increase of the mitochondria pool^{23,24} driven by mTORC1-dependent mitochondrial biogenesis²⁵.

In order to accomplish all these functions, mitochondrial morphology has been proven important to modulate their location, size, and distribution within the cell. In particular, mitochondrial fusion is controlled by the mitofusin-1, mitofusin-2, and OPA1 proteins, facilitating communication within mitochondrial compartments while elongating its total volume²⁶. On the other hand, mitochondrial fission is mediated by the Drp1 protein and produces small mitochondria that are more easily transported and distributed along microtubules²⁶. Interestingly, mitochondrial morphology has also been linked to particular T cell differentiation status. Buck et al. showed that effector T cells are mainly characterized by small, fissioned mitochondria dispersed all around the cytoplasm, while memory T cell populations possess fused, elongated mitochondria clustering together on one edge of the cell^{27,28}. Importantly, genetic- or pharmacological-driven mitochondrial fusion in effector T cells led to metabolic reprogramming, with enhanced oxidative metabolism, increased T cell survival, and improved anti-cancer activity *in vivo*²⁸, further strengthening the theory that T cell differentiation and metabolism are irrevocably intertwined.



Preferred metabolic pathways and mitochondrial morphology changes associated with T cell differentiation

(Taken from Corrado and Pearce, Journal of Clinical Investigation, 2022)

Several reports have also shed light on the role of mitochondria in T cell effector functions. The impaired mitochondrial activity in Tumor Infiltrating Lymphocytes (TIL) is directly correlated with diminished expression of PPAR-gamma coactivator 1 α (PGC1 α) and a block in their proliferation and IFN γ production. In fact, rescuing the mitochondrial biogenesis machinery in effector T cells significantly improves antitumor activity^{29,30}. Additionally, mitochondria have been implicated in T cell migration. Not only mitochondrial ATP production is necessary for the uropod to propel the leading edge extension of T cells^{31,32}; but also the subcellular location of mitochondria regulate the directionality of T cell migration. Mechanistically, mitochondria gather near the cell edge where they approach the extracellular chemokines to offer the necessary ATP supply so that T cells can move towards higher concentrations of chemokines and infiltrate tumor sites²⁸.

c. Mito-ER interactions

It is also important to highlight that mitochondria and ER are no longer considered isolated organelles with highly compartmentalized functions. Instead, more and more evidence supports the notion of mitochondria as a part of a bigger network that depends on physical contact or interaction with other organelles to efficiently carry out their functions^{33,34}. In particular, the physical contact between mitochondria and the ER is one of the better characterized. Considering that the ER is the main site for signal transduction, calcium storage, and nascent protein modification^{19,20}, it is not surprising that the extent of the mitochondrial-ER network could greatly impact T cell activation.

Mitochondria-ER contacts (MERC, ranging between 30 and 100µm) and Mitochondrial Associated Membranes (MAM, below 30 µm) are now known to be critical in maintaining cell homeostasis by allowing rapid exchange of biological molecules and modulating calcium homeostasis, autophagy, lipid metabolism, and apoptosis^{35,36}

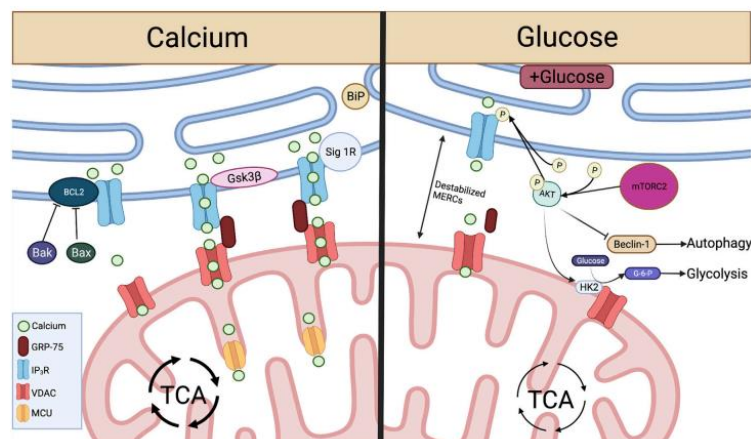
The junctions between mitochondria and MAM have been thoughtfully characterized and 8 different structural protein complexes have been identified. One, in particular, is thought to govern the ER-mitochondria bridging at MAM sites: the IP3R1-GRP75-VDAC1 complex. It is comprised of inositol 1,4,5-triphosphate receptors (IP3R1s) in the ER membrane and a Voltage-Dependent Anion Channel 1 (VDAC1) at the Outer Mitochondrial membrane (OMM). Additionally, the 75-kDa glucose-regulated protein (GRP75) links IP3Rs and VDAC1 to maintain the conformational stability of IP3Rs that participate in Ca²⁺ transport from the ER to the mitochondria³⁷.

Recent studies proved that uncoupling of the mitochondria-ER complexes or blocking Ca²⁺ transfer leads to impairment of the metabolic response of HeLa cells, rendering cells more vulnerable to ER stress³⁸. Moreover, alterations in the composition or abnormal induction of MAMs can lead to pathological outcomes with

a strong ER stress signature such as insulin resistance, cancer, neurodegenerative diseases, and inflammation³⁹.

Particularly, in T cells, the IP3R-GRP75-VDAC complex mediates crucial metabolite flow and nutrient sensing. In homeostatic conditions, B cell Lymphoma 1 (Bcl2) binds IP3R and hinders the IP3R-GRP75-VDAC complex formation. Upon Ca^{2+} depletion in the ER, Bcl2-IP3R interaction is inhibited by Bcl2 Associated X protein (Bax) and Bcl-2 homologous antagonist killer (Bak). Simultaneously, Sigma 1 Receptor (Sig-1R) dissociates from Binding Immunoglobulin Protein (BiP, also known as GRP78) and binds IP3Rs, enabling Ca^{2+} influx to the mitochondrial intermembrane space. Calcium ions then move into the mitochondrial matrix through the Mitochondrial Ca^{2+} Uniporter (MCU) and promote the Tricarboxylic Acid (TCA) cycle increasing mitochondrial respiration^{40–43}.

On the other hand, when glucose is available, MERCs are disrupted by mTOR-Akt-mediated inhibition of IP3R, leading to reduced ER Ca^{2+} release. The phosphorylation of Akt promotes hexokinase 2 (HK2) activity converting glucose to glucose-6-phosphate (G-6-P) and inhibits Beclin-1, a promoter of autophagy at MERCs^{41,42,44}.



Regulation of MERC structure and function in T cells.

(Taken from Hunt et.al. Frontiers in Cell and Developmental Biology. 2022)

A seminal work by Bantug and collaborators showed that mitochondrion-ER junctions are particularly enriched in memory T cells with respect to their effector

counterparts and that they are essential to the rapid recall response in memory CD8⁺ T cells. The work also confirmed the direct link between MERC structural integrity and the metabolic reprogramming occurring in memory T cells to elicit effector functions. In fact, increased MERC was correlated with glycolysis engagement to fuel IFN γ production; whilst pharmacological disruption leads to decreased mitochondrial respiration and reduced inflammasome activation²².

T cell differentiation status influences anti-tumor response

Cancer represents a particularly challenging setup for immune cells to provide antigen clearance. On one hand, the intrinsic characteristic of tumor cells to avoid the immune system's recognition hinders anti-tumor responses. On the other hand, once an antigen has been successfully recognized, repeated and continuous exposure leads to chronic stimulation and consequent loss of function in T cells^{28,45–47}. Nevertheless, CTL remain one of the key mediators of protective anti-tumor immunity and thus, special attention has been dedicated to the study of T cell activation and differentiation patterns in oncologic setups.

As previously described, upon antigen encounter, T cells change their phenotype from naïve, to effector, to memory. However, “effector” and “memory” are nowadays considered wide phenotype categories, and several studies have focused on the deep and detailed characterization of particular subpopulations resulting in an array of multiple types of effector and memory T cells with highly specific transcriptional programs, cytotoxic functions, memory pool formation capabilities, and lifespan⁴⁷.

Four models have been postulated to explain the wide heterogeneity in effector and memory populations: the separate-precursor, the decreasing-potential, the signal-strength, and the asymmetrical cell fate. All of these, along with several novel T cell differentiation statuses have been extensively described in the literature^{47–49} and have gained special attention in the study of anti-tumor immunity.

a. Progenitor T cells

Considering that CD8⁺ T cell phenotypic, functional, and transcriptional diversification can occur over multiple rounds of cell division⁴⁷, it is not surprising that special attention was given to the timely progression of those changes leading to the formation of specific T cell subpopulations. And so, the concept of progenitor cells was conceived as a way of referring to a cell subset that is not terminally differentiated and can give rise to one or multiple other subsets. For instance, memory precursor effector cells (MPEC) have been reported to produce a high number of long-lived memory cells upon antigen encounter^{49,50}. Similarly, CD8⁺ effector progenitors have been described in chronic viral infection as responsible for the generation of an effector pool with increased cytokine production that ultimately leads to antigen clearance⁵¹. Interestingly, one feature seems to be common to several progenitor lineages reported in the literature: TCF1-mediated stem-like phenotype. T cell factor 1 (TCF1) is a transcription factor that confers self-renewal and is recognized as a marker for both effector and memory progenitor lineages in CD8⁺ and CD4⁺ T cells^{49,52,53}. The link between TCF1 expression and T cell metabolism is yet to be studied but it's fascinating to speculate that the rapid cell expansion driven by TCF1 could affect the metabolic landscape of progenitor cells.

In particular, the identification of a progenitor-exhausted T cell (T_{PEX}) made a significant impact in the field of cancer immunology. This T cell subpopulation is characterized by high expression of TCF1 and low levels of CD127 and CD62L, thus conferring them the ability to self-renew whilst showing an effector-like phenotype. In fact, T_{PEX} are responsible for the expansion of terminally differentiated T_{EX} ^{50,54} and have shown superior response to blocking the interaction of PD1 with Immune Checkpoint Blockade (ICB) as well as increased and long-lasting anti-viral activity in murine models⁵⁵. However, the identification of rigorously defined T_{pex} in human cancer was only confirmed last year⁵⁶ and more studies are required to better understand their origin and potential in cancer immunotherapies.

b. Terminally differentiated T cells

Contrary to progenitors, terminally differentiated cells represent the last differentiation status in the lifespan of a T cell. The transcriptional profile of terminal differentiation is mainly governed by B lymphocyte-induced maturation protein 1 (BLIMP1). BLIMP1 is defined as a genetic switch leading to increased formation of KLRG1^{high}CD127^{low} terminal effector cells with enhanced CTL functions including increased migration towards sites of inflammation and the production of effector cytokines IFN γ and granzyme B^{47,57}. Numerous studies have highlighted the importance of a Blimp1-induced terminal differentiation profile in T cell biology since it provides sustained effector functions during persistent infection^{58,59}. Ultimately, in physiological conditions, antigen clearance and the subsequent formation of a memory pool is the result of an orchestrated balance between terminally differentiated effector cells and progenitors, thus highlighting the importance of strongly regulated transcriptional profiles in the development of immune responses.

Of note, terminally differentiated effector cells are more prone to develop an exhausted phenotype and become dysfunctional^{54,60}, further suggesting that the balance between Blimp1 and TCF1 is the key to mounting strong and effective immune responses while sustaining the effector cell pool renewal.

c. Memory T cells

By the time a human reaches 20 years of age, memory T cells (T_M) represent up to 35% of circulating T cells. However, immunological memory maintenance is a dynamic equilibrium where the frequency and activity of T_M cells goes from memory generation, to homeostasis, and, lastly, immunosenescence⁶¹.

In humans, T_M cells are classically identified by the expression of the CD45RO and the lack of CD45RA (CD45RO⁺CD45RA⁻)⁶¹⁻⁶³. However, this population is now known to be highly heterogeneous and new T_M sub-sets have emerged as important players in immune responses⁶¹.

The first evidence of heterogeneity within the memory population came to light when Sallusto and collaborators described a T_M sub-population expressing CCR7 that are retained in lymphoid tissues. This novel CD45RA-CCR7+ T cells population was coined central memory T (T_{CM}) cells, while their counterpart CD45RA-CCR7-, which can migrate to multiple peripheral tissue sites, where named effector memory T (T_{EM}) cells^{64,65}.

The study of the diverse sub-sets within T_M has been expanded over the years and into the clinic. For instance, T_M cells with high expression CD95 (also known as FAS) and CD122 (also known as IL-2R β) shows a superior survival potential with high stem-like proliferative capacity^{66,67}. This novel population was named memory stem cells (T_{SCM}) and is nowadays correlated with best responses and superior anti-cancer activity in ACT. In particular, transcriptomic analysis performed in patients treated with CD19 CAR-T cells showed enrichment of the T_{SCM} population was correlated with complete response⁶⁸.

Moreover, in the last several years, mouse studies have discovered a new tissue-resident memory T cell (T_{RM}) subset with long-term persistence in peripheral tissues and capable of rapid *in situ* protective responses⁶⁹. One of the best examples of the clinical relevance of T_{RM} cells is that of skin infections and cancer. In fact, The infiltration of T_{RM} cells in melanoma has been correlated with improved survival^{70,71}, suggesting an important role for T_{RM} cells in anti-tumor immunity.

Of note, T_M cells have been highly successful in ACT approaches. Originally, The T effector (T_{EFF}) phenotype was considered ideal for ACT therapies due to their potent killing capacity. However, T_{EFF} cells cannot expand and persist *in vivo* providing limited anti-tumor activity. On the contrary, memory T cells (T_M) can provide unique advantages to efficiently fight cancer cells in addition to persistence and expansion potential. In particular, T_M cells are characterized by enhanced metabolic fitness including higher aerobic glycolytic rates and amino acid uptake compared with primary effector cells that provides significant advantages in the TME (see

discussion below). Accordingly, secondary effector T cells -descending from T_M precursors - display a stronger ability to secrete IFN γ than primary effector T cells⁷². Indeed, studies performed on non-human primates proved that antigen-specific CD8⁺ T cells derived from T_{CM}, but not T_{EM}, were able to persist long-term *in vivo* following ACT. Additionally, TCM-derived cells reacquired phenotypic and functional properties of T_M cells, occupied T_M cell niches, and effectively controlled tumor growth⁷³.

Altogether, these pieces of evidence show that the establishment of a T_M phenotype enhances the efficacy of ACT approaches, and underscores the importance of selecting specific T cell subsets for the clinic.

d. Exhaustion

In general terms, T cell exhaustion describes a particular T cell status characterized by the expression of inhibitory receptors and reduction of cytokine production ultimately leading to an anergic-like metabolism and loss of cytotoxic activity⁷⁴.

Transcriptionally, Exhausted T cells (T_{EX}) are characterized by a very particular program that includes regulation of inhibitory receptors, TCR and signaling cytokines pathways, migratory potential, chemokine expression, and metabolism⁷⁵ strongly suggesting that the exhaustion state in T cells ticks all the boxes to be considered a differentiation status on itself. Of note, T_{EX} exhibit reduced NFAT expression that is associated with low effector cytokine production even though their degranulation capacities are not affected⁷⁶. This piece of evidence suggests that exhaustion might also hinder Ca²⁺ signaling and its downstream processes including proliferation and mitochondrial biogenesis.

Indeed, T_{EX} cells are characterized by substantial changes in genes involved in metabolism. T_{EX} cells display metabolic derangements, including suppressed cellular respiration, reduced glucose uptake, and abnormal mitochondrial structure^{30,77}. Of note, the decrease of glycolysis and mitochondrial respiration in T cells occurs at the early stage of exhaustion development, even before T cell

dysfunction occurs, suggesting that metabolic dysfunction is an early driver, rather than a consequence, of T cell exhaustion^{29,78,79}. Besides the intrinsic metabolic programs established during the development of exhaustion, the tumor microenvironment (TME) poses serious constraints in nutrient availability that contribute to the dysfunctional activity of TIL (See discussion below).

T_{EX} are also reported to exhibit particular signaling pathways and epigenetic landscapes, which derange effector immunity⁸⁰. In fact, Tex cells differ from other CD8+ T cells by ~6,000 differentially accessible chromatin regions, consistent with the scale of difference between other distinct hematopoietic lineages^{80,81}. These findings support the idea that exhaustion is a particular cell-fate commitment associated with robust changes in chromatin landscapes.

Recent studies have demonstrated that the DNA-binding protein Thymocyte selection-associated HMG BOX (TOX) plays a critical role in the development of exhausted T cells by driving epigenetic modifications. Even when ablation of TOX does not preclude the formation of effector and memory T cells; TOX deficiency does lead to superior T cell antiviral and anti-tumor activity as well as decreased expression of inhibitory receptors. Interestingly, TOX-deficient CD8+ T cells cannot persist during chronic viral infection and tumor development, thus indicating that TOX-mediated signaling and epigenetic programming are necessary to maintain the survival of exhausted T cells in the context of prolonged antigen exposure^{82–84}.

T_{ex} phenotype and surface markers expression has also been extensively studied. One of the most important phenotypic characteristics of T_{ex} is the increased expression of inhibitory receptors, namely Programmed Death 1 (PD1), T-cell immunoglobulin and mucin domain 3 (Tim3), Lymphocyte Activating 3 (Lag3), and cytotoxic T-lymphocyte–associated antigen 4 (CTLA4, also known as CD152). These proteins, expressed in the surface of T cells, have been historically described as key players in the maintenance of peripheral tolerance by inhibiting immune activatory signals, thus acting as an immune checkpoint and preventing exacerbated immune reactions and their associated adverse effects⁸⁵.

Mechanistically, inhibitory receptors are activated upon engagement with their counterpart co-activatory receptor in neighboring cells. This binding promotes the dephosphorylation of key molecules in the TCR signaling pathway, attenuating T cell activation and reducing proliferation and effector cytokine production⁸⁶.

Unfortunately, this strategy of immune response attenuation is exploited by tumor cells to prevent T cell cytotoxic activity and induce an anergic-like phenotype^{87,88} which creates a permissive environment for tumors to grow and spread to other organs. Based on these observations, the expression of inhibitory receptor ligands is now considered a marker of poor prognosis in cancer patients, while the increased expression of inhibitory markers in T cells has become a hallmark of exhaustion.

In this scenario, the generation of Immune Checkpoint Blockade (ICB) by monoclonal antibodies (mAb) completely revolutionized the field of immunotherapies. By binding to inhibitory receptor -on T cells- or its ligands -on tumor cells-, mAb prevent their dimerization and allow for T cells to exert their cytotoxic functions freely⁸⁹. This approach has been a great success in the clinic, not only for cancer but also for infectious diseases and autoimmune diseases⁸⁹. Nonetheless, a large number of patients still fail to respond to ICB and clinical data shows a high frequency of immune-related adverse events in patients undergoing ICB therapy⁹⁰. For these reasons, a great number of efforts are still dedicated to studying and characterizing the processes leading to T cell exhaustion and new strategies to avoid it and improve antitumor activity (see discussion below) or response to chronic viral infections.

Metabolism is a driving force of T cell differentiation

Every single differentiation state is characterized by the engagement of specific metabolic pathways that directly correlate with their energetic demands²⁸.

The activation of naïve into effector T cells relies mainly on glycolysis and, thus, requires a significant increase in the uptake of glucose, amino acids, and iron to sustain its cytotoxic

functions^{23,24,27}. Once the infection is cleared, a small sub-population of effector T cells differentiates into memory T cells, which are responsible for immunosurveillance and characterized by a metabolism mostly based on FAO and oxidative phosphorylation (OXPHOS) boosted by increased mitochondrial mass^{23,24,91}.

Considering the tight interconnection between T cell differentiation and metabolism, and keeping in mind the continuous crosstalk between signaling pathways, transcriptional programs, and metabolic landscape, it becomes clear that T cell activation and differentiation require not only a tightly regulated signaling network but also the continuous evolution of the cellular machinery required to sustain energetic and biosynthetic demands as well as environmental changes.

Here we provide a summary of some of the most important metabolic pathways and morphologic changes engaged during T cell activation and the roles they play in shaping the diverse differentiation profiles in CD8⁺ T cells:

a. Glucose metabolism

One of the first metabolic cues in T cell activation is the upregulation of both glycolysis and OXPHOS levels. However, the outstanding energy demands induced by the activation program require ATP to be produced in a swift and continuous manner, making glycolysis the preferred metabolic pathway for newly activated T cells. In the case of CD4⁺ T cells, glucose intake -and the success of the activation program- is highly dependent on the Glucose Transporter 1 (GLUT1) which is significantly upregulated upon TCR engagement⁹². In addition to GLUT 1, CD8⁺ T cells have been shown to express also GLUT3 and GLUT6, and the increase in glucose uptake is tightly regulated by the PI3K-mTOR pathways⁹².

Importantly, a seminal report by Chang and collaborators, proved that glycolysis is not necessary for activation or proliferation, but it is required to support effector functions in CTL. In fact, impaired glycolysis leads to reduced mRNA translation of cytokines IFN γ and IL2⁹³. Since one of the hallmarks of cancer is glucose depletion (see discussion below), this report has had a great impact on the way we understand

anti-tumor immunity and is fundamental when addressing possible therapeutic approaches to improve it.

Once an antigen has been cleared, the contraction phase of the immune response is initiated. During this phase, activation of AMPK and the MAPK lead to decreased mTOR signaling and the consequent activation of autophagy. T cells that are able to disengage glycolysis and switch to Fatty Acid Oxidation (FAO)- and Oxidative Phosphorylation (OXPHOS)-based metabolism will persist as long-term memory T cells⁹⁴. Hence, metabolic switches play an important role in defining T cell differentiation states and represent a potent tool to armor T cells against chronic infections and cancer.

b. FAO, OXPHOS, and *De novo* lipid synthesis

Fatty acids (FA) play key roles in all stages of T cell activation and differentiation as (1) key components of biological membranes, (2) signaling molecules or intermediates, and (3) energy substrates.

As essential components of the T cell plasma membrane, FA are deeply impacted by T cell activation. In particular, TCR engagement is known to drive significant changes in the composition, distribution, and dynamic of plasma membrane lipids⁹⁵. In this scenario, one of the main roles of FA is to coordinate the formation of the immunological synapse. Indeed, high cholesterol levels in the plasma membrane are known to facilitate TCR clustering, maturation of the immunological synapse, and degranulation in CD8⁺ T cells⁹⁶. Plasma membrane lipids also participate in the initiation of several signaling pathways by modifying the anchor properties Signal Transducer and Activator of Transcription 3 (STAT3), AMP-activated protein kinase (AMPK), Linker for Activation of T cells (LAT), and Histone deacetylases (HDAC) that are implicated in T cell proliferation and transcriptional control⁹⁷.

Besides their role as biological membrane components, lipids also have essential functions as secondary messengers importing TCR-derived signals, through the cytosol and into the cell's organelles. An important example is that of

phosphatidylinositol 4,5-bisphosphate (PIP₂), a membrane phospholipid that is hydrolyzed by phospholipase C (PLC) upon TCR engagement. PIP₂ is transformed into inositol triphosphate (IP₃) and diacylglycerol (DAG) which are responsible for mediating Ca²⁺ release and activation of Protein Kinase C (PKC) respectively⁹⁸. These processes have been correlated with cytoskeleton reorganization and actin polymerization⁹⁹ which are of paramount importance for T cell effector functions.

From the metabolic perspective, both FA anabolic and catabolic processes play an important role through all the diverse phases of T cell activation and development. Once activated, T cells upregulate *de novo* synthesis to produce FA- and mevalonate-derived lipids. This process is tightly regulated by the activation of Sterol Regulatory Element-Binding Proteins (SREBP) by SREBP cleavage activation protein (SCAP)^{98,100}. SREBP activation is indispensable for immune responses since it drives the early metabolic reprogramming required for T cells to exit quiescence and enter the expansion phase⁹⁸. In fact, newly synthesized lipids resulting from SREBP activation can be directly used by T cells as building blocks to increase biological membrane surface extension -thus sustaining proliferation- or as energy storage substrate -thus promoting the formation of lipid droplets¹⁰¹.

The role of SREBP in T cell differentiation, proliferation, and effector functions was thoughtfully investigated by Kidani and collaborators. SREBP loss of function in CD8⁺ T cells abrogated lipid synthesis upon activation and led to reduced growth and proliferation *in vitro* and *in vivo* following lymphocytic choriomeningitis (LCMV)¹⁰². Interestingly, the inactivation of SREBP activity also caused various metabolic defects in murine CD8⁺ T cells *in vitro*, including decreased glycolytic activity on top of the expected reduction of mitochondrial respiration¹⁰². These data unveil the intricate nature of the crosstalk between different metabolic pathways and serve as an example of how metabolic rewiring is tightly connected with T cell differentiation status and functions.

FA can also be used as an energy source in the mitochondria where the catabolic processes FAO and OXPHOS take place. FAO can be fueled by exogenous lipids

or intracellular FA reservoirs. Extracellular lipids are imported via FA transporters such as CD36 or Low-density lipoprotein receptors (LDLR), while endogenous lipids are sourced from lipid droplets via lysosomal acid lipase (LAL)-mediated lipolysis. Once inside the cytosol, FA are mobilized to the mitochondria by FA-binding proteins (FABP) and translocated into the mitochondrial matrix through the Carnitine palmitoyltransferase I-alpha (CPT1 α). Inside the mitochondria, FA enter FAO to produce acetyl-CoA that will fuel the Tricarboxylic acid (TCA) cycle to produce NADH that will fuel OXPHOS in the mitochondrial intermembrane space⁹⁸.

Mechanisms of FA import to FAO and TCA cycle.

Importantly, the preferential usage of FAO and OXPHOS has been linked to specific T cell fates and functional characteristics⁹¹. Although higher reliance on OXPHOS is a common metabolic trait of all memory T cells, different cell subpopulations have particular nuances in their metabolic profiles⁹⁴. For instance, effector memory T cells retain a higher glycolytic rate than central memory or resident memory T cells¹⁰³. On the other hand, although central memory and resident memory T cells share their dependency on OXPHOS, they differ in terms of substrate utilization, with resident memory T cells being able to uptake larger quantities of FA from the

microenvironment than central memory T cells that rely mostly on lipogenesis to fuel FAO¹⁰⁴.

More in-depth analysis of the use of FAO in memory T cells was performed by O'Sullivan and collaborators, who demonstrated that memory development relies on cell-intrinsic lipolysis to feed FA into FAO in a process fueled by glycolysis¹⁰⁵. These data, other than revealing the mechanisms leading to the boost of FAO in memory T cells, highlighted the essential -albeit unnoticed- contribution of glycolysis in the development of the memory phenotype, which added another important piece to the metabolic landscape complexity.

However, there's conflicting evidence on the role of FA in inflammation. For instance, in atherosclerosis saturated fatty acids are generally associated with pro-inflammatory responses with increased cytokine production, whereas unsaturated fatty acids induce anti-inflammatory responses in T cells¹⁰⁶. On the contrary, several studies in cancer have shown that the accumulation of saturated FA is strongly correlated with T cell dysfunction and reduced anti-tumor activity^{107–112}. These data clearly highlight the need for new studies that focus on the mechanistic programs behind the FA metabolism in T cells and uncover the unlimited FA potential to act as potent immunomodulators in health and disease.

c. Amino acid metabolism

Given that amino acids are the basic building blocks of proteins, it comes as no surprise that T cell activation-driven increase in size and proliferation requires significant amounts of amino acids to act as biosynthetic precursors. For instance, glutamine -the most abundant amino acid in serum- has been proven essential for T cell activation and, in fact, its consumption is significantly increased upon TCR engagement¹¹³. Moreover, *in vitro* glutamine deprivation compromised activation-induced T cell growth and proliferation¹¹³ further confirming its essential role in immune functions. In this scenario, the solute carrier transporter 1a5 (Slc1a5, also called ASCT2) plays a crucial role as responsible for glutamine uptake¹¹⁴ that is then

metabolized into alpha-ketoglutarate and glutamate through glutaminolysis. In fact, murine Slc7a5-deficient CD8⁺ T cells failed to metabolically support the clonal expansion or effector functions in response to antigenic challenge¹¹⁴. Relevant to these findings, c-Myc depletion inhibited glutaminolysis by reducing the surface expression of glutamine antiporter CD98 -a heterodimer of Slc1a5- and compromising the activation-induced T cell growth¹¹³.

Other than glutamine, Slc7a5 mediates the transport of other large neutral amino acids including leucine. Indeed, Slc7a5 deficiency results in impaired leucine uptake and leads to metabolic catastrophe by hindering mTORC1 activation¹¹⁴ in a Rag GTPase-dependent manner^{115,116}. These findings strongly suggest a central role for leucine in regulating the mTORC1 signaling pathway and downstream cellular processes.

Another example of the importance of amino acids in T cell activation and effector functions is that of L-arginine. *In vitro* studies using human primary CD4⁺ and Jurkat T cells, proved that L-arginine depletion in the culture media causes cell cycle arrest at G0/G1, accompanied by an inability to upregulate cyclin D3 and cyclin-dependent kinase 4 (CDK4)¹¹⁷. Moreover, arginase 1 levels are considered a prognosis factor in the clinic, since cancer patients with increased activity of arginase 1-cleaving L-arginine- also exhibit decreased T cell proliferation and impaired anti-tumor activity¹¹⁸. Importantly, exogenous L-arginine supplementation in the diet of mice was able to increase the response of adoptively transferred human CD4⁺ T cells against cognate antigen-expressing *Influenza* virus¹¹⁹. This evidence highlights the importance of diet in the development of immune responses and represents a potent tool in the development of new approaches to potentiate immune responses against infections and cancer.

Although several reports have gathered evidence regarding amino acid metabolism in regulating CD8⁺ T cell activation, more-comprehensive probing of amino acid profiles and dynamics is needed to provide mechanistic insights into the impact of amino acids on the modulation of T cell metabolism and fate decision.

Epigenetic-metabolic axes regulate T cell differentiation

As previously mentioned, the transcriptional changes induced by T cell activation play a pivotal role in establishing and developing diverse differentiation and metabolic landscapes. In this scenario, the ability of T cells to employ epigenetic changes to maintain these expression patterns following signal withdrawal becomes essential^{81,120}. Interestingly, metabolic pathways and epigenetic regulation are crosslinked in bidirectional crosstalk.

The activities of many chromatin-modifying enzymes are regulated, at least in part, by the concentration and gradient of metabolic substrates or cofactors. Therefore, the availability of metabolic intermediates may directly impact the epigenome of CD8⁺ T cells. For instance, the availability of acetyl-CoA can be rate-limiting for protein acetylation, thus modulating the transcription of genes involved in growth and proliferation¹²⁰. A recent study showed that memory CD8⁺ T cells took up acetate and converted it into acetyl-CoA, which in turn drove glyceraldehyde 3-phosphate dehydrogenase (GAPDH) acetylation. Accordingly, memory CD8⁺ T cells generated in the presence of acetate showed enhanced IFN γ production and greater recall capacity, concomitant to increased glycolytic flux and oxygen consumption¹²¹, suggesting that exposure to higher concentrations of acetate enables the reprogramming of T cell differentiation.

In the case of CD4⁺ T cells, lactate dehydrogenase (LDHA) deficiency led to decreased Th1 differentiation accompanied by reduced IFN γ expression without impairing global gene expression. This study further reported that decreased INF γ expression by LDHA deficiency was due to lower acetyl-CoA generation and reduced histone acetylation. Notably, excess acetate could restore histone remodeling at the IFN γ locus as well as mRNA expression in CD4⁺ T cells. Thus, these findings reveal that aerobic glycolysis can promote effector CD4⁺ T cell differentiation via epigenetic regulation. On the other hand, sirtuin 6 (SIRT6) -a histone H3K9 deacetylase- deficiency has been reported to increase glucose uptake with upregulation of glycolysis and diminished mitochondrial respiration in murine embryonic stem (ES) cells¹²². These reports provide evidence that metabolic pathways could be potential druggable targets to modulate T cells' epigenetic landscape and viceversa.

Nevertheless, the molecular connections between cellular metabolism and epigenetic remodeling in terms of CD8⁺ T cell differentiation and function remain explored in greater detail. Understanding the link between metabolism and epigenetic landscape in tumor-infiltrating CD8⁺ T cells would help develop new immunotherapy strategies based on epigenetic reactivation of metabolically exhausted infiltrating CD8⁺ T cells.

Cancer, the tumor microenvironment, and its implications for anti-tumor activity

Cancer is characterized by the abnormal growth of cells with the potential to go beyond their usual boundaries, spread, and invade other organs forming metastasis, which is one of the major causes of death from cancer.

During cancer progression, tissues and cell populations go through a remodeling process leading to the formation of the Tumor Microenvironment (TME). Hence, the TME is defined as the primary location in which tumor cells and the host immune system interact. Tumors are aggregates of cells, among which are not solely found malignant cells, but also a vast variety of other cell populations, mainly immune cells, fibroblasts, and blood and lymphatic vessels. Importantly, the crosstalk between cancer and host cells within the TME is crucial for the progression of several hallmarks of the disease; including angiogenesis, tumor-promoting inflammation, and immune escape^{123–125}. The production of cytokines and chemokines within the TME recruits diverse immune populations, each with differential effects on tumor progression and therapeutic outcomes^{125,126}. As oncology research moves forward, the study of the tight correlation between immune cells, the TME, and the clinical outcome of the disease has become essential for developing new therapeutic strategies. The TME is indeed a complex and continuously evolving entity, where the type and availability of nutrients, metabolites, and other biomolecules are regulated by malignant cells¹²⁷. Regardless of the increased interest in the study of the TME, the chain of events leading to the establishment of the tumor's metabolic program is yet to be fully understood. In fact, a study by Tsai and collaborators recently proved that IFN γ exposure during early-

stage tumorigenesis cues tumor cells to engage in aerobic glycolysis at the expense of FAO in a Myc-dependent manner¹²⁸.

Certain commonalities across cancer types are considered hallmarks of cancer progression including low glucose and oxygen availability, increased lactate and ion concentration, and fatty acid import from plasma or neighboring cells¹²⁹.

a. Glucose depletion and the Warburg effect

Under physiological conditions, cells exhibit low glycolytic rates and convert most glucose into carbon dioxide and water. On the contrary, cancer cells are characterized by an exceptionally high glycolytic rate converting the majority of incoming glucose into lactate even with ample oxygen in present in the TME¹³⁰.

Further studies demonstrated that high glucose consumption was necessary for cancer cells to maintain high division rates^{131,132}. Importantly, even when OXPHOS is significantly more efficient than glycolysis in the production of ATP, glycolysis is the fastest way to meet the energetic demands of rapidly proliferating cancer cells^{133,134}. This phenomenon was later coined the “Warburg effect” and refers to the tumor-induced depletion of glucose in the TME driven by this increased glycolytic rate.

The effects of glucose deprivation on T cell activity are catastrophic since the cytotoxic activity is heavily dependent on the glycolytic program. In fact, two seminal works were published in 2015 addressing the role of glucose deprivation in anti-tumor immune responses. On one hand, Chang and collaborators demonstrated that glucose deprivation in the TME dampens mTOR activity and downstream targets in TIL. Moreover, they also showed that ICB antibodies commonly used in the clinic can restore glucose influx within the TME, allowing TILs to engage in glycolysis to fuel cytokine production and tumor killing⁴⁶. Similarly, Ho and collaborators proved that limited glucose availability in the TME suppressed CD4+ T cell effector function and immunosurveillance¹³⁵. Interestingly, the reduced cytotoxic activity in CD4+ T cells was correlated with glycolysis-mediated

attenuation of Ca^{2+} -NFAT signaling¹³⁵; further providing evidence of the strong interconnection between metabolic programs, signaling pathways, and effector functions.

b. Oxygen deletion and hypoxia

As the rapid and uncontrolled proliferation of tumor cells continues, insufficient blood supply and oxygen depletion lead to intra-tumoral oxygen gradients which are a typical microenvironment feature found in most solid tumors¹³⁶. The development of hypoxic regions within the tumor can cause necrosis. However, while adapting to hypoxia, tumor cells can also become more aggressive, therapeutically resistant, and metastatic. These processes are driven by increased expression of hypoxia-inducible factor 1 α (HIF1 α) in tumor cells and lead to drug resistance and poor prognosis¹³⁷.

In the case of TIL, the evidence seems to point to a differential effect among T cell sub-types. On one hand, Clever and collaborators (2016) demonstrated that upon migration to the lungs, metastatic cells increased HIF1 α expression triggering Treg cell expansion, and the consequent impairment of IFN γ production¹³⁸. On the contrary, several studies on CD8 $^{+}$ T cells show that HIF1 α expression favors cytotoxic function. Genetic deletion of HIF1 α in adoptively transferred murine CD8 $^{+}$ T cells accelerates melanoma growth by promoting tumor vascularization¹³⁹. Accordingly, ectopic HIF expression in murine and human CD8 $^{+}$ T cells was able to increase tumor killing and drive the expression of a memory-related gene signature¹⁴⁰; further reaffirming the interconnection between nutrient availability, metabolism, and T cell differentiation.

c. Accumulation of K $^{+}$ ions

Based on the observation that solid tumors display dense areas of cellular apoptosis and necrosis, which alter the extracellular milieu by releasing intracellular ions, Eil and collaborators investigated the effect of elevated [K $^{+}$] on T cell function. The results show that an increased concentration of [K $^{+}$] on the TME significantly

reduces the production of IFN γ by T effector cells in both mice and humans, thus inhibiting cancer cell killing¹⁴¹.

In recent years, new mechanistic evidence has emerged in the study of [K⁺] on T cell proliferation and effector functions. On one hand, the inhibition of effector programs in T cells is driven by impaired nutrient uptake, leading to autophagy and reduction of histone acetylation at effector loci^{141,142}, thus reducing cytotoxic capabilities. However, the same reduction of histone acetylation also modulates exhaustion loci, endowing CD8⁺ T cells with improved *in vivo* persistence, multipotency, and tumor clearance¹⁴². This new evidence underscores the intricate and complex molecular pathways involving ion homeostasis in T cells and provides potential new approaches to armor them against cancer.

Of note, the effects of [K⁺] on T cell functions are specific to each particular differentiation profile and these differences are mainly mediated by the differential expression of plasma membrane [K⁺] channels. For instance, activated T_{EM} cells (CD45RO⁺CCR7⁻) express high levels of voltage-gated [K⁺] (Kv1.3) channels, while calcium-dependent [K⁺] (KCa3.1) channels are more abundant in naïve (CD45RO⁻CCR7⁺) and T_{CM} cells (CD45RO⁺CCR7⁺)^{64,143}. These structural differences add-up to the level of complexity underlying the effects of [K⁺] in T cells and further studies are required to elucidate the possible exploitation of these differences to increase T cell functionality.

d. Lactic acidosis

As a direct consequence of the elevated glycolytic rates, there is a massive accumulation of lactate in the tumor's interstitial fluid leading to the development of lactic acidosis. This phenomenon is common in many tumor types and has been reported to rescue cancer cells from apoptosis by arresting cells at G0/G1 phase and activating autophagy¹⁴⁴.

Lactic acidosis has been correlated with poor prognosis and suppression of the anti-cancer immune response¹⁴⁵. In fact, pre-clinical studies using bicarbonate monotherapy to reduce lactic acidosis showed significant improvement in anti-tumor

responses in anti-CTLA-4, anti-PD1, or adoptive T cell transfer in melanoma and pancreatic cancer¹⁴⁶. In this regard, a novel study provided evidence that lactic acid increases PD1 expression in regulatory T (T_{REG}) cells in a c-Myc-dependent manner. Thus providing an insight on the mechanisms underlying PD1 regulation within the TME and a novel prognosis marker for the success of ICB.

Paradoxically, Feng and collaborators proved that subcutaneous administration of sodium lactate to MC38 tumor-bearing mice results in increased CD8⁺ T cell-dependent tumor control. Mechanistically, increased lactate concentrations inhibit histone deacetylase activity and leads to increased TCF1 expression in CD8⁺ T cells. Of note, these effects of lactate were also observed when adoptively transferring *in vitro* pre-treated CD8⁺ T cell into tumor-bearing mice¹⁴⁷ providing a new *ex vivo* strategy to increase ACT efficacy.

e. Lipid accumulation

Lipid accumulation within the TME is frequently accompanied by perturbation of TIL's metabolic landscape and -consequently- hinders T cell cytotoxic functions^{148–150}. In fact, our group recently showed a previously unappreciated role of specific saturated Long-Chain Fatty Acids (LCFAs) in CD8⁺ T cell biology. We demonstrated that palmitamide accumulation in the TME harms TIL's mitochondrial integrity and function, and leads to metabolic exhaustion and defective anti-tumor response¹⁴⁹. Our results were further validated by other reports showing that oxidized lipid uptake leads to defective effector functions in TIL¹⁴⁸. Interestingly, this study also showed that the inhibition of TIL effector function was dependent on CD36¹⁴⁸ thus providing a potential strategy to avoid T cell dysfunction. Moreover, lipid-induced defects in the cytotoxic machinery were also observed in NK cells, where obesity induces peroxisome proliferator-activated receptor (PPAR)-driven lipid accumulation and drives a state of paralysis of their metabolism and trafficking¹⁵¹.

However, in the case of CD4⁺ T cells, high-fat content in the diet seems to induce a more inflammatory profile. Indeed, Mauro and collaborators showed high palmitate

concentration promotes the activation of effector memory T cells as opposed to central memory by modulating the PI3K-Akt axis and inducing metabolic reprogramming via FAO¹⁵². This evidence suggests that the effects of FA on metabolism, effector function, and cell fate could be driven by cell-specific factors. However, whether all lipids are detrimental or differentially regulate fate decisions on immune cells remains unanswered and requires detailed mechanistic dissection.

Overcoming exhaustion through cancer immunotherapies

Cancer immunotherapies harness the anti-tumor activity of CD8⁺ T cells and are a cornerstone in the treatment of cancer patients. Even with a success rate of 15-20%, immunotherapies have revolutionized the field of oncology by providing long-lasting tumor control in multiple solid and hematological malignancies.

Adoptive Cell Therapy (ACT) is a treatment designed to enhance the immune system's capacity to deal with diseases by infusion of immune effector cells. Depending on the cell type, the effectors can be obtained from donors (allogenic) or from the patients themselves (autologous) and can be armed to exert specific functions by ex vivo activation and/or genetic modification, after which they are re-infused to patients leading to the elimination of tumors¹⁵³.

Effector cells currently used in the clinic for ACT include Tumor-Infiltrating Lymphocytes (TIL), and TCR-based therapies.

TIL cells were identified in 1986 as a subpopulation of -mostly- CD8⁺ T lymphocytes that migrate and infiltrate into growing tumors. These cells are isolated from the tumor mass of patients and expanded ex vivo to later perform re-infusion into the patient. Clinical studies using TIL cells for the treatment of patients with melanoma achieved promising clinical benefits¹⁵⁴. Nonetheless, the biggest drawback of the use of TIL cells as ACT is that not all patients are eligible for this approach, since their TILs might not expand sufficiently or because their tumors might have lost expression of antigens or MHC molecules¹⁵⁵.

TCR based therapies is a broader category of therapeutic approaches including Chimeric Antigen Receptor (CAR)-modified T cells (CAR-T) and TCR-redirected T cells (TCR-T). Both approaches are based in the *ex vivo* genetic modification of the patient's own T lymphocytes that are later infused back to exert their anti-tumor activity. The difference between these two products resides in the mechanisms used for recognizing antigens.

CAR-T cells (now available in marketed products) are synthetic or hybrid molecules expressing the antigen recognition domain of the B-cell receptor, and the co-stimulatory domains of the T cell receptor. CARs provide T cells with the ability of specifically recognize defined antigens in the cell surface of tumor cells and thus allow highly specific T cell targeting. One of the advantages of using CAR-T cells is the targeting of Tumor-Specific Antigens (TSA), which are not significantly expressed in normal tissues, thus reducing the possibility of off-target side effects¹⁵⁶.

On the contrary, TCR-engineered effector cells are instructed with naturally occurring (or minimally modified) TCR coming from the genetic screening of the patient-specific tumor sample. This method has a broader application potential since it allows engineered T cells to recognize intracellular TSA which are not available for CAR-based approaches¹⁵⁷. However, contrary to CAR-T cells, the efficiency of TCR-T cell therapy depends on MHC molecules presentation to recognize targets and activate T cell function. This particular characteristic of TCR-T cells represents one of its greatest disadvantages since tumors may escape immune surveillance by downregulating MHC molecules in the cell's surface¹⁵⁸.

In all, antigen selection is crucial for both CAR-T and TCR-T therapy efficiency, since the selection of Tumor-Associated Antigens (TAAs), which expression on normal tissues varies among patients, can cause engineered immune cells to attack both the tumor tissues and normal tissues or organs. Thus the discovery and/or selection of novel antigens is fundamental to securing the efficacy of TCR-based therapies.

ACT approaches hold the promise to enhance cancer treatment by instructing the patient's immune system to recognize and attack specific cancer cells¹⁵⁹. In fact, it has given rise to unparalleled clinical outcomes in the treatment of cancer^{154,160,161} but its efficacy - especially in solid tumors - is often limited by the scarce intra-tumor infiltration, persistence, and

function of adoptively transferred T cell^{162–165}. Thus, there is an urgent need to identify factors able to improve immunotherapy efficacy.

Metabolic rewiring to potentiate ACT approaches

As previously described, metabolic constraints imposed by the TME greatly influence CTL anti-tumor responses. So it is not surprising that these same metabolic programs hinder the success of adoptively transferred immune cells^{48,166,167}. In this scenario, T cell metabolism has been investigated as a potential target for enhancing immunological responses against tumors¹⁶⁸. Here we review the three main approaches used for T cell metabolic rewiring:

a. Systemic treatments

Great efforts have been made to increase CTL anti-tumor responses through systemic treatments. The main reasoning behind this approach is the potential to simultaneously boost both the patient's intrinsic CTL and adoptively transferred population with a single intervention. However, attention should be made to the undesired secondary effects that could result from such systemic approaches and that represents a major hurdle on the road to clinical approval (

One of the most relevant examples is that of IL10 which was first described to induce metabolic reprogramming of macrophages by increasing OXPHOS and driving strong anti-inflammatory effects¹⁶⁹. More recent studies tested the role of IL10 in modulating T_{EX} metabolism. Results showed that peritumoral administration of IL10 onto tumor-bearing mice significantly increased CD8⁺ TIL counts and proliferation, promoting tumor regression¹⁷⁰. Importantly, the effects of IL10 in anti-tumor activity seemed to be mediated by the substantial increase in OXPHOS at the expense of glycolysis¹⁷⁰. This metabolic program was accompanied by the enrichment of tumor-specific memory T cells with enhanced recall response upon ex vivo re-stimulation¹⁷⁰, proving once more that T cell differentiation and metabolic pathways are deeply intertwined.

b. Gene therapy

Given the wide range of enzymes correlated with T cell metabolic dysfunction and fitness, genetic approaches have emerged as potential tools to overcome metabolic exhaustion in the TME and increase anti-tumor responses. A good example is the overexpression of Glutathione peroxidase 4 (GPX4), which inhibits CD36-mediated lipid peroxidation and restores effector functions in adoptively transferred CD8⁺ T cells in a murine melanoma model¹⁴⁸. Similarly, Phosphoenolpyruvate Carboxykinase 1 (PCK1) overexpression in CD4⁺ T cells can reverse the effects of glucose deprivation on Ca²⁺ flux, restore NFAT1 nuclear localization, and boost the production of IFN γ upon adoptive transfer; thus increasing tumor control¹³⁵.

c. *Ex vivo* metabolic instruction

Amongst the different approaches reported, *ex vivo* T cell metabolic instruction provides the added advantage to minimize potential off-target and consequent undesired secondary effects for the patients¹⁷¹.

A very promising approach for use in the clinic was first described by Sukumar and collaborators. They reported that glycolysis blockage -by glucose analog 2-deoxy-n-glucose (2-DG) treatment- during T cell *in vitro* expansion, primed CD8⁺ T cells to exert increased glycolytic activity upon adoptive transfer in melanoma-bearing mice¹⁷². Moreover, 2-DG-treated cells showed increased production of IFN γ and TNF α after adoptive transfer, leading to effective tumor regression¹⁷².

Importantly, *ex vivo* metabolic instruction of CTL represents a powerful tool with high clinical potential, since they don't pose any implications in the patient's journey and are fairly simple to scale up following Good Manufacturing Protocols (GMP).

All things considered, the present project was conceived with the aim of dissecting the role of Linoleic acid (LA; C18:2n-6) in shaping CD8⁺ T cell metabolism and differentiation and, in turn, its impact on anti-tumor immunity.

LA is the most abundant ω -6 poly-unsaturated fatty acid (PUFA) assumed through diet^{173,174}, with essential functions in health and disease¹⁷⁵. However, its specific role on CTL immunity remains to be underscored and, in light of the impact of diet on immune function^{176–178}, decoding its modulatory effect on CTL response might have important implications for cancer therapies.

We provide evidence that LA is a major positive regulator of CD8+ T cell functionality *in vitro* and *in vivo*, in a murine model, and also in human CTL. We provide a mechanism that effectively links the LA-induced metabolic landscape with a memory-like transcriptional program, and the signaling pathways driving increased effector function and improved tumor control. Our observations may pave the way for highly effective ACTs targeting a wide range of malignancies in which LA is routinely applied *ex vivo* as part of T cell manufacturing to achieve metabolic reprogramming and promote long-term functionality for improved therapeutic efficacy

MATERIALS AND METHODS

CD8+ T cell isolation and activation

Spleen and lymph nodes (inguinal and axillary) were harvested from mice under sterile conditions, mechanically dissociated into single cell suspension and red blood cells were lysed. Cells were then sorted by negative selection following the manufacturer's instructions. After sorting, cells were resuspended at 1×10^6 cells/mL in complete RPMI media (10% FBS, 1% NaPy, 1% Glutamine, 1% Pen/Strep, 0,1% β -mercaptoethanol) supplemented with 100U/mL IL2. For OT1 mice, media was further complemented with 2 μ g/mL of SIINFELK peptide, while for C57J/B6 mice polyclonal activation was achieved using 5 μ g/mL plated-bound α CD3 and 0,5 μ g/mL soluble α CD28. Cells were further treated with BSA, linoleic acid, oleic acid or arachidonic acid. Unless other-wise specified, the final concentration for each lipid was kept at 100 μ M. For high glucose experiments, T cell media was supplemented with 12 mM D-()-glucose (twice as physiological conditions). Unless other-wise specified, cells were activated for 48 hours at 37°C, 5% CO₂ and 90-95% humidity for a pH neutral environment.

Immunophenotype and flow cytometry

Characterization of the activation and differentiation profile of LA-CTL was performed by flow cytometry following standard protocols. Briefly, for staining of surface markers 1×10^5 – 2×10^6 cells were harvested at the specified post-activation time points, washed with PBS, resuspended in FACS buffer (PBS, 1% FBS, EDTA 2mM) and stained for 20 minutes at 4°C. Intracellular staining for cytokine production was performed following a 4 hours incubation with complete RPMI and 1:1000 dilution of GlogiPlug in the presence or absence of a secondary stimulation with SIINFELK peptide (5 μ g/mL) or PMA/Ionomycin (20 ng/mL and 1 μ g/mL respectively) at 37°C. Cells were fixed and permeabilized following the manufacturer's instructions. Intracellular staining was then performed in permeabilization

buffer for 2 hour at 4°C. CFSE; MitoTRK Orange and Green; and HCS LipidTOX stainings were performed as per manufacturer's instructions. The expression of GD2.CAR on T cells was detected using a specific anti-idiotypic antibody (1A7) while for the CD30 and CD19.CAR detection a biotinylated human CD30 or CD19 protein has been used (Acro Biosystems, Newark, DE – USA). Samples were acquired with a BD FACS Canto II or Celesta.

tSNE analysis

FCS data was analysed with software FlowJo v10.2. RPhenograph package was used to perform computational analysis of multiparametric flow cytometry data. 3000 events per sample were concatenated by the "cytof_exprsMerge function", after manual gating isolation of singlet, LD negative CD4+5+CD3+CD8+ T cells. All samples were concatenated by the "cytof_exprsMerge function". /). The number of nearest neighbours identified in the first iteration of the algorithm, K value, was set to 100. UMAP and tSNE representation were generated and visualized using FlowJo version 10.2. Under-represented clusters (<0.5%) were discarded in subsequent analysis.

Immunofluorescence and confocal microscopy

Confocal microscopy with fixed cells was performed on a LEICA SP8 FSU confocal (Leica Microsystems GmbH, Wetzlar, Germany) microscope (Imaging Unit, IEO) with a 40X/1.3, 63X/1.4 or 100X/1.47 oil- immersion objectives. Cells were pre-stained with MitoOr. and a fixable viability dye; allowed to attach by gravity on culture slides coated with poly-d-lysine, and then fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. IP3R IC staining was performed overnight at 4°C in blocking buffer (PBS, Triton 0,1%, 3% BSA, and 5% FBS) and afterward cells were counterstained with DAPI and the indicated secondary antibody for 2 hours at room temperature.

For static killing experiments, B16-OVA cells were plated on a Lab-Tek II chamber slide and left to adhere for 6 hours prior to co-culture with activated CD8+ T cells. OT1 CTL activated in the presence or absence of LA for 48 hours were then counted and added to the chamber

in a Effector: Target (E:T) ratio of 1:1. 12 hours post-co-culture, chambers were washed, stained with a fixable viability dye and α CD8+ for 30 min at 4°C in PBS; fixed and permeabilized as previously described. Cells were then stained with primary antibody α Caspase 3 overnight at 4°C, labeled with the appropriate secondary antibody, and counterstained with DAPI for 2 hours at room temperature.

For colocalization analysis, a multi-channel Z-stack of 2.5 μ m of the central part of the cells was acquired for each field of view with a 63x/1.4NA oil immersion objective lens and a voxel size of 60x60x180nm. DAPI, anti-IP3R labelled with an AlexaFluor488 labeled secondary antibody, MitoOr. and DRAQ7 signals were excited with 405 nm, 488 nm, 568 nm, 633 nm laser lines and the emitted fluorescence acquired in the following acquisition windows: 410-512, 495-545, 566-668, 638-775 nm, respectively. The Z-stacks were deconvolved with Huygens software and the central section of each cell was used to calculate the percentage of colocalization. A macro in Fiji/ImageJ ¹⁷⁹ has been created. Briefly, regions of interest (ROIs) including only one cell were split in the single fluorescent channels, the IP3R and MitoOr. channels were segmented using the Moments and Otsu algorithm, respectively, and the area occupied by the two signals was calculated. The colocalization area was calculated as the interception between the IP3R and the MitoTracker Orange binary images and expressed as percentage respect to the total area occupied by the mitochondria.

Time-lapse microscopy

For time-lapse killing experiments B16-OVA cells were pre-stained with Hoechst33342 solution at 37°C for 30 minutes before plating on a pre-coated Ibidi chamber slide and left to adhere for 8 hours. OT1 cells were activated in the presence or absence of LA; with or without TG for 12 or 48 hours and stained using 1 μ M Calcein AM at 37°C for 30 minutes. CTL were then put in co-culture with tumor cells at E:T ratios of 1:1 or 1:4 as specified. DRAQ7 dye was added directly to the chambers to follow cell death in real time using a Leica Thunder Imager microscope equipped with a Okolab incubation system (Okolab S.r.l. Naples, Italy). Acquisitions were made every 15 minutes for a total of 5 hours and 8-9

randomly assigned fields were acquired for each chamber. Time-lapse hyper-stacks were processed with Fiji/ImageJ and a custom macro. Briefly, Hoechst, calcein and DRAQ7 channels were processed for shading correction using the BaSiC tool ¹⁸⁰. Then, tumor cells were segmented thanks to the Hoechst staining and the DRAQ7 signal was measured in the segmented cells. Results tables were analyzed with the R software. The number of dead cells at each time-point was corrected for the number of dead cells at the first time-point and the percentage of dead cells calculated respect to the total number of cells.

Fluorescence lifetime imaging microscopy (FLIM)

OT1 cells were sorted with >95% purity as previously described, activated for 48 hours in the presence or absence of LA and 5x10⁵ cells were seeded on a Poly-Lysine coated coverslip and let to adhere for 30 min at 37°C. Fixation was performed using following manufacturer's instructions. NAD(P)H Fluorescence Lifetime Imaging Microscopy ^{181,182} was performed using an OLYMPUS Fluoview FV3000 system (Shinjuku, Tokyo, Japan) equipped with a pulsed 372 nm laser from PicoQuant (Berlin, Germany). The laser was operated at 40 MHz and photons were detected by a photon counting detector (Photomultiplier Detector Assembly Hybrid Series, PicoQuant, Berlin, Germany). Photons were collected from two randomly chosen fields from each well of each condition (10 minutes each, 60x magnification, 256x256 pixels). Fluorescence decay data were acquired using SymPhoTime 64 software. The raw data were converted to TIFF format, imported into MATLAB and processed using a custom script. A triexponential curve was fitted to the averaged decay curve of all pixels with a photon count greater than 100. The resulting decay coefficients were 1.645 ns (assigned to protein-bound NAD(P)H), 0.400 ns (assigned to free NAD(P)H), and 6.814 ns (unassigned, but observed also in control NADH + lactate dehydrogenase solutions). NADH and NADPH have the same spectral properties and thus the acronym "NAD(P)H" was used to indicate their combination (Datta et al., 2020). Fluorescence lifetimes were consistent with the lifetimes previously reported for bound and free NAD(P)H (Datta et al., 2020). The pre-exponential factors of the averaged decay curve of each field (obtained from pixels with a photon count >100) were fitted with a triexponential

curve, using the global decay coefficients as fixed parameters. The ratio between the pre-exponential coefficients of protein-bound NAD(P)H and free NAD(P)H was calculated for each field. An increase in the protein bound/free NAD(P)H ratio corresponds to a shift from a glycolytic towards an OXPHOS metabolism ¹⁸¹. Protein bound/Free NAD(P)H ratios referring to the same condition of each mouse were averaged.

Metabolic assays

A Seahorse XF-96e extracellular flux analyzer (Seahorse Bioscience, Agilent) was used to determine the metabolic profile of cells. OT1 CD8⁺ T cells were plated (2x10⁵ cells/well) onto cell culture microplates coated with poly-lysine. Mitochondrial perturbation experiments were carried out using the MitoStress Test Kit by the sequential treatment with oligomycin (Oligo, 1 μ M), FCCP (1.5 μ M), and rotenone (Rotenone A + Antimycin, 0,1 μ M). Oxygen consumption rates (OCR, pmol/min), proton efflux rate (PER, pmol/min), and extracellular acidification rates (ECAR, mpH/min) were monitored in real time after injection of each compound. A variation on the standard MitoStress Test protocol was used to assess the role of FAO in OCR and SRC levels of CD8⁺ T cells. Briefly, etomoxir (200 μ M) acute injection was performed ahead of the regular MitoStress Test. OCR, PER and ECAR levels were calculated as previously described in treated and non-treated cells (with complete media as acute injection for control). Glycolytic Rate Assay was performed by the sequential injection of Rotenone A + Antimycin A (0,5 μ M) and 2-deoxy-D-glucose (2-DG, 50nM) to discriminate between the glycolysis- and mitochondrial-dependent PER.

Electron microscopy

Transmission electron microscopy was performed at the ALEMBIC Facility (San Raffaele Hospital). Cells were pelleted and fixed in 2,5% glutaraldehyde 0.1M sodium cacodylate pH 7.4 for 1 hour at RT. After fixation cells were rinsed with 0.1 M Cacodylate and post-fixed in 1% OsO₄, 1.5% K₄Fe(CN)₆, 0.1M sodium cacodylate for 1h at 4°C. After washes steps cells were dehydrated using ethanol and embedded in Epon. Ultrathin sectioning (70 nm) was performed on a Leica EM UC7 ultramicrotome and collected on 300 mesh grids. Thin

sections were contrasted with uranyl acetate and Sato lead stain. Samples were observed with a TALOS L120C Transmission Electron Microscope (ThermoFisher Scientific) and images were acquired with a CETA 4x4k CMOS camera (ThermoFisher Scientific).

MAM's and ER-mitochondria contact analysis

From the EM images, the endoplasmic reticulum and mitochondria masks are manually segmented. These masks are analysed with a MATLAB pipeline to identify the contact sites. We first extract information on the single mitochondria: for each mitochondrion mask, the area is expanded in each direction of 30 nm. These contact sites (ER-MAMs) are identified as the intersections of the ER mask that follow within this new area. The ER-MAMs extension is calculated and normalized to mitochondrion perimeter and expressed as percentage. For broad ER-mitochondria contacts the same analysis is performed within a range from mitochondria of 30 to 100nm.

RNA-seq and analysis

OT1 cells were sorted with >95% purity as previously described, activated for 48 hours in the presence or absence of LA, counted and resuspended in RLT lysis buffer (Qiagen). RNA was isolated from purified cells using RNeasy Mini kits, and RNA concentrations were determined using Nanodrop. Fastqc software was used on the fastq files to generate the quality control report, and Tophat software was used to align the sequences to the mouse genome mm10. HTSeq was used to count the read number of genes from each sample. Genes with read counts <10 in all the samples were considered to be noise and therefore removed. The obtained read counts are analogous to the expression level of each gene across all the samples. Differential expression analysis was performed using DESeq2¹⁸⁴. Genes with raw mean reads >10 (i.e., ~9,225 genes) were used for normalization and differential gene expression analysis using DESeq2 package in R. The Wald test defined in the DESeq function of the package was used for differential expression and further downstream analysis. PCA, hierarchical clustering plots (hclust), and XY plots between replicates of the same genotypes were used to examine for nominal amounts of nontechnical variation and other latent factors. PCA and volcano plots were generated

using ggplot2 package, and all the heatmaps were generated using pheatmap or heatmap2 package in R environment. Gene overlap analysis was performed against the Molecular Signatures Database ^{185,186} Investigate Gene Sets web program under GSEA.

WGCNA network analysis

Weighted gene co-expression network analysis (WGCNA) is one of the most commonly employed algorithms to construct gene co-expression networks across gene expression data, exploring the association between gene networks and external phenotypic/clinical traits of interest. The algorithm builds co-expression networks as undirected, weighted gene networks where nodes correspond to genes and edges between them are determined by the pairwise correlations between their gene expression profiles. By raising the absolute value of the correlation to a power (soft thresholding), the weighted gene co-expression network construction emphasizes high correlations at the expense of low correlations. A reasonable choice to properly set the value of this power is the one that guarantees an approximate scale-free network topology that is typical of most of biological networks [3,4]. Scale-free networks are extremely heterogeneous with a topology characterized by a few highly connected nodes (hub nodes) and many less connected nodes (peripheral nodes). Then, WGCNA identifies modules (labeled by colors) of highly interconnected, or co-expressed, genes within the weighted network by grouping together the most similar nodes. The similarity measure between two nodes is expressed in terms of their direct connection strength as well as connection strengths “mediated” by shared neighbors. The relationship among modules and their associations with external traits can be studied by correlating the corresponding module eigengenes (MEs). The ME is defined as the first principal component of a given module and can be considered a representative of the gene expression profiles in that module. For each gene, a measure of module membership (MM) is defined by correlating its gene expression profile with the module eigengene of a given module and can be computed for all input genes (irrespective of their original module membership). If MM of a given gene with respect to a given module is close to 0, that gene is not part of that module. On the other hand, if MM is close to 1 or -1, the gene is highly

connected to the genes of that module. The sign of MM encodes whether the gene has a positive or a negative relationship with the module eigengene. Finally, to incorporate external information into the co-expression network, WGCNA makes use of gene significance (GS) measures computed as the correlations between gene expressions and external sample traits. Abstractly speaking, the higher the absolute value of GS of a given gene, the more biologically significant is that gene. The gene significance of 0 indicates that the gene is not significant with regard to the biological question of interest. The gene significance can take on positive or negative values.

Calcium influx

Intracellular changes in Ca^{2+} levels were measured using the ratio metric calcium-sensitive dye Fura-2-AM ¹⁸⁷. A total of 9×10^5 cells were incubated in 0.5 mL imaging buffer (HBSS, supplemented with 2mM CaCl_2 , 2mM MgCl_2 , 2% FCS) supplemented with 5 μM Fura-2-AM for 15 min at 37°C , washed twice with 10 ml of imaging buffer and resuspended in 135 μl imaging buffer. Cells were kept at RT for a maximum of 30 minutes prior to starting the experiment. Right before imaging, SLBs buffer was exchanged to imaging buffer prewarmed to 37°C . T-cells were pipetted into the imaging buffer and allowed to sink for 30 seconds after which 510/80 nm emission was recorded with alternating 340 nm and 387 nm excitation every 15 seconds for 20 minutes.

An in-house custom-built MATLAB software was used to track cells in each frame using a particle tracking algorithm published by Gao and Kilfoi ¹⁸⁸. Tracking parameters were chosen so only single cells in contact with the SLB were included. We used the MATLAB software to create ratio images for each frame. 'Methods for automated and accurate analysis of cell signals' or MAACS was used for population analysis as described in Salles et al., 2013 ¹⁸⁹. For each trajectory within a population the ratio was normalized frame-wise to that of the population median of T cells in contact with antigen-free SLBs. Cells that were above the threshold for at least 80% of their trajectory were counted as activated and plotted in a dose-response curve. The calcium histograms were compiled from the measured population values of the median Fura-2-AM ratio corresponding to the first 10 frames after

the peak Fura-2-AM ratio value within the trajectory. The latter was normalized frame-wise to the population median of the negative control, i.e. cells confronted with antigen-free SLBs.

Metabolomics and lipidomic assays

Liquid chromatography–MS lipidomic analyses were performed on sorted OT1 cells using reverse-phase liquid chromatography (RPLC) coupled with positive- and negative- mode electrospray-ionization high-resolution MS. Lipids from the samples were extracted using the Folch extraction method. Briefly, cell pellets were resuspended in 100 µl ammonium formate buffer, 50 mM, pH 6.8, followed by the addition of 400 µl ice-cold methanol. Each sample was vortexed and sonicated for 10 pulses at 30% power using a sonication probe while cooling on ice. 800 µl chloroform and 100 µl water were added to each sample to a final ratio of 4:2:1 chloroform: methanol: water. Samples were vortexed, incubated on ice for 10 min, and spun down at 1,000 rpm for 10 min. The bottom layer (organic layer containing the lipids) was transferred to a clean Eppendorf tube, and the extraction with chloroform of the aqueous layer was repeated once. The organic layers (lipid layers) were coupled and dried down under vacuum. Before MS analysis, dried lipids were reconstituted in chloroform: methanol (50:50, vol/ vol) containing an internal heavy labelled lipid standard (Splash Lipidomix Mass Spec Standard; Avanti Polar Lipids) and centrifuged for 5 min at 15,000 rpm. Samples were volume adjusted to ensure that cell count per volume (µl) was normalized. Global untargeted lipidomic analyses were performed using full MS and data-dependent acquisition analyses on a Q-Exactive HF hybrid quadrupole- Orbitrap mass spectrometer (Thermo Fisher Scientific) equipped with a Vanquish UHPLC binary system and auto-sampler (Thermo Fisher Scientific). Extracts (5-µl injection volume for RPLC-POS and 6 µl for RPLC-NEG) were separated by RPLC on a Hypersil Gold 1.9-µm, 2.1 × 100-mm column (Thermo Fisher Scientific) held at 40°C. Reverse-phase separation was performed at 250 µl/min using solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in isopropanol: acetonitrile: water [60:36:4]) with the following gradient: 30% B for 0.5 min, 30–70% B over 1.5 min, 70–99% B over 13 min, 99–100% B over

1min,10%Bheld4min,10–30%Bover1min,and30%Bheld over 5 min (gradient length 30 min).

Ultra-performance liquid chromatography/tandem MS raw data were imported, processed, normalized, and reviewed using Progenesis QI v.2.1 (Non-linear Dynamics). All sample runs were aligned against a quality control pool reference run, and peak picking was performed on individual aligned runs to create an aggregate dataset. Features (retention time and m/z pairs) were combined using both adduct and isotope deconvolution. Data were normalized to the spike-in lipid standards as an abundance ratio. Statistically significant changes were identified using multivariate statistical analysis, including PCA, and P values were generated using ANOVA or pairwise comparison. Tentative annotations¹⁹⁰ were made within Progenesis using accurate mass measurements (<5 ppm error), isotope distribution similarity, and assessment of fragmentation spectrum matching (when applicable) from the Lipid Maps database¹⁹¹. Four biological replicates and two technical replicates from each sample type were used to calculate the fold change and P value, and features were considered differentially expressed only if they met both criteria of fold change $\geq |2|$ and significance ($P \leq 0.05$). Heatmap visualization of individual lipid features was performed using Metaboanalyst 4.0 software¹⁹² using Pearson average clustering algorithm. To assess instrument performance and reproducibility throughout our experimental run sequence, the retention times and peak areas of SPLASH Lipidomix standards were monitored.

Total cell lysate preparation and cell fractionation

To prepare cell lysates for protein extraction, cells were activated for 48 hours in the presence or absence of LA. For the total cell protein extract, cell pellets were washed with cold PBS and lysed using RIPA buffer containing protease and phosphatase inhibitors. The subcellular fractionation was performed as previously described by Wieckowski et. al, 2009. Briefly, 100 M of OT-I T cells were homogenized in hypotonic buffer containing mannitol and sucrose and centrifuge at 600g for 5 min at 4°C to separate nuclei. The supernatant was collected and centrifuged at 7000g at 4°C to collect the MAMs/mitochondrial fraction.

The supernatant, containing ER microsomes and lysosomes fraction, was firstly centrifuged at 20000g for 30 min, to discard lysosomes and plasma membrane debris and, subsequently at 100000g for 1 h to obtain pure ER fraction. Pure mitochondria and MAMs were obtained via density-gradient separation with percoll. Pellet was resuspended in cold Mitochondria Resuspension Buffer (MRB), composed as described by Wieckowski et. al, 2009 and centrifuge at 95000g for 30 min at 4°C. Different fractions are collected and washed at 6300g for 10 min at 4°C in MRB, then MAMs fraction was concentrated by a further centrifugation at 100000g for 1h at 4°C.

Immunoblots

Protein concentrations were determined by BCA protein assay kit. Total cell lysates, cytoplasmic, mitochondria– ER junction-enriched mitochondrial fractions or pure mitochondrial fractions were separated using 10% acrylamide gel, and transferred to nitrocellulose membranes. Membranes were probed with antibodies against GR78, VDAC, IP3R or OXPHOS kit. Blots were stained with appropriate secondary antibodies and revealed using peroxide solution.

Lactate assay

Cell media was collected following 48 hours of T cell activation in the presence or absence of LA centrifuged at 2000 rpm for 10 minutes to remove any cell debris and lactate concentration in the TCM was detected using a colorimetric assay according to manufacturer's instructions.

Acetyl Co-A assay

CD8+ T cells were collected and washed in PBS to perform analysis of total and cytoplasmic AcCoA. For extraction of cytoplasmic AcCoA from 1x10⁶ T cells sample were homogenized in 1% Triton X-100, 20 mM Tris-HCl, pH=7.4, 150 mM NaCl on ice for 10 min and centrifugated at 20000 g for 10 min at 4°C to pellet nuclei and mitochondria. Supernatant obtained after centrifugation was used to measure the nucleocytosolic fraction. For total cellular AcCoA quantification, cells were wash in PBS and the pellet was extracted with two

freezing-thawing cycles on liquid nitrogen 80% methanol or 5% sulfo-salicylic acid with 50 μ M DTT. Samples were deproteinize with a 10 kDa MWCO spin filter prior to the assay. AcCoA was quantified with Acetyl-Coenzyme A Assay Kit following manufacturer instructions.

Adoptive cell transfer (ACT) experiments

In vivo experiments were performed using antigen specific melanoma cell line B16-OVA (Bellone reference) that was maintained in culture in RPMI, 10%FBS, 1%PenStrep, 0,4mg G418 up to 5 passages prior to injection. C57B6/J mice were subcutaneously injected with 2×10^5 cells and tumor growth was monitored daily. 10 days post-injections, and once tumors had reached a measurable size, ACT was performed intravenously using 5×10^6 OT1 cells previously activated in the presence or absence of LA as previously described. Tumors were inspected and measured daily to construct a growth curve using the formula: Tumor volume= length x width²/2, where length represents the largest tumor diameter and width represents the perpendicular tumor diameter. On the day of analysis spleen, draining and non-draining lymph nodes and tumor were collected from each mouse, disaggregated to single cell suspension and used for flow cytometry analysis.

Isolation and culture of primary human T lymphocytes

Peripheral blood mononuclear cells (PBMCs) were isolated from samples obtained from healthy donors or anonymous buffy coats (purchased as discarded material from the blood bank) of healthy donors using Ficoll density separation. CD8⁺ T cells were obtained from PBMCs by negative magnetic selection. T lymphocytes were activated with immobilized Anti-human CD3 (2 μ g/ml,) and anti-CD28 (2 μ g/ml) antibodies and then expanded in complete RPMI 1640 medium (10% FBS, 1% NaPy, 1% Glutamine, 1% Pen/Strep) supplemented with 100 U/mL IL2. Cells were cultured for 48 hours in the presence or absence of LA. Resting cells were kept as control with no further activation stimulus provided.

CAR-T cell engineering and GMP protocols

Cell lines. Neuroblastoma (NB)-derived cell line SHSY5Y, Burkitt's lymphoma cell line Raji and the immortalized human embryonic kidney 293T were obtained from DSMZ. The non-Hodgkin's Lymphoma (NHL)-derived cell line Karpas 299 was instead purchased from ECACC (Salisbury, United Kingdom). SHSY5Y and the 293T cell lines were maintained in culture with DMEM medium; the Karpas 299 and Raji cell lines were cultured with RPMI 1640 medium. All the cell lines were supplemented with 10% foetal bovine serum (FBS) and 2 mM glutamine. Cells were maintained in a humidified atmosphere containing 5% CO₂ at 37°C. All cell lines were routinely tested for mycoplasma and for surface expression of target antigens as well as authenticated by STR analysis (Eurofins Genomics, Ebersberg – Germany).

Isolation, generation and transduction of CAR T cells. Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats obtained from healthy donors (University Hospital Würzburg, Würzburg, Germany) who signed a written informed consent, in accordance with rules set by the Institutional Review Board (Approval number 250/20-am), using Lymphocyte separation medium Ficoll. T lymphocytes were activated with immobilized OKT3 (1 mg/ml,) and anti-CD28 (1 mg/ml) monoclonal antibody (mAb) in complete medium consisting of CTS OpTmizer T Cell Expansion medium supplemented with 2.5% human AB serum, 2 mM glutamine and with or without 100 µM/ml of LA, in a humidified atmosphere containing 5% CO₂ at 37°C. The day after activation, T cells were fed with recombinant human interleukin-7 (IL7, 500 U/ml) and -15 (IL15, 50 U/ml). Activated T cells were transduced on day 3 in 24-well plates pre-coated with recombinant human RetroNectin using a specific retroviral supernatant and the above-described cytokines. On day 5 after transduction, T cells were removed from RetroNectin and expanded in complete media.

Retroviral constructs. Three CAR constructs have been used in this study: a third generation CD30.CAR and GD2.CAR encoding for CD28.4-1bb.zeta-chain (ζ) and a second generation CD19.CAR encoding for 4-1bb.ζ. An additional retroviral vector encoding eGFP-

Firefly-Luciferase (eGFP-FFLuc) was used in selected experiments to label tumor cells for in vitro co-culture and in vivo studies as previously described.

Co-culture assay. For co-culture experiments, NT, CD30.CAR, GD2.CAR or CAR.CD19 T lymphocytes cultured in presence or absence of LA were plated in 24-well plates at the indicated E:T ratios with GFP modified tumor cell lines (Karpas 299, CD30+ tumor cell line; SHSY5Y, GD2+ tumor cell line; Raji, CD19+ tumor cell line). Following 5 days of incubation at 37°C, co-cultures were collected and analysed by FACS to detect residual tumor cells (GFP+) and T cells based on CD3 expression.

Live cell imaging staining. NT and CAR-T cells were collected and washed twice with phosphate buffered saline (PBS) supplemented with 1% of bovine serum albumin (BSA). T cells were then staining with CD4+5 BV421. CAR-T cell population was visualized by staining with 1A7 anti-idiotypic CAR mAb (1 mg/ml), followed by APC-conjugated goat anti-mouse secondary antibody (1:500 in 1% BSA/PBS). In specific setting GFP modified tumors were used to redirect CAR T cells.

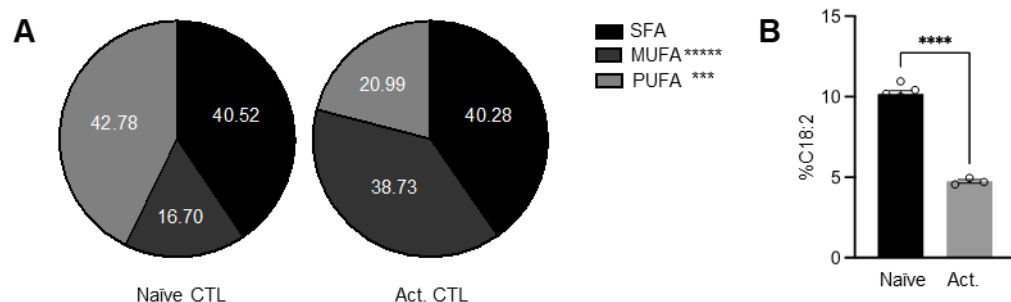
Quantification and statistical analysis

Flow cytometry data was analysed using FlowJo v10.2. Western blots were quantified with ImageJ software. Statistical Analysis was performed using GraphPad Prism. Results are represented as mean $\pm$ standard error (SEM). Unless otherwise specified, paired t-Student test was used to compare LA-CTL and CTRs. Normal distribution was evaluated using the Shapiro-Wilk test. Metabolomics data were normalized to cell count and/or protein content. Comparisons of more than two groups were calculated using one-way ANOVA with Tukey multiple comparison correction. All graphs show mean $\pm$ SEM were **** p-value < 0.0001, *** p-value < 0.001; ** p-value < 0.01, * p-value < 0.05.

RESULTS

1. LA levels are modulated during T cell activation.

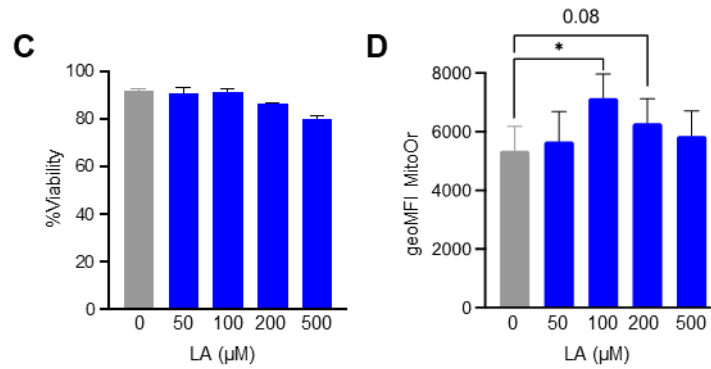
Long Chain Fatty Acids (LCFAs) are essential regulators of T cell activation, proliferation, and function^{149,193,194}. We investigated if different types of lipids regulate CTL responses by quantifying the composition and abundance of LCFAs during T cell activation. When compared to naïve, activated CTL contained similar levels of SFA (saturated FAs) but were different in terms of unsaturated FAs; in particular, MUFA (mono-unsaturated FAs) were increased, while PUFA (poly-unsaturated FAs) were significantly decreased (Figure 1A). Being LA an essential PUFA associated with a plethora of beneficial effects for human health¹⁷⁵, we assessed its abundance upon T cell activation. LA levels were significantly lower in activated CTL (Figure 1B), suggesting that it might be used or consumed during T cell activation. Thus, we decided to study the role of LA in tuning CTL fate and activity.



Lipid composition is significantly impacted by T cell activation.

Lipid content was assessed by LS-MS on CTL before and after 48h activation. **A.** quantification of SFA, MUFA, and PUFA. **B.** Linoleic acid (C18:2) was quantified in naïve (n=5) and activated (n=3) CTL.

We then proceeded to evaluate the effects of LA on OT1 T cells activated in the presence or absence of LA. We observed that, after 48 hours in culture, there was no significant modulation of cell viability across all tested concentrations (Figure 1C). Similarly, titration experiments showed that LA was able to maximally regulate T cell mitochondrial potential at the concentration of 100μM (Figure 1D). We thus decided to use LA at 100 μM which is within the range of the concentrations present in human plasma^{152,195,196}



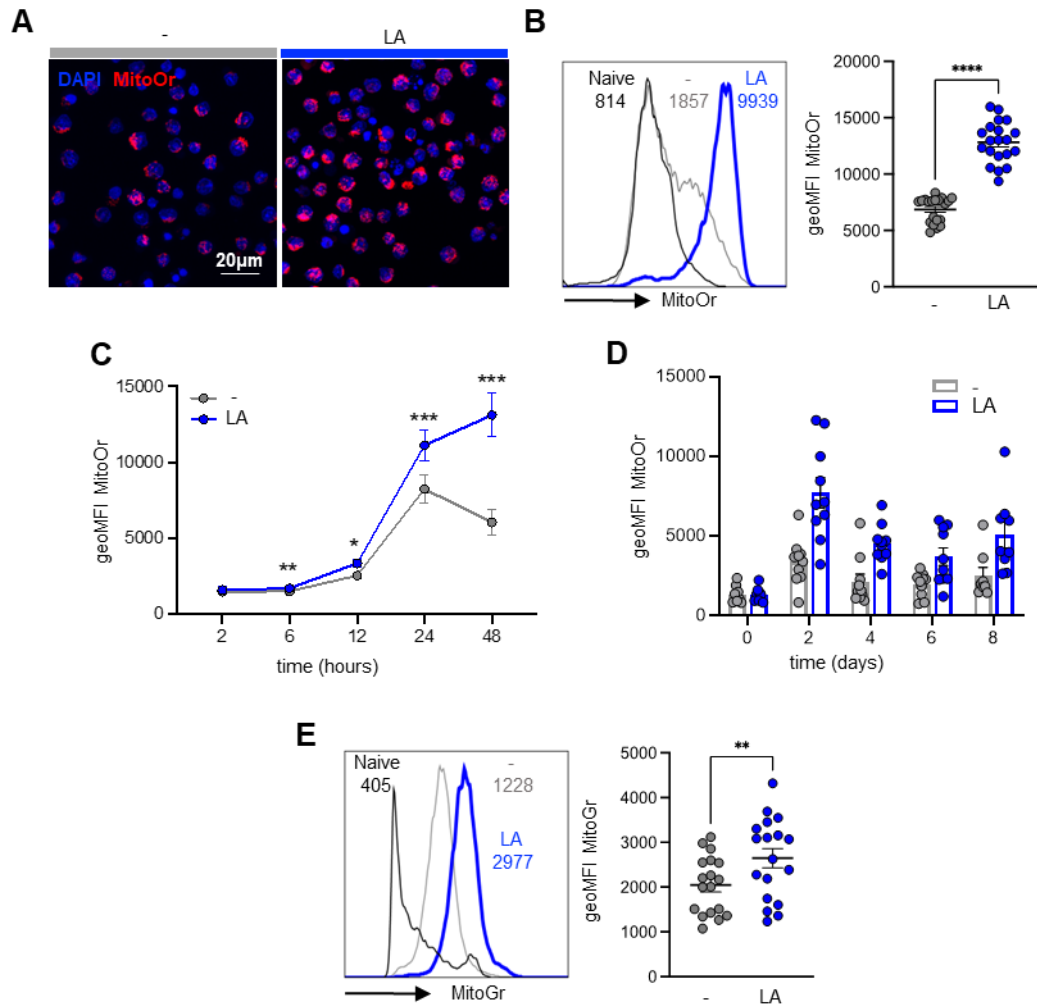
LA increases CTL mitochondria membrane's potential without influencing viability

C. Percentage of viable OT1 cells 48 hours post-activation in the presence of LA at the indicated concentrations.

D. Assessment of mitochondria's membrane potential through MitoOr. staining (n=4)

2. LA enhances mitochondrial content and activity in CTL.

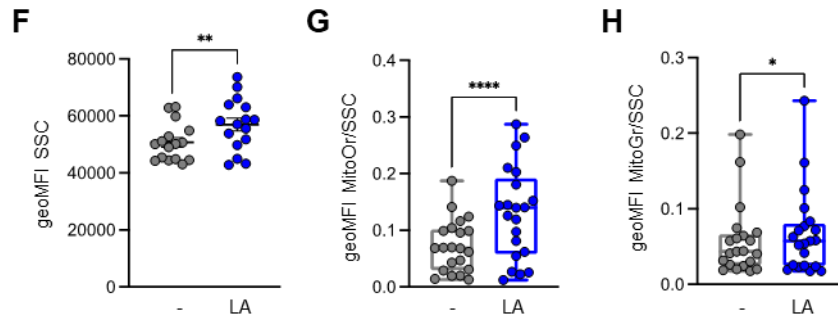
To decode whether LA has a role in regulating CTL metabolism, we measured mitochondrial content and function by staining with MitoTracker Green (MitoGr.) and MitoTracker Orange (MitoOr) in CTL activated in the presence of LA and compared them with controls (LA- vs CTR-CTL). Analyses of CTL stained with MitoOr. confirmed our preliminary results with a significant increase in mitochondrial potential on LA-treated cells measured as both immunofluorescence (Figure 2A) and flow cytometry (Figure 2B) assays. The LA-induced mitochondrial burst is rapid -reaching statistical significance 6 hours after stimulation- (Figure 2C) and sustained -even after 8 days in culture- (Figure 2D). The higher mitochondrial activity is accompanied by an increased mitochondrial content in LA-treated CTL, which held more mitochondrial mass as detected by increased MitoGr. staining (Figure 2E).



LA induces rapid and sustained mitochondrial burst in activated CTL

OT-I cells were activated with OVA peptide and IL-2 for 2 days and their metabolic landscape was subsequently analyzed. **A**. Representative immunofluorescence images of MitoOr. On CTR-CTL (left panel) or LA-CTL (right panel). MitoOr. (red) was detected by immunofluorescence and nuclei (blue) were stained with DAPI. Images are representative of 3 experiments with three mice in each group. **B**. Representative histogram and relative quantification of MitoOr. Staining by flow-cytometry on LA-CTL (blue), CTR-CTL (grey), and naïve (black). Results show MitoOr MFI pooled from 5 independent experiments, n=20. MFI. **C-D**. Kinetics of MitoOr. On CTR and LA-CTL over a 48 hours *ex vivo* culture (**C**, pooled from 2 independent experiments, n=6) or after long-term culture (**D**, pooled from 3 independent experiments, n=10). **E**. Mitochondrial mass (MitoGr.) was also assessed and quantified by flow cytometry, we show a representative histogram, and relative MFI quantification (pooled from 5 independent experiments, n=25).

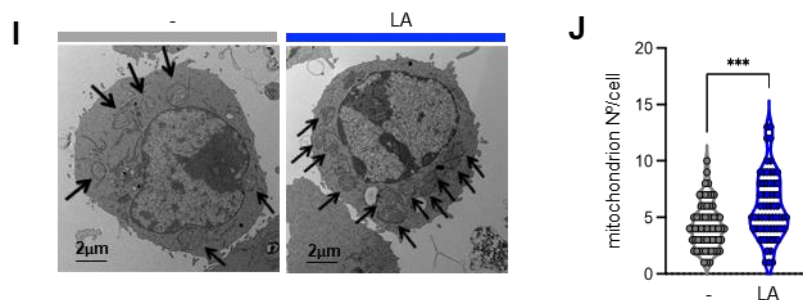
Although LA-treated cells exhibited a significantly higher SSC when compared to control cells (Figure 2E), the effects observed on mitochondria in LA-treated cells were not due to changes in cell size (Figure 2F-G).



LA increases CTL size.

F. Morphology of OT1 cells activated in the presence or absence of LA was assessed by flow cytometry and quantified as the MFI of Side Scatter (SSC, pooled from 4 independent experiments, n=16). In order to rule out a size bias on LA-CTL, mitochondrial activity (**G**) and mass (**H**) were normalized to CTLs SSC.

Since metabolic functions are strongly dependent on structural organelle integrity, we next performed ultrastructural analysis of LA-treated CTL. Quantification of the mitochondria number per cell (Figure 2I-J) further confirmed that the administration of LA increased CTL's mitochondrial content.

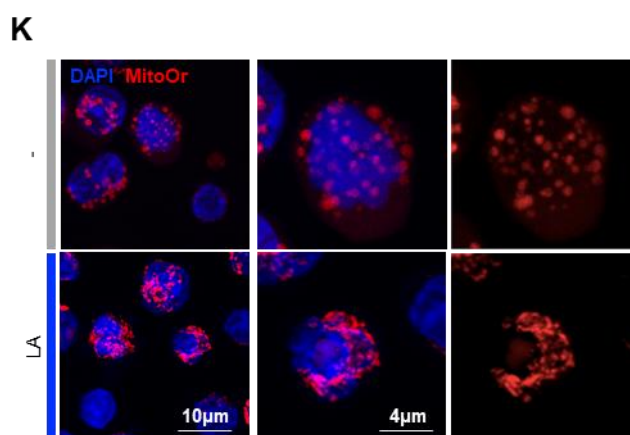


LA treatment increases mitochondrial number and distribution in CTL

I. Representative electron microscopy (EM) image from CTR-CTL (left) and LA-CTL (right, n= 3 per group, scale bar 2 μ m). **J.** Graph showing the quantification of the mitochondrion number per cell as determined by TEM analysis. Data are representative of one experiment with 3 mice/each group with ≥ 60 cells counted in each experimental condition.

Qualitatively, LA-treated cells prevalently exhibited rod-shaped and highly condensed mitochondria, placed predominantly at one edge of the cell (Figure 2I, right panel). Mitochondria from this pool were distinct from the rounded and rather discrete mitochondrial dispersed evenly across the cytoplasm of untreated controls (Figure 2I, left panel). These qualitative observations fit well with the elongated tubular shape of mitochondria seen by confocal microscopy upon LA treatment (Figure 2K, lower panel), which have previously been described in cells heavily relying on oxidative phosphorylation (OXPHOS)¹⁹⁷. Overall,

these data indicate that LA treatment upholds mitochondria function, quantity, and quality in CTL, suggesting that they may have improved metabolic performance.

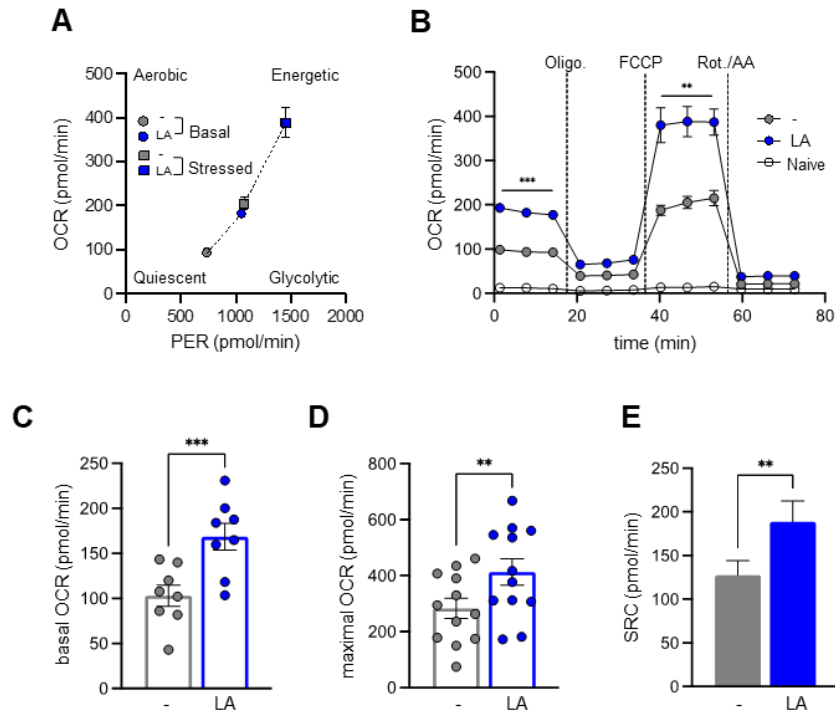


Mitochondria from LA-CTL have an elongated morphology

K. Confocal microscopy assessing mitochondria distribution and shape using MitoOr. Staining. High-resolution images were obtained using the 40X (left panel) and 100X (right panels) objectives.

3. LA reinforces CTL mitochondrial bioenergetics and metabolic fitness.

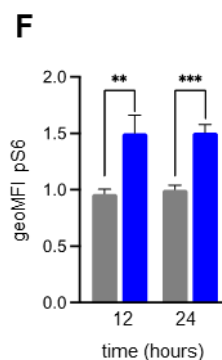
Given the above findings, we employed extracellular flux analysis on SeaHorse to characterize the metabolic state of LA-treated cells both at basal levels and in response to stress. The energetic map revealed LA-treated CTL as more energetic under both resting and stressed conditions (Figure 3A). Indicative of their augmented OXPHOS and in line with the more active and elongated phenotype of the mitochondria, LA-CTLs displayed a higher O₂ Consumption Rate (OCR) in the resting state in comparison to CTR-CTL (Figure 3B-C). Most importantly, upon disruption of mitochondria complexes with FCCP, LA-treated cells showed a significant increase in maximal respiration levels (Figure 3B and D) and Spare Respiratory Capacity (SRC, figure 3E), suggesting that LA-treated cells may respond better to increased energetic demands and adapt to stress conditions.



LA treatment induces CTL metabolic reprogramming with enhanced OXPHOS levels

SeaHorse platform was used to analyze the metabolic landscape of OT1 cells activated in the presence or absence of LA in real-time. The representative energetic map (**A**) was constructed using OCR (Oxygen Consumption Rate) and PER (Proton Efflux Rate) values registered during basal (before any perturbation) and stressed (after FCCP injection) conditions. OCR was measured in real-time by metabolic flux analysis (**B**) and bars show the calculated basal (**C**) and maximal respiration (**D**) levels, as well as spare respiratory capacity (SRC, **E**). Results are pooled from 4 independent experiments with 3-4 mice per group.

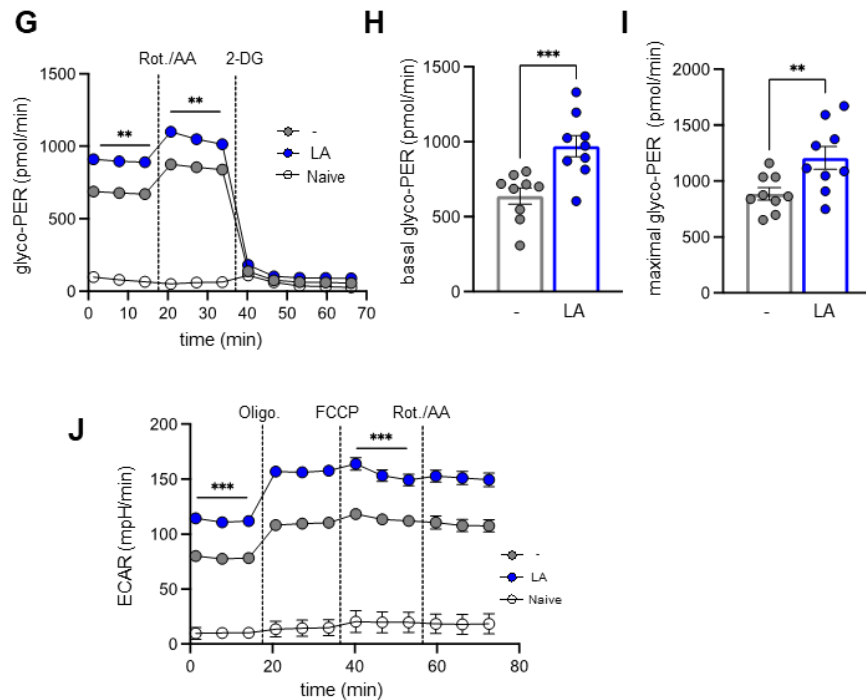
Since the mammalian target of rapamycin complex 1 (mTORC1) is a known regulator of metabolic activity in T cells ¹⁹⁸, we next evaluated the phosphorylation of S6 ribosomal protein (pS6), as a downstream target of the mTOR pathway. Our results are best explained by LA-treated CTL upregulating pS6 levels (Figure 3F) with kinetics that matches that of the mitochondrial activity (Figure 2C).



LA-CTL show increased pS6 level

F. Phosphorylated S6 ribosomal protein (pS6), a downstream target of mTOR, was assessed by FACS and bars show the normalized Mean Fluorescent Intensity (MFI) at 12- and 24-hours post-activation.

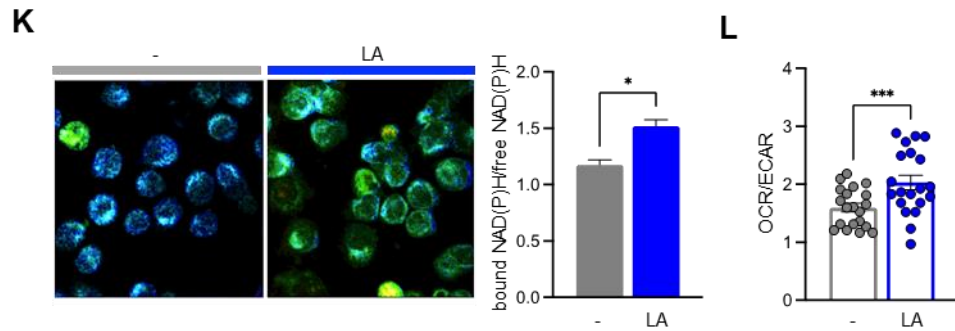
Because mTORC1 in CD8⁺ T cells sustains glycolysis ¹⁹⁸, which is essential for the execution of effector functions under stress, we evaluated the glycolytic activity by analyzing the proton efflux rate (PER) and the extracellular acidification rate (ECAR). As predicted, both parameters were higher in LA-CTL compared to CTR-CTL under basal and stressed conditions (Figure 3G and 3H-I), indicating that LA-treated CTL were metabolically more active than CTRs.



LA-CTL show increased glycolytic activity

G. Glycolytic Rate Assay was performed by the sequential injection of Rotenone + Antimycin A (0,5 μ M) and 2-deoxy-D-glucose (2-DG, 50nM) to calculate the glycolysis-dependent Proton Efflux Rate (PER) in both basal (**H**) and stressed (**I**) conditions (pooled from 3 independent experiments, n=12). Glycolysis levels results were further confirmed by Extracellular Acidification Rate (ECAR, **J**, pooled from 4 independent experiments with 3-4 mice per group).

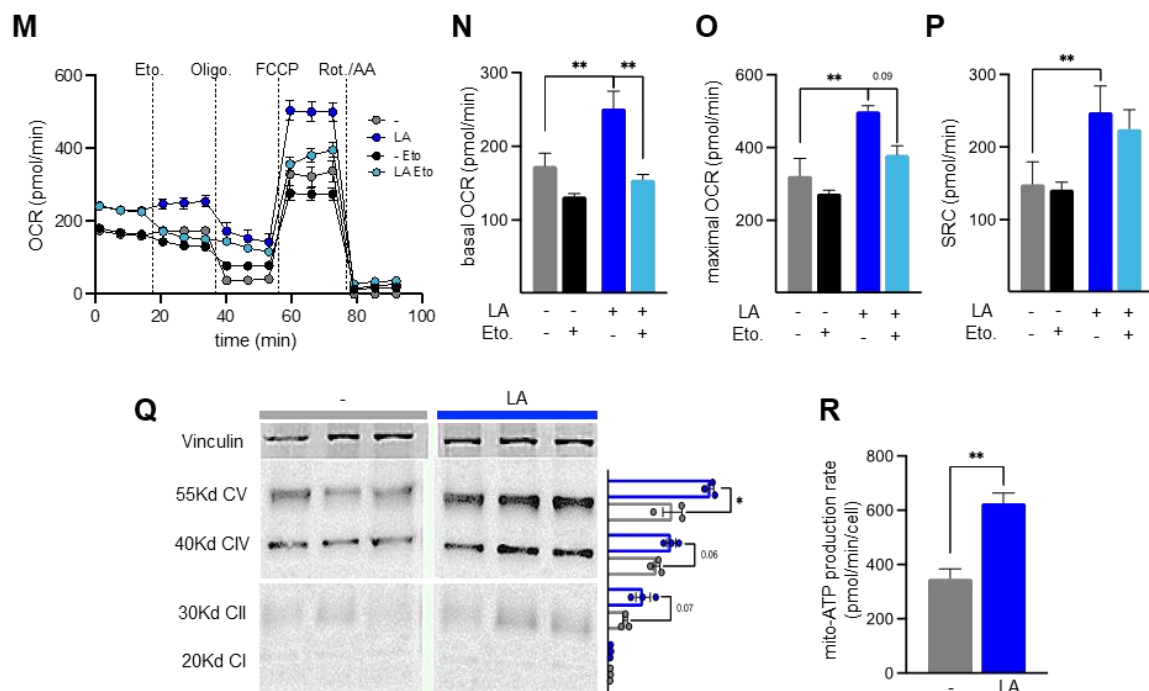
To examine the relative use of OXPHOS and glycolysis in CTL undergoing LA treatment, we employed fluorescence lifetime imaging microscopy (FLIM). LA-CTL displayed a pronounced increase in the protein-bound NADH/free NADH ratio (Figure 3K), implying a shift towards OXPHOS metabolism. This finding was independently supported by a higher OCR/ECAR ratio (Figure 3L), which indicates that LA-CTL preferentially use OXPHOS metabolic pathway, in line with their increased mitochondrial activity and SRC.



LA treated CTL preferentially use OXPHOS as energy source

K. Fluorescence lifetime imaging microscopy (FLIM) was performed in order to assess LA-CTL preferred metabolic pathway, and results are reported as the ratio between protein-bound NADH and free NADH (n=3 mice per group). These results were further validated by calculating the OCR/ECAR ratio (**L**, pooled from 4 independent experiments with 3-4 mice per group).

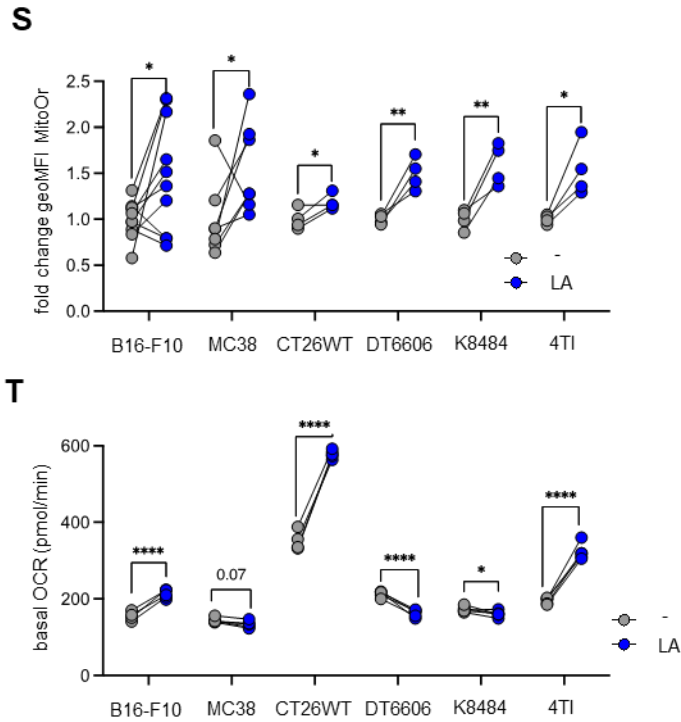
Finally, we investigated whether the improved ability of LA-CTL to respond to stress conditions was dependent on fatty acid oxidation (FAO). When LA is combined with etomoxir (Eto.), a small molecule that inhibits FAO, benefits induced by LA on basal (Figure 3M-N) and on maximal OCR (Figure 3M and O) as well as in SRC (Figure 3P) are overruled, suggesting FAO dependency in LA-CTL. In accordance with enhanced mitochondrial respiration, LA-CTL displayed increased levels of all components of the electron transport chain (ETC, Figure 3Q). Complex V (ATP synthase) was found the most upregulated, and this is of particular importance for CTL because it utilizes the mitochondrial membrane potential to generate ATP, fuelling in this manner cell proliferation, survival, and effector functions¹⁹⁹. Accordingly, ATP levels in LA-CTL exceeded those measured in CTR-CTL (Figure 3R).



The LA-induced metabolic phenotype is dependent on FAO

The effect of Fatty Acid Oxidation (FAO) in OXPHOS metabolism was assessed using a MitoStress Test preceded by an acute injection of Etomoxir (Eto, n=4) and we report **(M)** OCR kinetics, quantification of **(N)** basal and **(O)** maximal OCR, as well as **(P)** SRC. Additionally, western blot for mitochondrial complexes was performed in LA- versus CTR-CTLs. **(Q)** and results were independently validated by the ATP production rate, measured using the glycolytic rate assay in the Seahorse platform **(R)** that allows the discrimination of glycolytic and mitochondrial ATP production (pooled from 3 independent experiments, n=12).

Notably, when tested on different tumor cell lines, LA was able to raise their mitochondrial potential (Figure 3S) but with variable effects on their bioenergetic profile (Figure 3T), postulating the existence of cell type-specific LA-dependent wiring.



LA has differential effects in the mitochondrial metabolism of diverse tumor cell lines

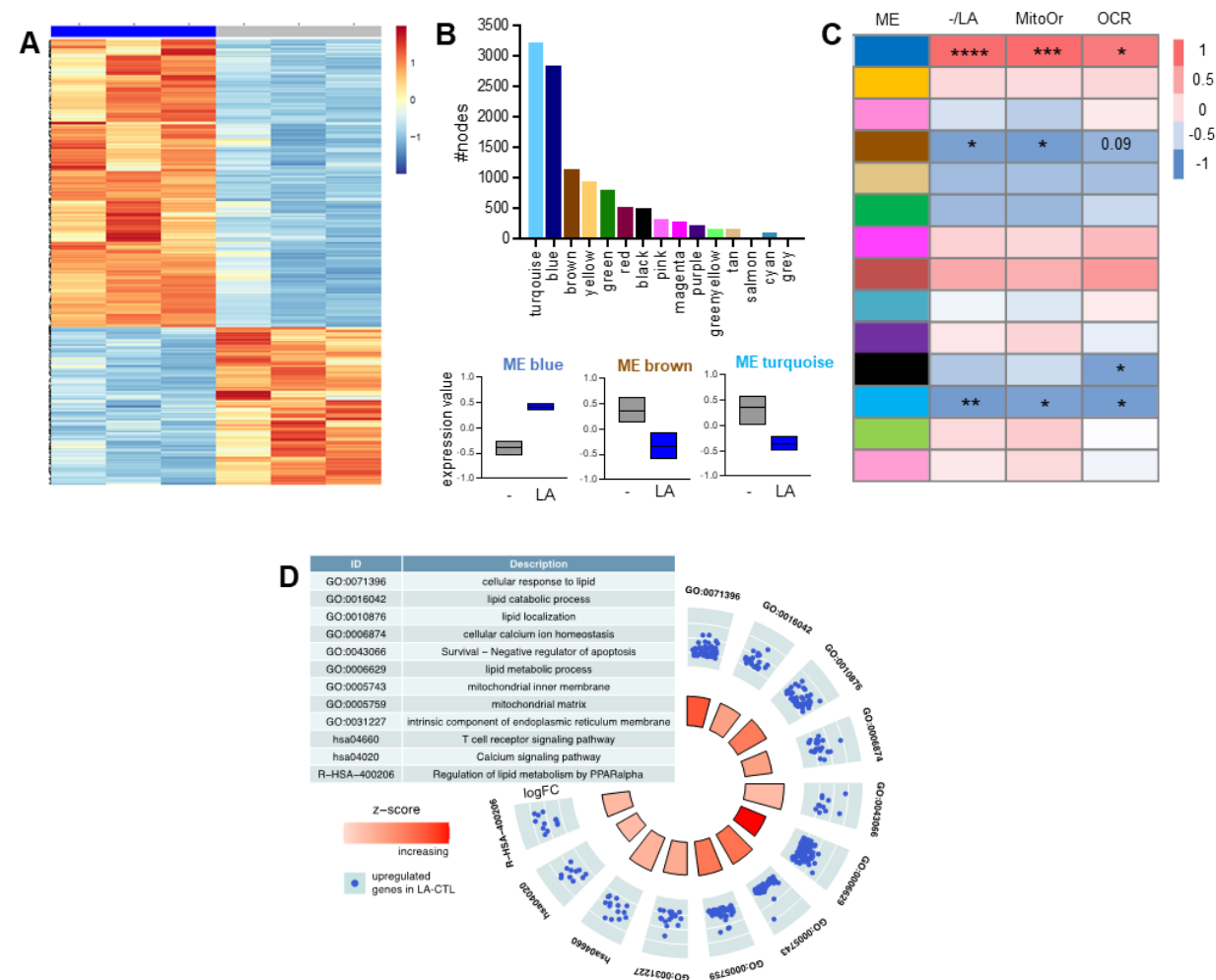
LA effect on tumor cell lines was assessed by flow cytometry with MitoOr. staining (S) and using the SeaHorse platform to assess basal OCR levels (T).

Combined, these data suggest that LA endowed CTL with enhanced metabolic fitness by augmenting the quantity and quality of mitochondria and their bioenergetic profile. We consider it likely that such LA-induced mitochondrial advantage provides CTL with a selective advantage to adapt to increased energetic demands.

4. LA treatment strengthens the ER-mitochondria network.

To provide more mechanistic insights on the effect of LA treatment on CD8⁺ T cells, we performed a transcriptional analysis of LA-CTL in comparison with CTR-CTL, which highlighted 1970 differentially expressed genes (DEGs, Figure 4A). Thus, in order to identify groups of potentially co-regulated genes (modules, ME), we integrated information from the whole transcriptomic dataset with the most significant hallmarks induced by LA treatment using Weighted Gene Co-expression Network Analysis (WGCNA, see Methods for details)²⁰⁰. Focusing on the three ME with the highest correlation score (Figure 4B), we found genes included in ME-1 (labeled in blue) are positively correlated with features dependent on LA treatment, while those in ME-4 and ME-12 (labeled in brown and turquoise, respectively)

show a negative correlation (Figure 4B, bar charts). In particular, genes in ME-1 had a strong positive correlation with an increase in MitoOr., and basal OCR. Functional enrichment analysis confirmed the central involvement of mitochondria (i.e. intracellular membrane-bounded organelles, mitochondria membranes, respiration, Figures 4C), in agreement with the pivotal role of LA in boosting CTL's mitochondrial activity.

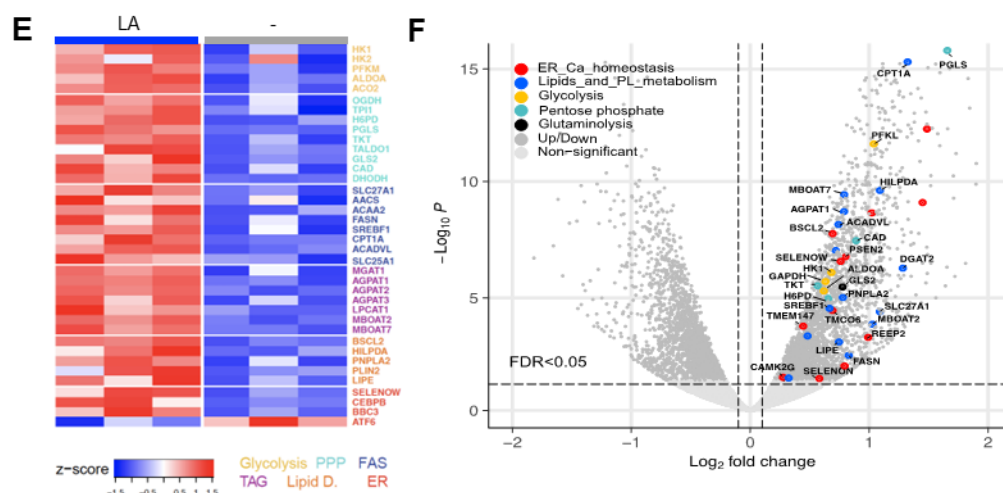


Transcriptomic profile of LA-CTL reveals the interplay between mitochondria and ER

A. Unsupervised clustering analysis of the transcriptome of CTL $\pm$ LA ($n = 3$). Top-most variable genes were used to plot the heatmap. **B.** Modules identified from Weighted Gene Co-expression Network Analysis (WGCNA). For each column (module), the number of DEG is listed (top panel) The three modules exhibiting the higher statistically significant correlation ($p\text{-value} \leq 0.05$) with the condition LA-/LA+ are highlighted (bottom panel). **C.** Heatmap from WGCNA. Each row corresponds to a module gene, and each column to a trait of interest. Each cell contains the corresponding correlation and p-value.. Expression values (log scale) of the module eigengenes (right panel) of the three selected modules in LA- (grey) and LA+ (blue). **D.** Top biological processes upregulated within total Differentially Expressed Genes (DEG).

On the other hand, LA regulated several ER-related transcripts ^{201–206}linked to its composition and structure (i.e. *Tmem147*, *Selenow*, *Cebpb*, *Bbc3*, *Atf6*; Figure 4E), as well

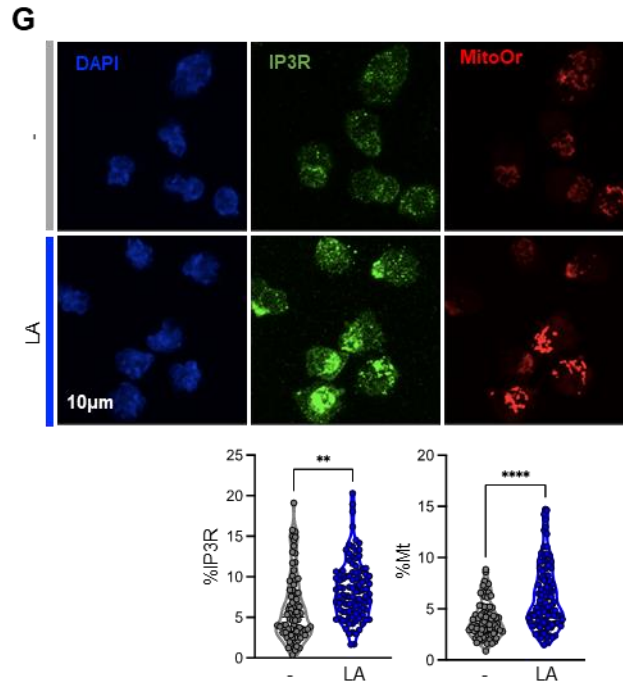
as genes of calcium influx regulation (such as *Camk2g*, *Tmco6*, *Psen2*, *Selenon*, Figure 4E-F), as instrumental to contain ER stress^{207–210}.



LA upregulates genes involved in glycolysis, lipid synthesis, glutaminolysis and ER homeostasis.

E. Heatmaps representing genes related to glycolysis, PPP, FAS, TAG, lipid droplets and ER from total DEG.
F Volcano plot highlighting most representative genes for ER-Ca²⁺ homeostasis, lipids and PL metabolism, glycolysis, PPP and glutaminolysis pathways.

These data suggested a tight co-regulation of mitochondrial fitness and ER homeostasis, in agreement with recent studies revealing that ER stress response controls T cell functionality by regulating mitochondrial activity^{211,212}. Therefore, the paired involvement of the ER and mitochondria, in response to LA, was independently validated by quantifying the ER-resident Inositol 1,4,5-trisphosphate receptor type 1 (α -IP3R1), while simultaneously monitoring mitochondria using MitoOr staining. The marked increase of both markers in LA-CTL (Figure 4G) supported that LA regulated the mitochondria-ER network in CTL.

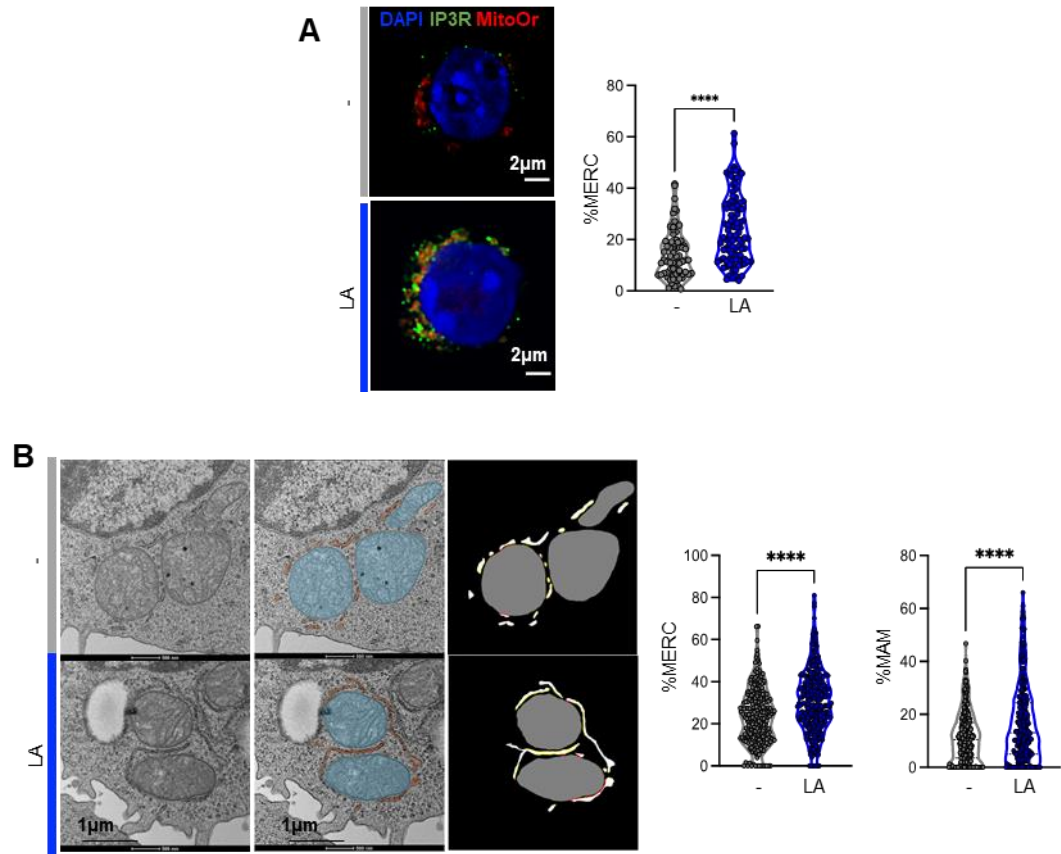


LA treatment promotes ER extension

G. Representative confocal microscopy images of multicolor immunofluorescence staining displaying aIP3R (green) and MitoOr. (red) staining in CTR- (upper panels) versus LA (bottom panels) CTLs. Nuclei (blue) were counter-stained with DAPI. Plots show the quantification of aIP3R and MitoOr. area percentage. Each dot represents one cell, and the expression level of ≥ 50 cells per condition were quantified on three independent samples.

5. LA promotes MERC formation and downstream processes in a Ca^{2+} -dependent manner.

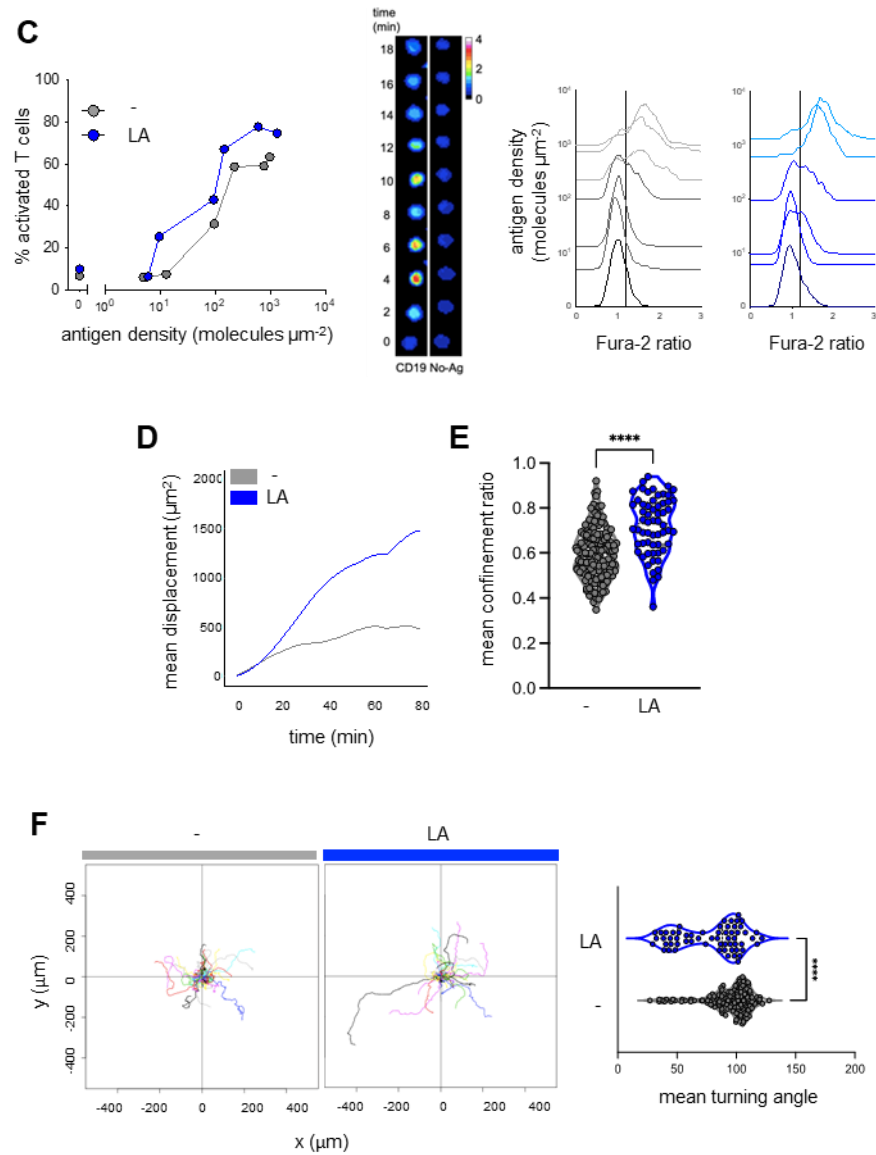
Since mitochondria-ER contacts (MERC) have been shown to modulate mitochondrial respiration in CTL ²¹³, we investigated whether LA affects MERC formation in CTL. Quantitative confocal microscopy (Figure 5A) and EM analysis (Figure 5B) confirmed an increased frequency of MERC (between 30 and 100 nm) and mitochondrial-associated-membranes (MAMs, below 30 nm) in LA-CTL; while fewer mitochondria were in close proximity to ER in CTR-CTL.



LA-CTL show increased MERC and MAM formation

A. High-resolution confocal imaging was used to calculate ER-Mitochondria contact points by quantifying the colocalization area of MitoOr. and IP3R. **B.** TEM representative images of CTR- (upper panels) versus LA (bottom panels) CTLs and relative quantification of numbers of MERC (between 30 and 100 nm) and MAMs (below 30 nm). Each dot represents one cell, and the expression level of ≥ 50 cells per condition were quantified on three independent samples.

MERC are dynamic structures controlling the transport of Ca^{2+} from the ER to mitochondria²¹⁴ that is crucial for T cell activation, migration, and TCR signaling²¹⁵. Accordingly, Fura-2-based time-lapsed calcium imaging demonstrated that LA-CTL responded more vigorously and with increased sensitivity in antigen-limiting conditions, compared to CTR-CTL (Figure 5C). Likewise, LA-CTL migrated faster and further when compared to CTR-CTL as assessed by increased confined migration (Figure 5D-E) and decreased turning angle (Figure 5F).

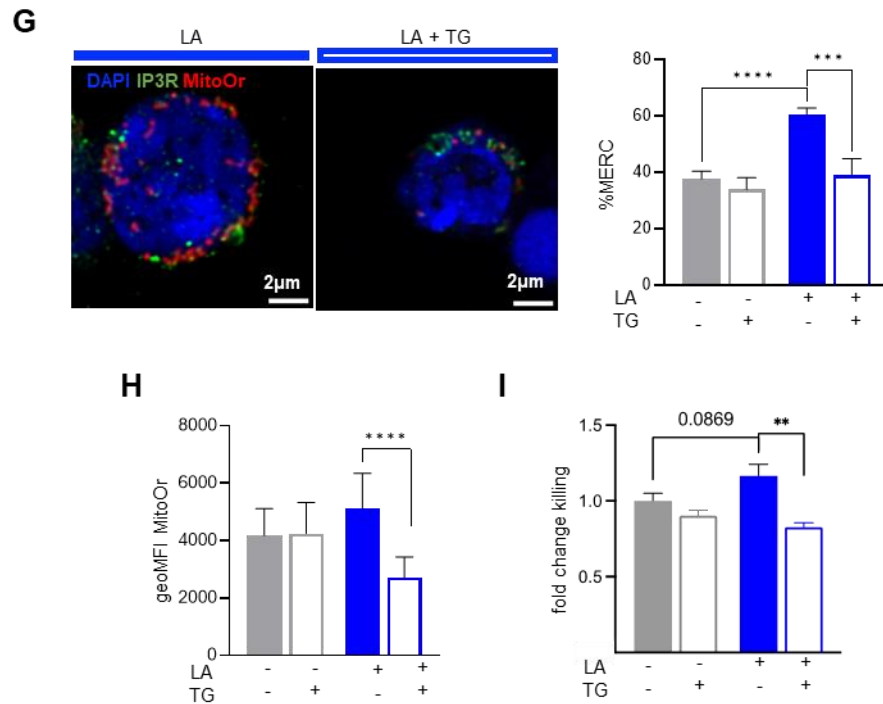


LA treatment enhances downstream processes of MAM formation

C. Assessment of Ca^{2+} response and antigen sensitivity performed by using Fura-2 time-lapse microscopy.

D. Chemokine-mediated T cell migration assay by live cell tracking and analysis with the CelltrckR software allowed for quantification of the mean displacement (**D**), mean confinement ratio (**E**), and mean turning angle (**F**) of CTLs $\pm$ LA.

In accordance, by disrupting Ca^{2+} homeostasis using thapsigargin (TG), an irreversible inhibitor of the sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA) (Iurlaro and Muñoz-Pinedo, 2016), LA-CTLs showed a significant reduction in MERC formation (Figure 5G), mitochondrial activity (Figure 5H) and cytotoxic capacity (Figure 5I). Of note, TG did not influence the cytotoxic activity of CTR-CTL (Figure 5I), which reflects the complexity of Ca^{2+} signaling in different T cell subsets.

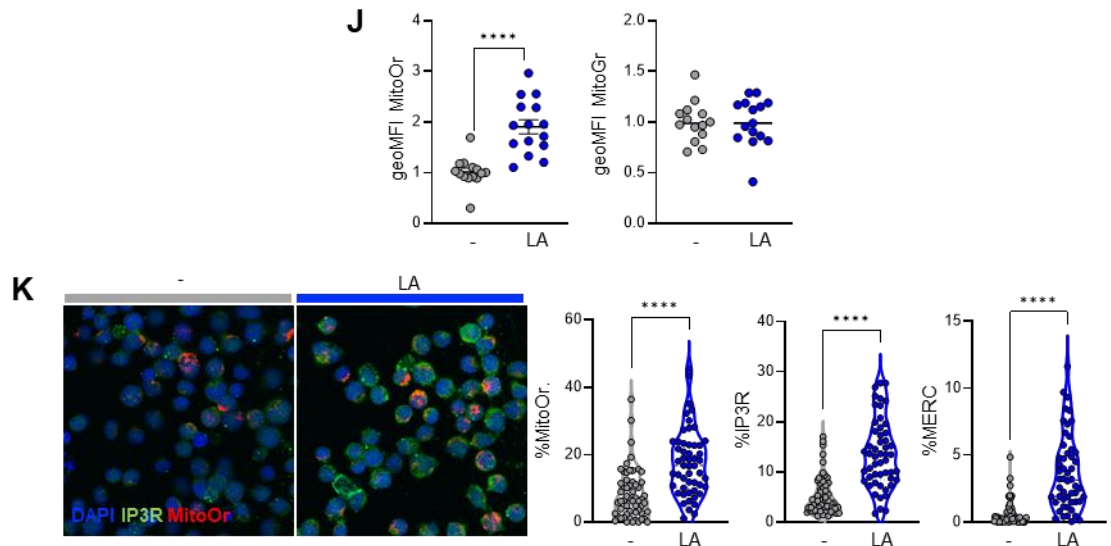


The LA-induced phenotype is dependent on calcium signaling.

Thapsigargin (TG) was used to assess the involvement of Ca^{2+} influx on the LA-induced phenotype. We measured MERC formation (**G**, $n=3$), mitochondrial activity (**H**, $n=13$), and *in vitro* killing capacity (**I**, $n=5/6$) in CTL $\pm$ LA upon TG treatment for 12 hours.

Overall, these results suggest that LA enhanced MERC formation and the downstream CTL processes associated with calcium signaling.

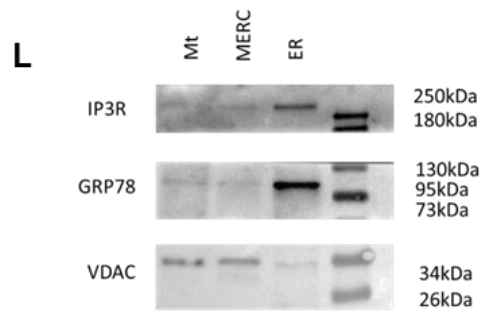
We next wondered whether LA integrates into the membranes of mitochondria, ER, and MERC by assessing the lipid composition of their enriched subcellular fractions. In order to successfully isolate the above mentioned cellular fractions, we adapted the protocol previously reported by Wieckowski and collaborators (2009)²¹⁷. However, the significant amount of cells required made this protocol particularly challenging; especially considering our experimental set-up is based on primary murine T cells. For this reason, we decided to validate our results using Jurkat cells, an immortalized line of T Acute Lymphoblastic Leukemia (T-ALL) that allows for the required cell expansion necessary to perform this protocol. Results show that the LA-induced phenotype was partially recapitulated in Jurkat cells with enhanced mitochondrial potential (Figure 5J) and area per cell, as well as increased ER area and MERC formation (Figure 5K).



The LA-induced phenotype is partially recapitulated by Jurkat cells.

Jurkat cells were kept in culture in the presence or absence of LA for 48 hours, after which mitochondrial potential and mass were assessed by FACS (**J**). Each point represents a technical replicate and the bar shows mean $\pm$ standard deviation, data is represented as normalized with non-treated controls (n=14m pooled from 3 independent experiments). MERC formation was assessed and quantified by confocal microscopy (**K**, n=3 technical replicates).

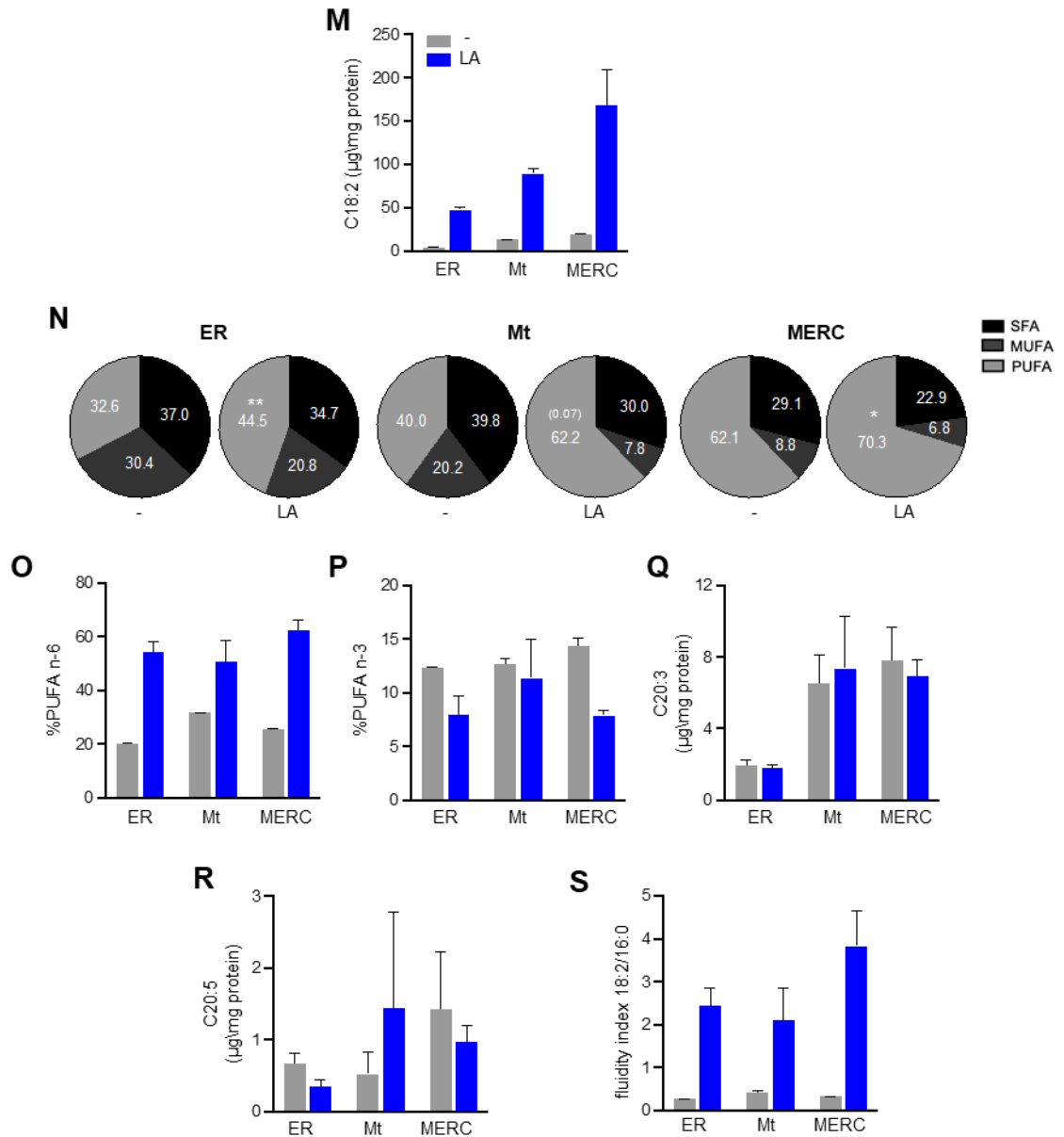
Therefore, centrifugation-based cellular fractionation was performed starting from 1×10^8 Jurkat cells. Quality control and fraction purity were assessed by western blot using IP3R as a surrogate marker for MERC, GRP78 to assess ER membranes, and VDAC as mitochondrial membrane resident protein (Figure 5L). Of note, while purified ER fractions contained negligible amounts of IP3R, MERC fractions were almost exclusively composed by IP3R and mitochondria with little traces of GRP78. These results confirm that the fractionation process successfully isolated cellular compartments enriched in mitochondria, MERC and ER.



Centrifugation-based cellular fractionation successfully produces mitochondria-, MERC-, and ER-enriched fractions

Cellular fractionation protocol was performed using 1×10^8 Jurkat cells. Quality control and fraction purity were assessed by western blot using IP3R as a surrogate marker for MERC, GRP78 to assess ER membranes, and VDAC as mitochondrial membrane resident protein

Once the fractionation protocol was successfully tested, we proceeded with the assessment of the lipid composition of each individual fraction using primary T cells. Results indicate that LA indeed integrates into the organelle's subcellular fractions (Figure 4A) and modified their lipid asset, inducing a drop of SFA and MUFA and a prominent rise in PUFA abundance (Figure 4B), with the specific increase of PUFA_n6 (Figure 4C) at the expenses of PUFA_n3 (Figure 4D). Of note, the increase in PUFA_n6 is entirely attributable to C18:2, since other PUFA_n6 metabolites - such as C20:3 and C20:5 - did not change (Figure 4E-F).



LA integrates and increases the fluidity of ER, mitochondria and MERC membranes.

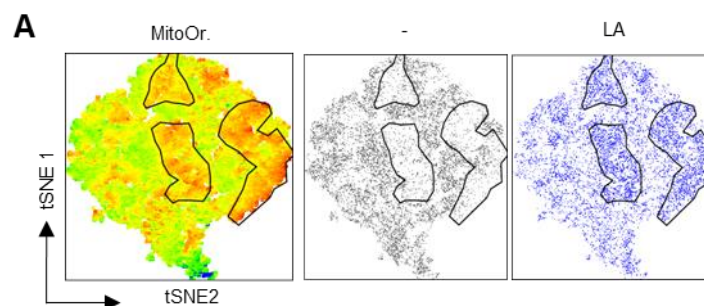
Lipidomics analysis on ER, mitochondria, and MERC fractions purified from CTL (n=2). Quantification of C18:2 (M), SFA, MUFA e PUFA (N), PUFAn=6 (O), PUFAn=3 (P), C20:3 (Q), C20:5 (R) and fluidity index (S).

The increase of PUFA and consequently increased unsaturation levels in the membranes has been reported to positively regulate ER, mitochondria, and MAM functionality^{218–220}. Accordingly, we showed increased MERC formation (Figure 5A-B), OXPHOS (Figure 3A-D), and Ca^{2+} signaling (Figure 5C). Moreover, LA-CTL membranes displayed a high 18:2/16:0 ratio (Figure 5P), which indicates an improved membrane fluidity. Thus we concluded that LA mediates a profound lipid remodeling of mitochondria, ER, and MERC membranes, thereby changing their chemical-physical properties and improving their functionality.

6. LA-induced metabolic reprogramming redirects CTL towards a memory-like phenotype with enhanced cytotoxic activity *in vitro*.

Since enhanced metabolic fitness and MAMs formation have been described as metabolic hallmarks of memory T cells²², we asked whether LA had a role in shaping CTL activation and fate decision by modulating their mitochondrial function.

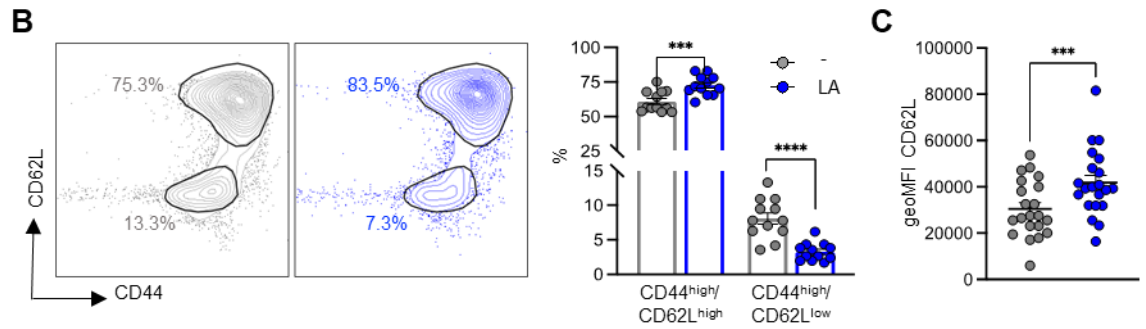
High-dimensional flow cytometry enabled a comprehensive assessment of the phenotypic diversity between LA- and CTR-CTL, including markers of memory (CD62L, CD127, TCF1, Eomes), activation (CD44, 41BB, CD69, CD25), inhibitory receptors (PD1, and TIM3), and effector molecules (GZM α , IFN γ , TNF α , IL2, and Perforin). t-distributed stochastic neighbor embedding (tSNE) analysis confirmed that CTL profiles were substantially different in the presence or absence of LA (Figure 6A). Based on the calculated mean fluorescence intensity (MFI) derived from MitoOr. staining, we identified three CTL clusters characterized by the highest mitochondrial membrane potential, which were specifically enriched in LA-CTL and underrepresented in CTR-CTL.



Enriched sub-populations in LA-CTL are defined by high mitochondrial potential.

A. t-distributed stochastic neighbor embedding (tSNE) representation of Phenograph algorithm generated from MitoOr expression of concatenated CTLs (3000cells/sample) in LA- vs. CTR-CTLs (n=5).

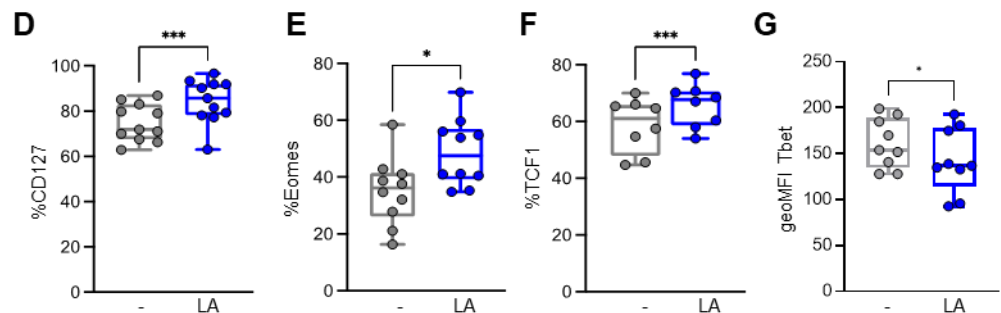
Manual gating strategy revealed that, compared to CTR-CTL, LA-treated CTL were enriched in the CD44^{high}/CD62L^{high} memory population (Figure 6B), which bears a higher expression of the canonical memory marker CD62L (Figure 6C). Conversely, CD44^{high}/CD62L^{low} effector cell frequencies were significantly decreased in LA-CTL.



LA-CTL are characterized by an increased memory population.

Memory-T cell phenotype assessment in CTR- and LA-CTLs was performed by flow cytometry. Representative dot-plot and quantification of CD44^{high}/CD62L^{high} and CD44^{high}/CD62L^{low} cells in CTR- and LA-CTLs (**B**, n=12), accompanied by CD62L MFI (**C**, n=21)

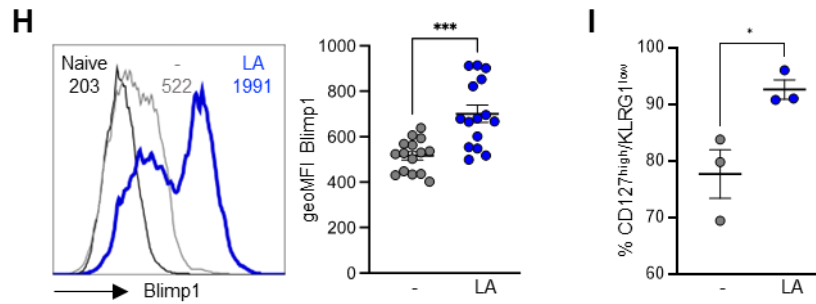
In line with a skewing towards a memory phenotype, we found that LA-CTL were enriched in cells expressing CD127 (Figure 6D) and the transcription factors Eomes (Figure 6E) and TCF1 (Figure 6F), which have been described to promote a central memory (TCM) differentiation profile²²¹, while Tbet expression was found decreased (Figure 6G) in LA-CTL when compared to CTR-CTL.



LA treatment increased the expression of canonical memory markers and transcription factors.

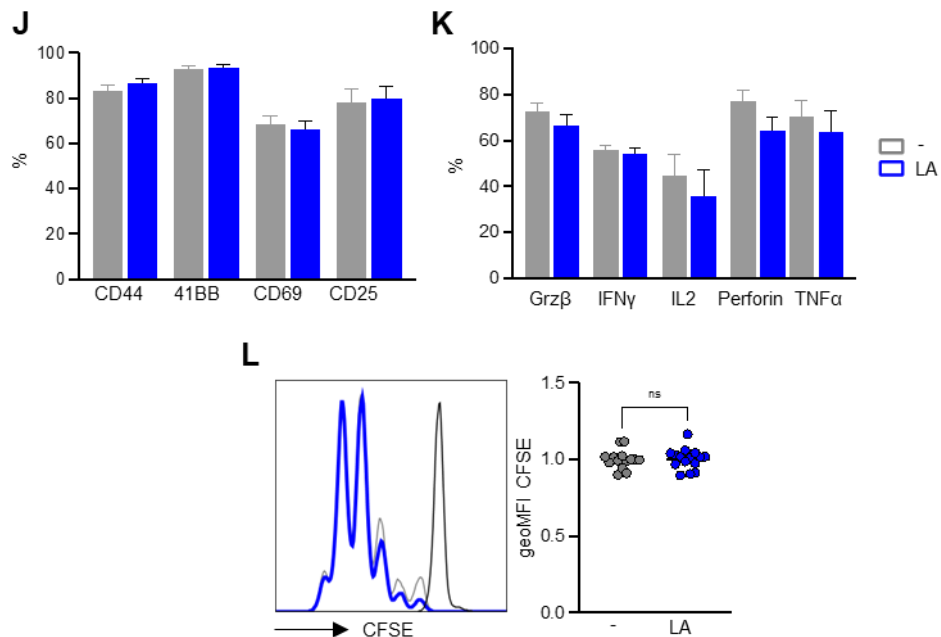
Memory phenotype was further assessed by the calculating frequency of canonical marker CD127 (**D**) and transcription factors Eomes (**E**), TCF1 (**F**), and Tbet (**G**).

Surprisingly, the expression of Blimp1, known to promote short-lived effector CTL^{54,59,60} and favor exhaustion⁵⁸, was significantly increased in LA-CTL (Figure 6H). Nevertheless, LA-CTL were predominantly defined by a CD127^{high} and KLRG1^{low} expression (Figure 6I), typical of long-lived memory phenotype associated with an enhanced recall response⁵⁷.



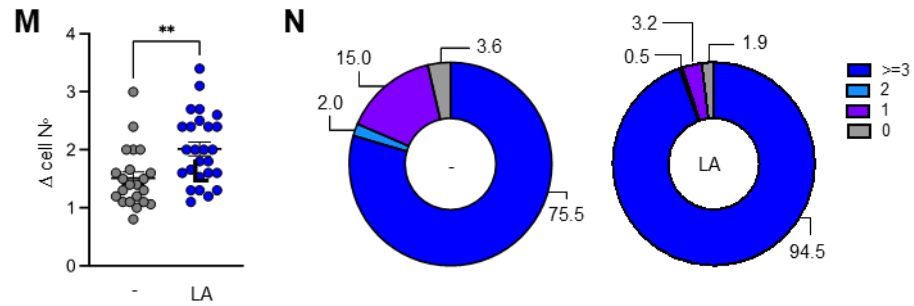
LA-induced phenotype resembles that of long-lived memory cells with increased Blimp1 expression. Representative histogram and relative quantification of transcription factor Blimp1 (H) and quantification of CD127^{high}KLRG1^{low} population in CTR- vs LA-CTL.

On the other hand, LA treatment did not affect frequency levels of canonical activation markers (Figure 6J) nor effector cytokines (e.g. GZM β , IFN γ , TNF α , IL2, and perforin, Figure 6K), suggesting that it did not influence CTL activation. In line with this evidence, LA-CTL did not harbor a proliferative advantage compared to untreated CTL (Figure 6L).



LA treatment does not influence activation status or independent cytokine production. Bar plots showing frequencies of the indicated activation markers (J) and cytokines (K) quantified in LA (blue) and CTR (gray) CTLs. L. CFSE profile and quantification of the percentage of CFSE diluted cells 72 hours post-activation.

However, LA treatment did lead to a significant increase in cell number (Figure 6M), suggesting a survival advantage with respect to CTR-CTL, and the ability to simultaneously produce up to 5 effector cytokines (Figure 6N); an ability known as Tcell polyfunctionality.

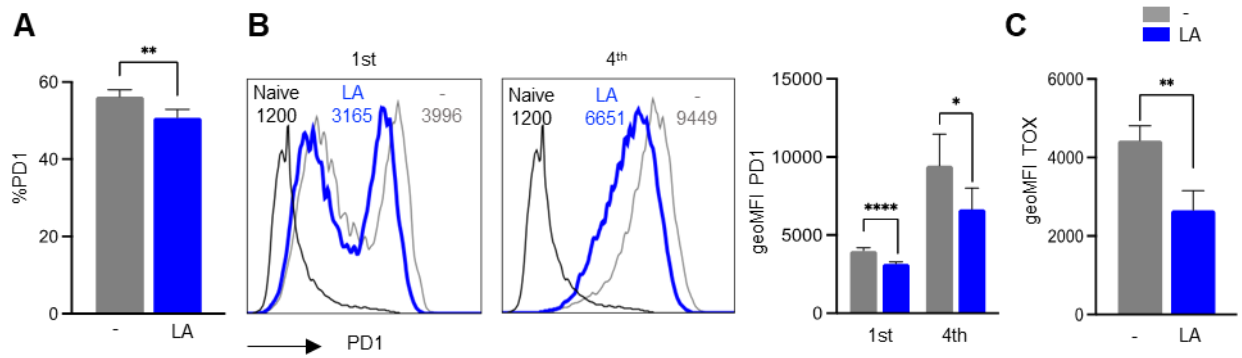


LA increases cell numbers and polyfunctionality in T cells.

M. Total numbers of LA- and CTR-CTLs expressed as fold-change 48 hours post-stimulation (n=26; 5 independent experiments). **N.** CTL polyfunctionality was calculated as the ability of CTR- and LA-CTL to simultaneously produce any of the tested cytokines (GZM β , IFN γ , TNF α , IL2, and perforin, n=3 representative biological replicates).

7. LA treatment attenuates exhaustion and drives increased anti-tumor activity in CTL

Strikingly, LA-treated cells showed a significant drop in PD1 expression- both as a percentage of positive cells (Figure 7A) and overall expression levels (Figure 7B). This particular finding could provide a considerable advantage upon infiltration in the TME, where chronic antigen expression could lead to T cell exhaustion²²². We thus performed an *in vitro* chronic stimulation assay and assessed whether LA supply in the cell media could prevent the development of an exhausted phenotype early after the first stimulus and, most importantly, after subsequent stimulations, where we mimicked the persistent antigen stimulation that T cells found in the TME. Our results showed significant and sustained downregulation of PD1 expression in LA-treated cells along the stimulation cycles (Figure 7B), suggesting a reduced likelihood to develop an exhausted phenotype upon LA treatment. In line with these results, we also observed a significant decrease in the expression of TOX (Figure 7C), which is a transcription factor associated with the establishment of the T cell exhaustion program^{82,84,223,224}.

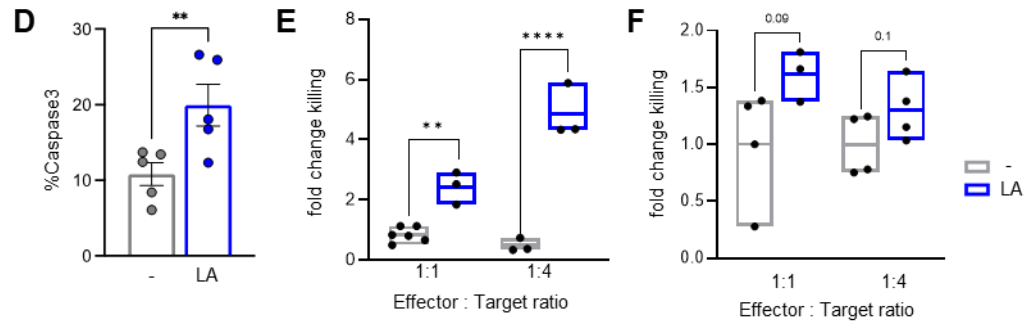


LA treatment attenuates exhaustion in CTL

PD1 expression was assessed as percentage of positive cells (**A**, 48 hours post-stimulation, n=20 biological replicates, pooled from 6 independent experiments) and as an expression level by MFI quantification (**B**, 48 hours post-stimulation -n=47, pooled from 10 independent experiments; and following 4 consecutive stimulations cycles -n=5, pooled from 2 independent experiments). **C**. Expression of TOX was assessed following 4 consecutive stimulation cycles (n=6, pooled from 2 independent experiments).

Finally, to test whether LA-induced changes are associated with an increased anti-tumor response, we evaluated the ability of LA-CTL to kill cognate antigen tumor cells in comparison to CTR-CTL (Figure 7D-F). At the effector: target (E:T) ratio of 1:1, LA-CTL killed target tumor cells more efficiently than CTR-CTL, as independently measured by Caspase 3 (Figure 7D) and live/dead (Figure 7E-F) staining. Notably, LA-CTL retained their cytotoxic ability at the unfavorable E:T ratio of 1:4 while CTR-CTL no longer showed anti-tumor activity (Figure 7E-F).

Of note, the effects of LA treatment on CTL effector functions were rapid, with significant differences showing only 12 hours post-activation (E). Later time points (F, here we reported 48 hours post-activation) were characterized by increased cytotoxic functions in CTR-CTL, thus suggesting that LA modifies the kinetic rate at which CTL develop their effector functions.



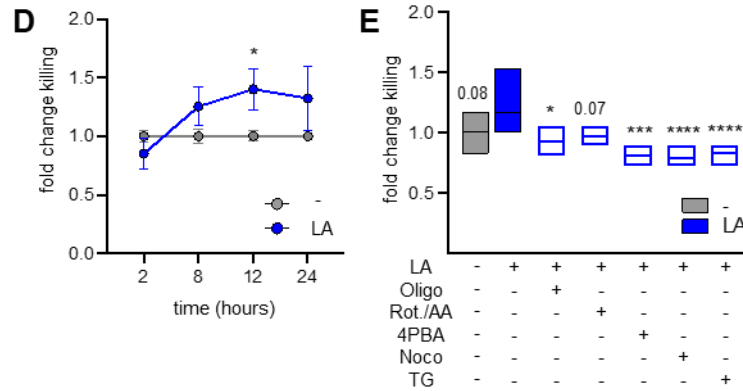
LA-CTL are endowed with superior tumor-killing capacities.

Tumor killing capacity was assessed in vitro by caspase 3 staining (**D**) or by live tracking of tumor cell death using time-lapse microscopy (**E-F**). Antigen-specific CTR- and LA-CTL were co-cultured with cognate antigen-expressing B16 melanoma cells and immunostaining for caspase 3 was performed after 24 hours (**D**). Similarly, CTR- and LA-CTL were activated for 12 (**E**) or 48 (**F**) hours and killing capacity was recorded after only 2 hours of coculture.

These results suggested that LA-induced metabolic reprogramming redirects CD8+ T cells away from exhaustion and endows them with faster and stronger cytotoxic.

8. Development of the LA-induced phenotype follows a sequential kinetics dynamic

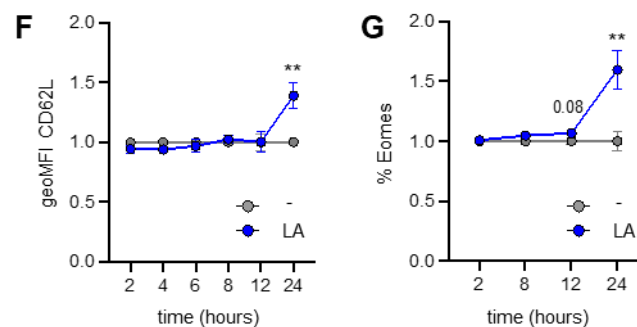
Thereafter, we dissected the time sequence of the LA-induced phenotypes. LA, at first, induced a strong and quick modulation of ER within just 2h (Figure 8A), followed at 6 h by the mitochondrial burst (Figure 8B), alongside with MERC formation (Figure 8C).



LA-induced MERC formation supports CTL anti-tumor activity through mitochondrial burst and calcium influx

D. The dynamic development of cytotoxic functions in CTL was assessed by flow cytometry (n=5, pooled from 2 independent experiments). **D.** Pharmacological intervention was employed to assess the effect of mitochondrial activity blockage (Oligo and Rot./AA) and MAM disruption (4PBA, Noco and TG) on CTL effector function (n=4).

The temporal gap between the increase in MitoOr (Figure 2C) and memory establishment (Figure 8F-G), which appeared at 24h, suggested that the memory shift is consequent to the ER-mitochondria regulation operated by LA in CTL.



Memory development is a late event in the kinetics of LA-induced phenotype

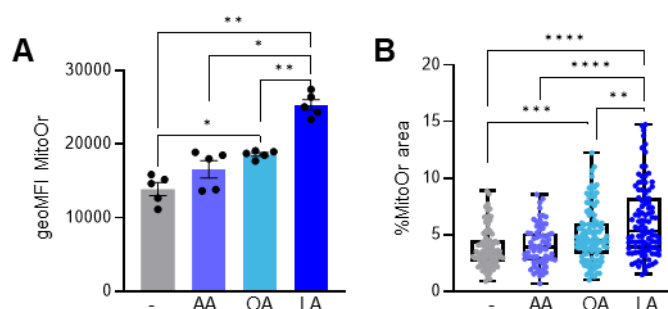
Memory differentiation dynamics was assessed in CTR- and LA-CTL by flow cytometry as expression of CD62L (**F**) and Eomes (**G**). Results are normalized against CTR-CTL and , graphs show mean $\pm$ SEM (n=7, pooled from 3 independent experiments).

Altogether, our data support a model in which LA, by promoting MERC formation, fosters mitochondrial metabolism and Ca^{2+} signaling, which in concert promote cytotoxic activity, followed by improved metabolic fitness and skewing towards memory CTL.

9. LA has a unique role in regulating CTL responses

Several studies have centered their attention on saturated LCFA and its detrimental effects on inflammatory responses^{151,152,226}. In particular, our recent study showed that the enrichment of saturated FAs, such as palmitic acid, impairs CD8+ T cell functions and leads to metabolic exhaustion in pancreatic cancer¹⁴⁹. However, the family of LCFAs is comprised of numerous lipid species divided into several classes and sub-classes that possess intrinsic chemical and physiological characteristics. And the question of whether all LCFAs are detrimental or have an impact on T cell metabolic landscape and differentiation patterns is still open.

In order to provide an answer to this question, we scanned a range of LCFAs varying in length and saturation levels. Mitochondrial mass and activity of T cells treated with oleic acid (OA, C18:1), linoleic acid (LA, C18:2), and arachidonic acid (AA, C20:4) were assessed by flow cytometry and confocal microscopy, 48 hours post-activation. Our analysis revealed disparities in mitochondrial function upon treatment with different lipids. While AA did not raise the mitochondrial potential of treated CTL, either OA or LA significantly increased mitochondrial function and extension (Figure 9A-B), with LA-CTL displaying the most prominent increase in MitoOr staining.

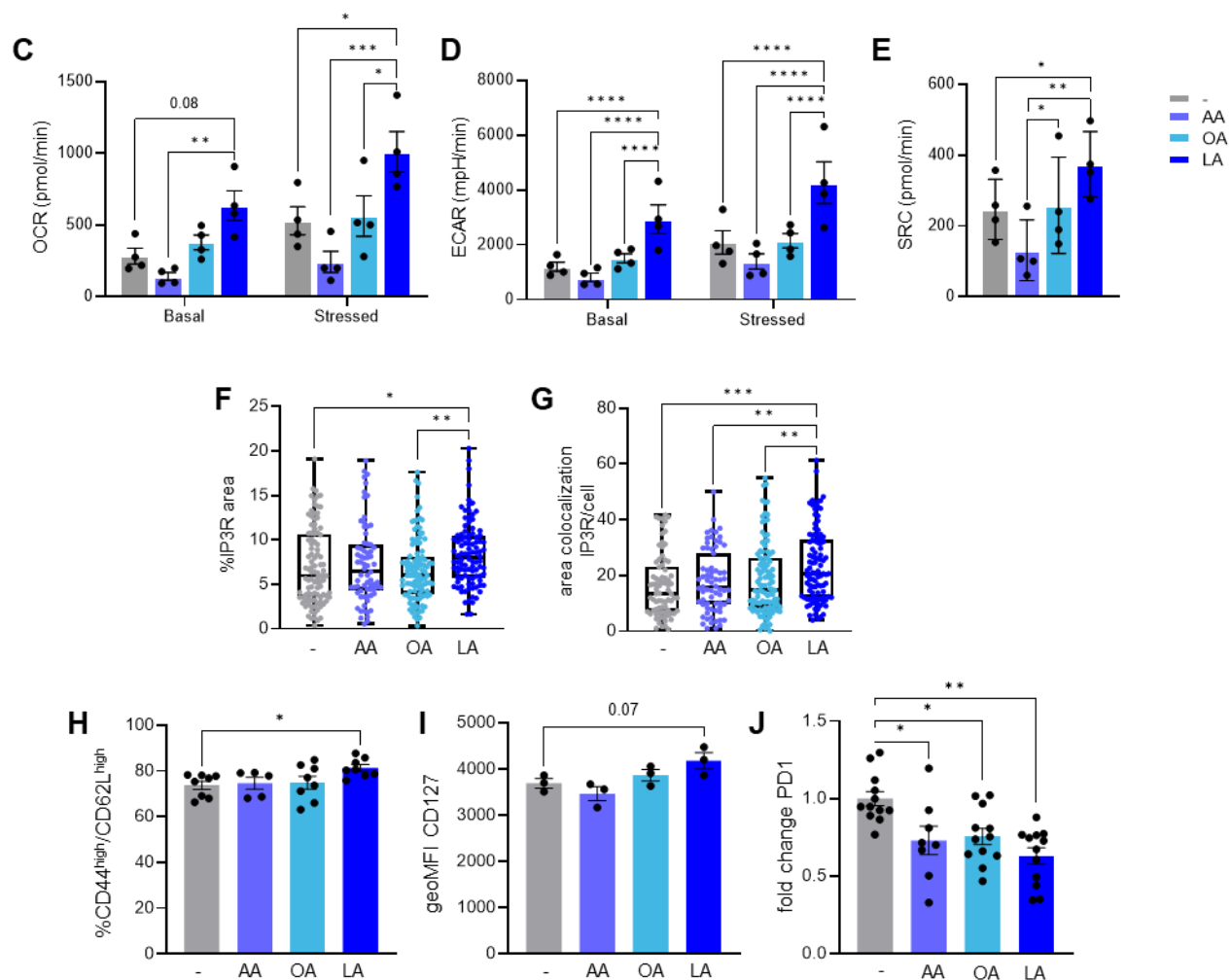


LA drives the most prominent mitochondrial burst among several SFA tested

MitoOr. staining MFI -calculated by FACS - (A, n=5) and area -calculated by confocal microscopy (B, n=5) was assessed on CTL treated with oleic acid (OA, C18:1), linoleic acid (LA, C18:2), and arachidonic acid (AA, C20:4) 48 hours post-activation.

Despite these changes in mitochondrial activity, a noticeable increase in mitochondrial respiration and glycolysis, as well as in SRC, was observed only in LA-CTL and not in OA- and AA counterparts (Figure 9C-E). Furthermore, ER extension and MERC formation were

significantly induced only upon LA treatment (Figure 9F-G). Thus, LA is unique in its ability to simultaneously induce mitochondrial activity and MAM. As a matter of fact, LA solely was promoting memory phenotype (Figure 9H-I) and PD1 downregulation (Figure 9J).



LA is unique in promoting concerted increase in metabolic fitness, MERC formation, and memory phenotype with reduced exhaustion levels.

Metabolic profile of CTR- and LA-CTL was assessed using the Seahorse platform and we report : OCR (C) and ECAR (D) in both basal and stressed conditions, as well as the resulting SRC (E). n= 4. IP3R area (F) and MERC quantification (G) was performed by confocal microscopy (n=5; 2 independent experiments); while memory phenotype was assessed by flow cytometry and defined as CD4+4^{high}/CD62L^{high} and (H), CD127 (I). PD1 expression on CTL treated with different LC-FAs is reported as fold change with respect with CTR-CTL.

Together, these data provide evidences that LA has a unique ability in regulating CTL fate and functions because, differently from other lipids, it fosters a joint mitochondria-ER cross-talk in CTL, which in turn promotes metabolic fitness and memory phenotype coupled with increased cytotoxic activity

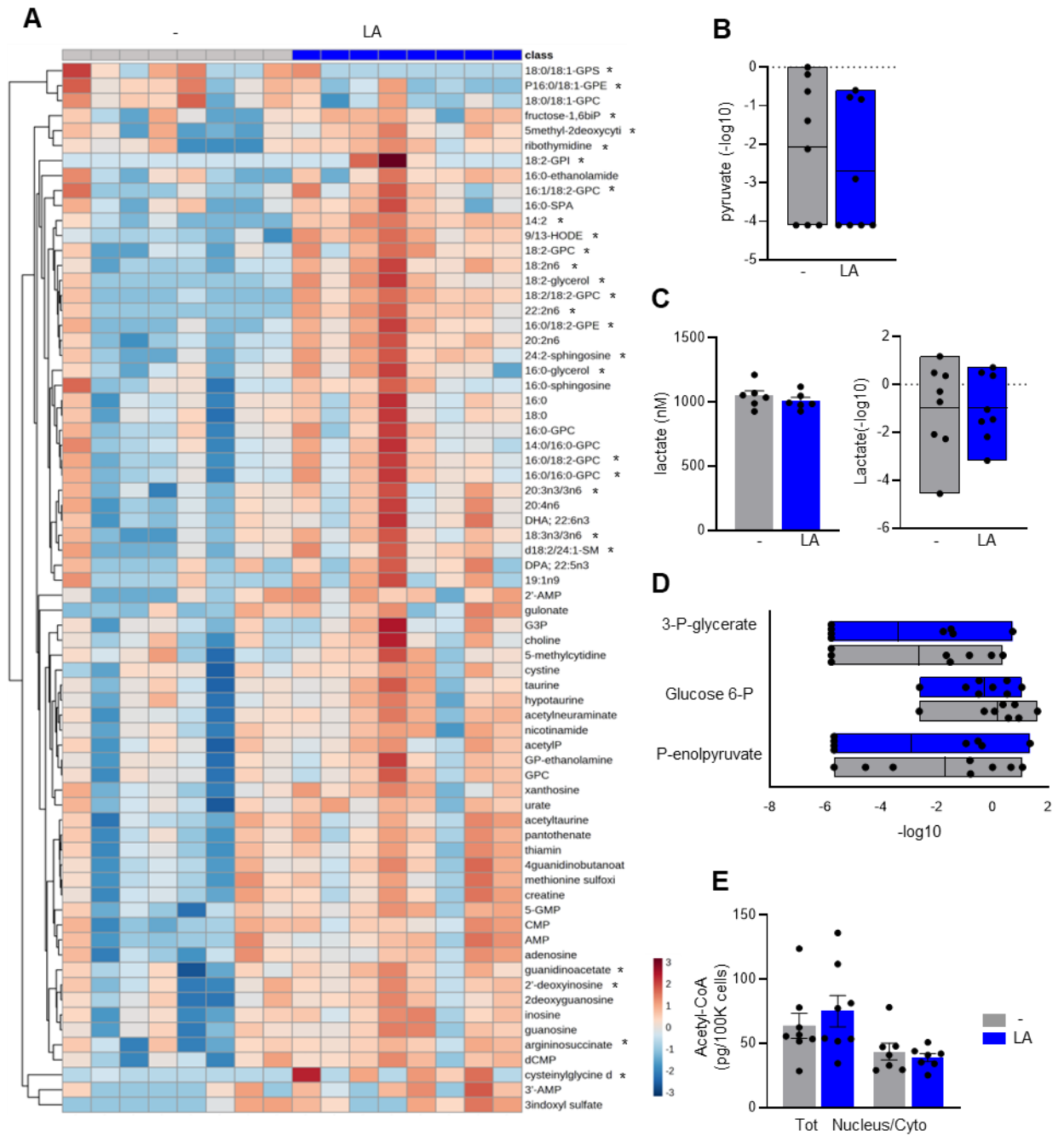
10. LA-induced reprogramming re-routes glycolysis to support FA synthesis.

To elucidate the metabolic program associated with LA treatment, we performed unbiased metabolomic profiling of LA- and CTR-CTL. This metabolic analysis revealed that, when compared with controls, LA-CTL had differences in 49 metabolites (with $p \leq 0.05$, 43 upregulated and 3 downregulated; Figure 10A).

The most striking differences detected between LA- and CTR-CTL were related to metabolic intermediates derived from LA metabolism²²⁷ (Figure 10A).

However, LA- and CTR-CTL showed the same abundance of glycolytic intermediates- such as pyruvate (Figure 10B) and lactate (Figure 10C) - as well as of pentose phosphate pathway intermediates (Figure 10D). The enhanced mitochondrial activity in LA-CTL led us to quantify acetyl-CoA (AcCoA), which enables carbons derived from carbohydrates, fats, and proteins to be utilized in the TCA for OXPHOS. However, we found the same abundance of total and nuclear AcCoA in LA and CTR-CTL (Figure 10E) and, indeed, metabolites related to TCA cycle - citrate, malate, and oxaloacetate - were also comparable regardless of LA treatment (Figure 10F). Suggesting, to our initial surprise, that LA-CTL do not accumulate intermediates resulting from its elevated metabolic rate.

At first glance, these results might seem incongruent with the increased glycolysis and OXPHOS rates recorded by real-time cell metabolic analysis. However, by integrating our metabolomic and transcriptomic data, we established a model in which both concepts can co-exist.



Metabolic profiling of LA-CTL shows increased levels of intermediates derived from LA metabolism

A. Metabolomics assays were performed on CTR- and LA-CTL 48 hours post-activation. The heatmap shows the 80 top most variable metabolites. Significant metabolites (with $p \leq 0.05$, 43 upregulated and 3 downregulated) are highlighted with “*”. Quantification of metabolites and metabolic intermediates: pyruvate (by metabolomics, B), lactate (by metabolomics and enzymatic assay, C), PPP (by metabolomics, D), acetyl-CoA (by enzymatic assay, E), urea cycle, and TCA cycle (by metabolomics, F).

Upon further analysis, we observed a significant enrichment of fructose 1,6 bisphosphate (F16BP), the urea cycle intermediate argininosuccinate, the oxidized derivate of the glutathione (GSH) cysteinylglycine disulfide (GSSR), a number of inosine, adenosine, uracil and cytidine containing nucleotides downstream of the pentose phosphate pathway (PPP), and intermediates of glycerol metabolism (Figure 10A). These findings were well matched at the transcriptional level where we found increased expression of glycolytic enzymes (*Hk*, *Hk2*, *Pfkm*, *Aldo1*, and *Alco1*), several key enzymes of the PPP (*Ogdh*, *Tpi1*, *H6pd*, *Pgls*, *Tkt*, *Taldo1*, *Gls2*, *Cad*, and *Dhodh*), other metabolic enzymes that promote NADPH generation (*Nadk*, *Mthfd*, *Aldh*, *Idh2*, and *Gsl2*). (Figure 4E-F).

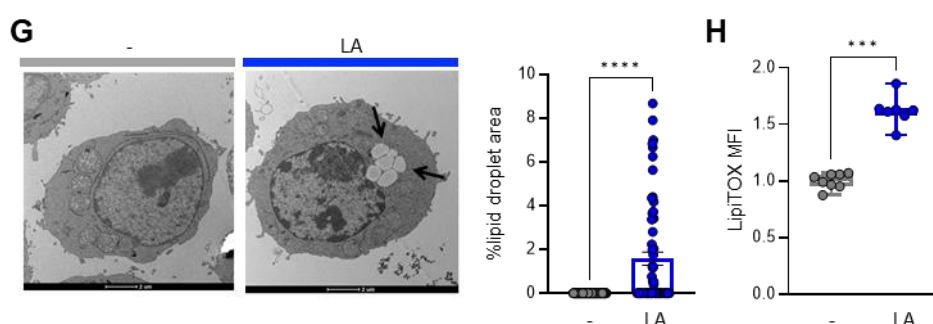
Indeed, increased expression of hexokinase 1 (*Hk1* and *Hk2*) and upregulation of ER-resident hexose-6P dehydrogenase (H6PD), suggest that glucose-6-phosphate (G6P) is rapidly diverted into the PPP. This process is likely to generate the reduced nicotinamide adenine dinucleotide phosphate (NADPH) needed for both lipid biosynthesis²²⁸, contributing to keeping the intracellular redox balance. In particular, Glutathione peroxidase 1 (GPX1), could be playing a key role in protecting cells against membrane lipid peroxidation (promoted by GPX4) and cell death.

Once channeled into the PPP, the G6P is converted to 6-phospho-gluconolactone and, from there, to 6-phospho-gluconate by the phosphogluconolactonase (PGLS), which is one of the top upregulated genes in LA-CTL compared to controls (Figure 4E). The subsequent step generates ribulose-5-phosphate (Ru-5P) and leads to purine and pyrimidine synthesis. Upregulation of glutaminase II (GSL2) indicated that the glutamine catabolism, besides producing additional NADPH²²⁹, contributes to the pyrimidine biosynthetic process by generating ammonium cations, which combines with the pool of CO₂ thanks to the action of

the trifunctional enzyme CAD (Carbamoyl-phosphate synthetase 2, Aspartate transcarbamoylase, and Dihydroorotase) -highly expressed upon LA treatment (Figure 4E)- to generate the carbamoyl-phosphate needed. Both the carbamoyl-phosphate and the glutamate generated from glutamine catabolism can contribute to the accumulation of arginosuccinate we observed in LA-CTL (Figure 10A).

On the other hand, the Ru-5P can also re-enter the glycolytic route as fructose-6P (F6P) thanks to the tandem activity of transketolase (TKT), which we found upregulated in LA-CTL, and transaldolase. The phosphofructokinase (Pfkf, upregulated in LA-CTL) further converts F6P into F16BP, which is the metabolic node where the glycerol synthesis falls into the route to triacylglycerides (TAG) synthesis.

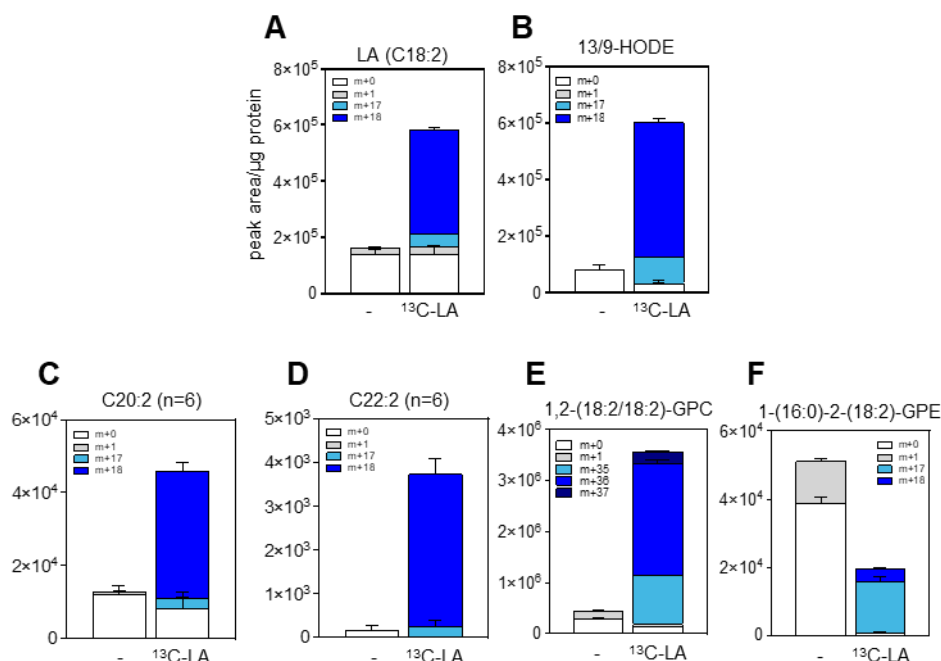
In line with this model, we registered upregulation of genes involved in FA import and synthesis (i.e. *Slc27a1*, *Aacs*, *Acaa2*, *Fasn*, *Srebf1*, *Cpt1a*, *Acadvl*, and *Slc25A1*, Figure 4E-F), mitochondrial citrate-malate antiport (*Slc25a1* or *Cic*) that feed FA synthesis by transporting citrate from mitochondria to cytoplasm producing NADPH, and *Cpt1a*, which transports LC-FAs into mitochondria thereby mediating FAO²³⁰ (Figure 4E). Additionally, LA-CTL displayed higher expression of key enzymes for TAG synthesis (*Dgat2*, *Mgat*, *Agpat1-3*), remodeling (*Mboat2* and *Mboat7*), and storage as lipid droplets (LD) (*Bscl2*, *Lipe*, *Plin2*, *Hilpda*, and *Pnpla2*) (Figure 4E). In fact, EM analysis highlighted an abundance of lipid droplets in LA-treated CTL (Figure 10G), a finding which was further supported by increased levels of total neutral lipids assessed by flow cytometry (Figure 10H) confirming our previous observations¹⁰⁸.



LA treatment promotes the formation of lipid droplets

G. Representative electron microscopy (EM) image from CTR-CTL (left) and LA-CTL (right, n= 3 per group, scale bar 2 μm) and relative quantification of the LD area per cell. **H.** Quantification of neutral lipids performed by flow cytometry. Results represent the MFI of LipiTOX staining normalized to CTR-CTL (n=7, pooled from 2 independent experiments).

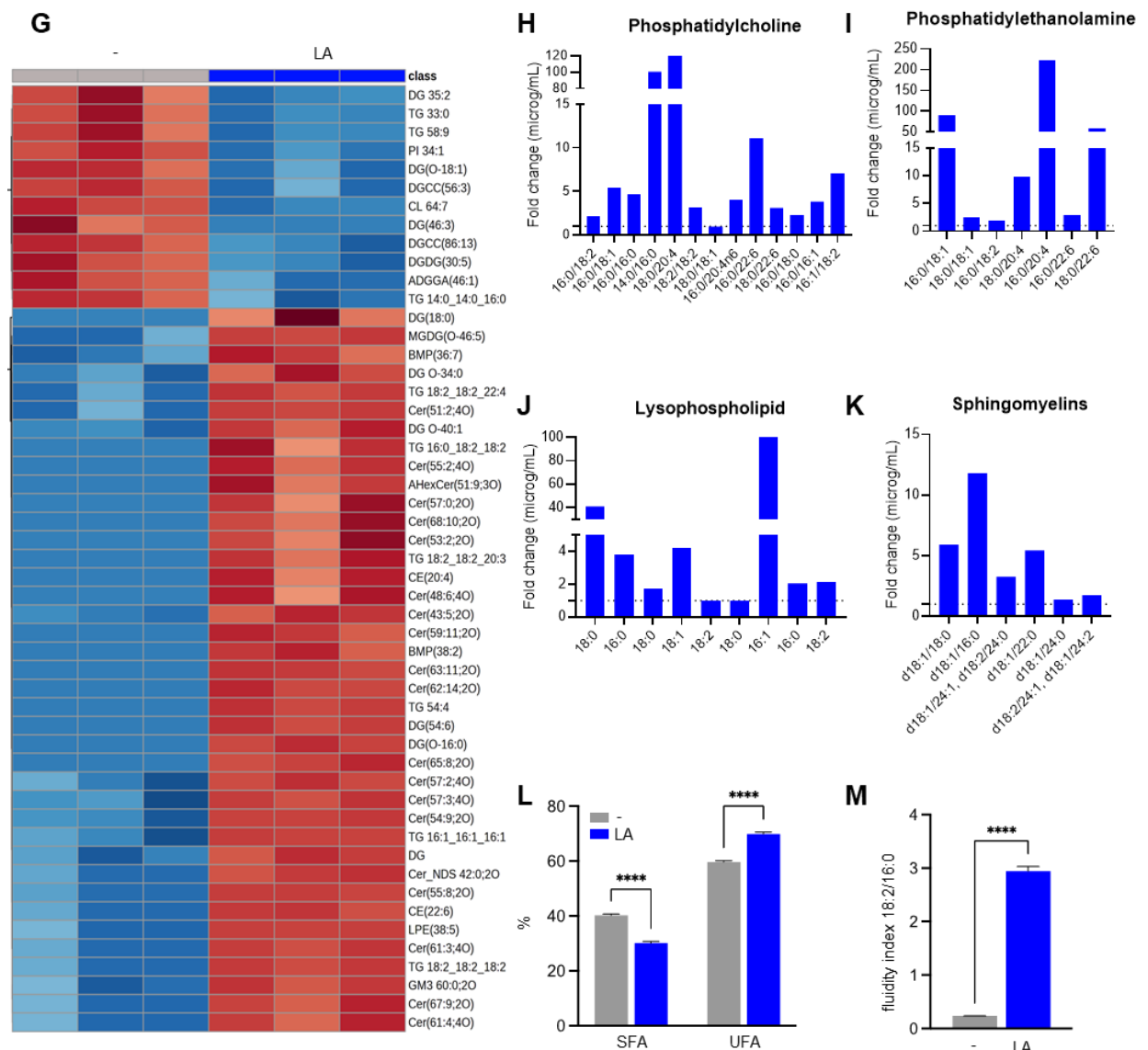
Figure 11D) levels, as well as more complex LA-derived lipids, such as 1,2-dilinoleoyl-GPC (18:2/18:2, Figure 11E) and 1-palmitoyl-2-linoleoyl-GPE (16:0/18:2, Figure 11F); confirming that LA is incorporated and elongated into LC-FAs.



LA is incorporated and elongated into poly-unsaturated LCFA

^{13}C labeled LA experiments were performed in CTR- and LA-CTL 48 hours post-activation (n=2). We show quantification of C18:2 (**A**), 13/9-HODE (**B**), C20:2n=6 (**C**), C22:2n=6 (**D**), 1,2-(18:2/18:2)-GPC (**E**) and 1-(16:0)-2-(18:2)-GPE (**F**).

Moreover, LC-MS based lipidomic analyses recorded broad changes in the abundance of several PL (Figure 11G), especially phosphatidylcholines (PC, Figure 11H), phosphatidylethanolamines (PE, Figure 11I), lysophospholipids (LPL, Figure 11J) and sphingomyelins (SPH, Figure 11K). Notably, LA-CTL displayed differences in the overall degree of saturation of LC-FA Figure 11L). The increased fraction of unsaturated lipids is predicted to decrease membrane rigidity²³¹. Indeed, membranes were more fluid in LA-CTL (Figure 11M), which might provide a mechanistic explanation of the reduced threshold for TCR clustering and activation in LA-CTL (Figure 5C), with the consequent improved cytotoxic activity (Figure 5H-I).



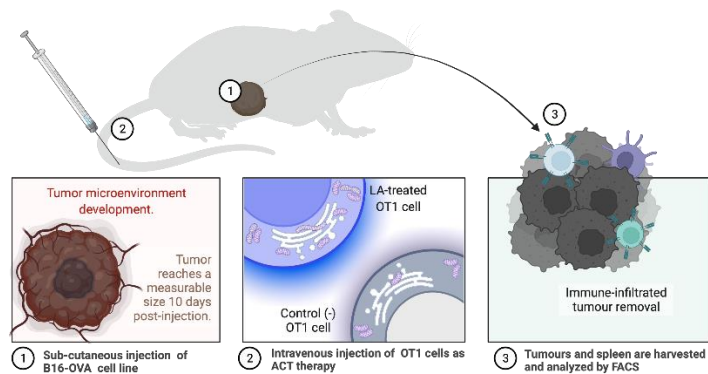
LA-CTL exhibit increased levels of polyunsaturated FA

LS-MS lipidomics was performed on CTR- and LA-CTL 48 hours post-stimulation. We reported a heatmap showing the 50 top most variable lipid species (**G**). Integration of lipidomics and metabolomics data allowed for the quantification of phosphatidylcholines (PC, **H**), phosphatidylethanolamines (PE, **I**), lysophospholipids (LPL, **J**), and sphingomyelins (SPH, **K**). Overall SFA and UFA (**L**) were also assessed and the fluidity index was calculated as the ratio between C18:2 and C16:0 (**M**).

Taken together, these data indicate that, while maximizing NADPH production to sustain FAO and quench reactive bioproducts, LA diverts available resources toward the synthesis of TAG, promoting PL remodeling by increasing unsaturation. In doing so, LA changes membrane fluidity, which ultimately endows CTL with improved and sustained functionality.

12. CTL metabolically reprogrammed by LA carry out a superior anti-tumor response *in vivo*.

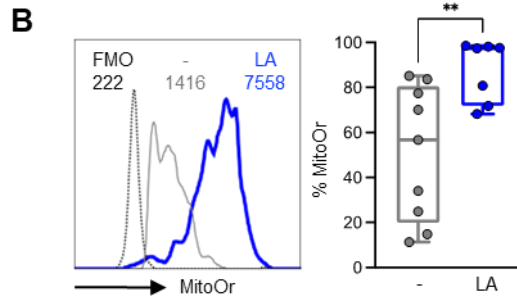
To demonstrate that features conferred by LA to CTL *in vitro* enable them to better adapt to the inhibitory TME, persist longer and mediate durable tumor control, we employed an ACT model for B16-OVA melanoma by generating OVA-specific T cells *ex vivo* in the presence or absence of LA (LA-ACT and ACT, respectively) and injecting them intravenously in tumor-bearing mice (experimental scheme in Figure 12A).



Scheme and time-line of ACT therapy treatment.

A. Mice bearing a 10-day-old B16-OVA melanoma tumors (approximately 200 mm²) were randomly assigned to either one of following treatments: No-ACT ($n = 17$), CTR- OT1 cells (6×10^6 /mouse; ACT; $n = 19$) or LA-OT1 (6×10^6 /mouse; LA-ACT; $n = 20$). Tumors were measured every 48 hours and 14 post-infusion (24 days after tumor injection), animals were sacrificed, organs (tumors, spleen, draining and non-draining lymph nodes) were harvested and single-cell suspensions were analyzed by flow cytometry. All analyses were performed within the adoptively transferred population defined as CD45+CD3+CD8+Vbeta+ cells.

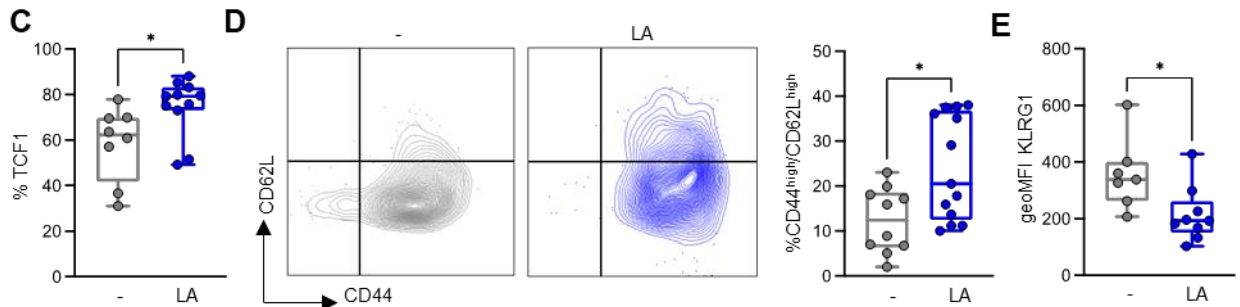
Analysis of the mitochondrial activity of tumor-infiltrating CTL showed that LA-CTL preserved their metabolic advantage by means of superior mitochondrial activity within the TME compared to CTR-CTL (Figure 12B). This confirmed our hypothesis that LA treatment induces metabolic reprogramming in CTL, allowing them to better cope with the metabolic constraints imposed by the TME.



LA-CTL promotes increased intratumoral metabolic fitness.

B. Representative histogram and quantification of MitoOr positive TIL

In line with their metabolic fitness, infiltrating LA-CTL harbored elevated expression of memory transcription factor TCF1 (Figure 12C), which resulted in increased frequencies of CD44^{high}/CD62L^{high} cells (Figure 12D) in mice infused with LA-ACT. We also detected a reduced expression of KLRG1 in the LA-ACT group (Figure 12E), indicative of low senescence levels²³². Combined, these data indicated that LA treatment endowed CTL with superior metabolic fitness coupled with a memory phenotype, and further suggested that LA-CTL can survive and operate for a longer time within the TME, an ability that has been associated with improved ACT efficacy in mice and humans.

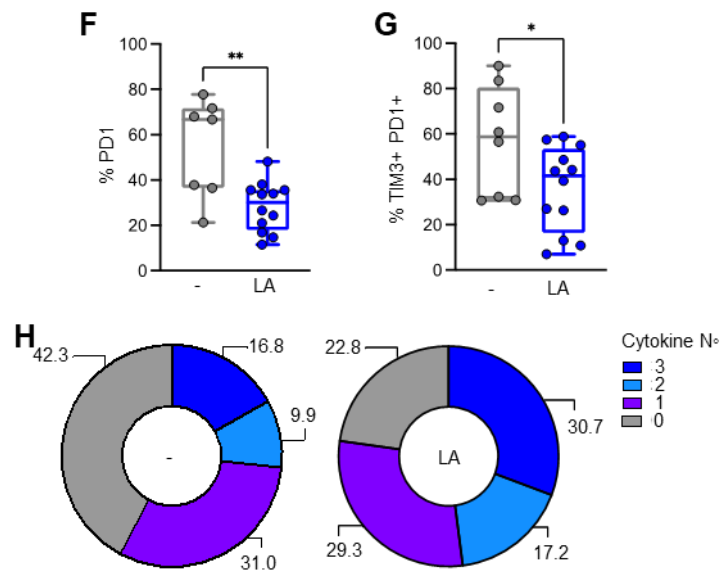


Adoptively transferred LA-CTL are able to maintain memory phenotype 14 days post-infusion

Frequencies of memory population markers TCF1 (C) and CD44^{high}/CD62L^{high} (D) as well as KLRG1 expression levels quantified as MFI (E).

Since our *in vitro* experiments suggested that LA treatment skewed CTL towards a memory phenotype and away from exhaustion, we measured if LA-CTL were prone to acquire hallmarks of exhaustion entering the TME. LA-CTL exhibited significantly lower levels of PD1⁺ cells (Figure 12F) as well as PD1⁺TIM3⁺ cells (Figure 12G), a clear indication of reduced exhaustion features in LA-treated CTL. Indeed, LA-CTL featured increased poly-functionality as indicated by increased frequencies of cells able to simultaneously produce

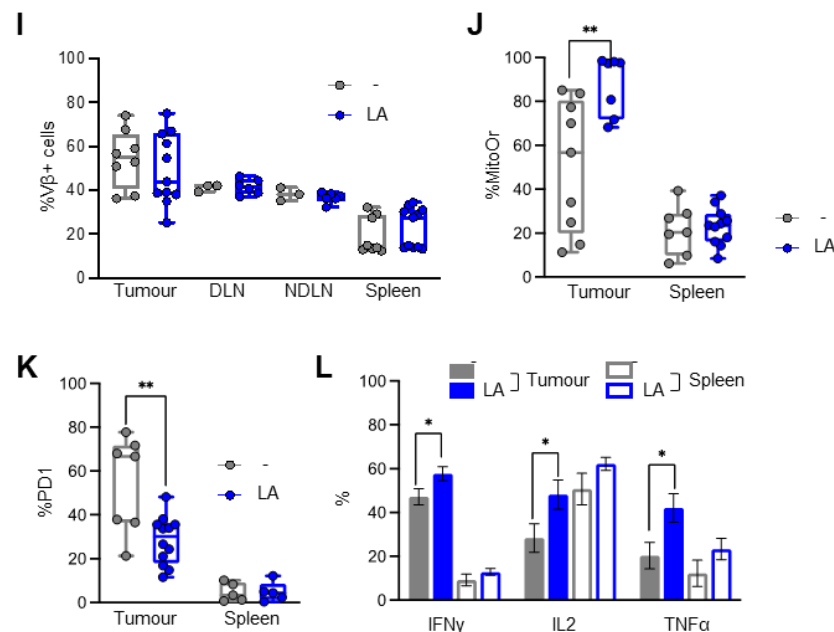
2 or 3 effector cytokines (IFN γ , IL2, and TNF α , Figure 12H). On the contrary, more than 40% of CTR-CTL were no longer able to produce effector cytokines (Figure 12H).



Ex vivo LA treatment ameliorates exhaustion and improves polyfunctionality in intra-tumoral CTL

Percentages of PD1 positive cells (F) and PD1/TIM3 double-positive cells (G) in the adoptively transferred T cell population (V β +). H. Pie charts show analysis of polyfunctionality in the intratumoral V β + population as the percentage of cells able to simultaneously produce 0/1/2 or 3 of the tested cytokines (IFN γ , IL2, and TNF α).

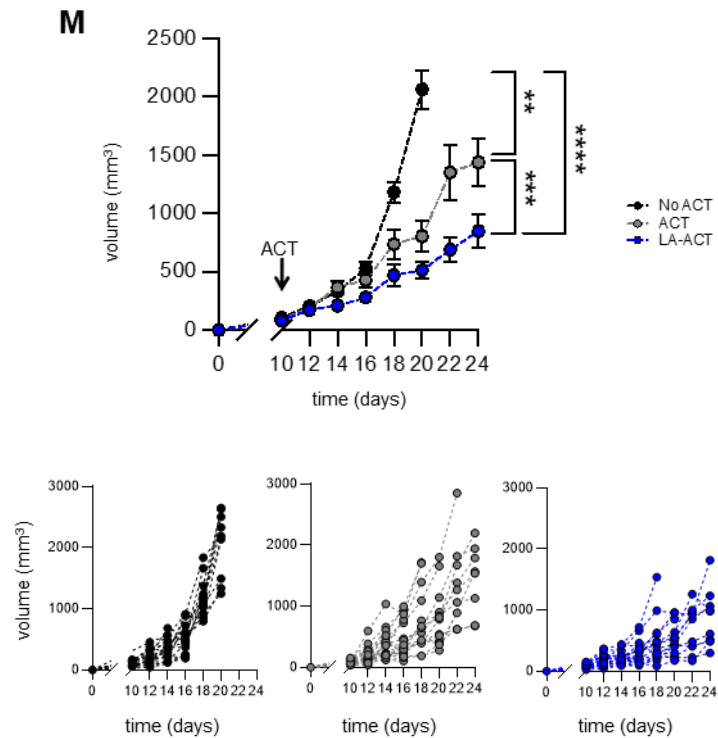
Notably, the advantages induced by LA treatment are specific of the TME and do not influence LA-ACT and ACT neither in the spleen nor in lymph-nodes (Figure12I-L).



LA-induced advantages are specific to the tumor site

Phenotypic analysis of the single cell suspensions from each tumor, spleen, draining (DLN), and nondraining (NDLN) lymph nodes as indicated. Plots show I. frequencies of V β positive T cells, J. quantification of MitoOr. positive cells, K. percentages PD1+ cells, and L. cytokine production.

Finally, all these features enable LA-ACT to control tumors more efficiently when infused in tumor-bearing mice (Figure 12M), demonstrating their superior therapeutic potential. Of note, CTR-CTL were able to significantly control tumor growth, in line with clinical data that prove the high efficiency of ACT approaches. However, LA was able to further increase the tumor-killing capacities of CTL with a rapid and sustained cytotoxic response that is highly specific in the tumor site.



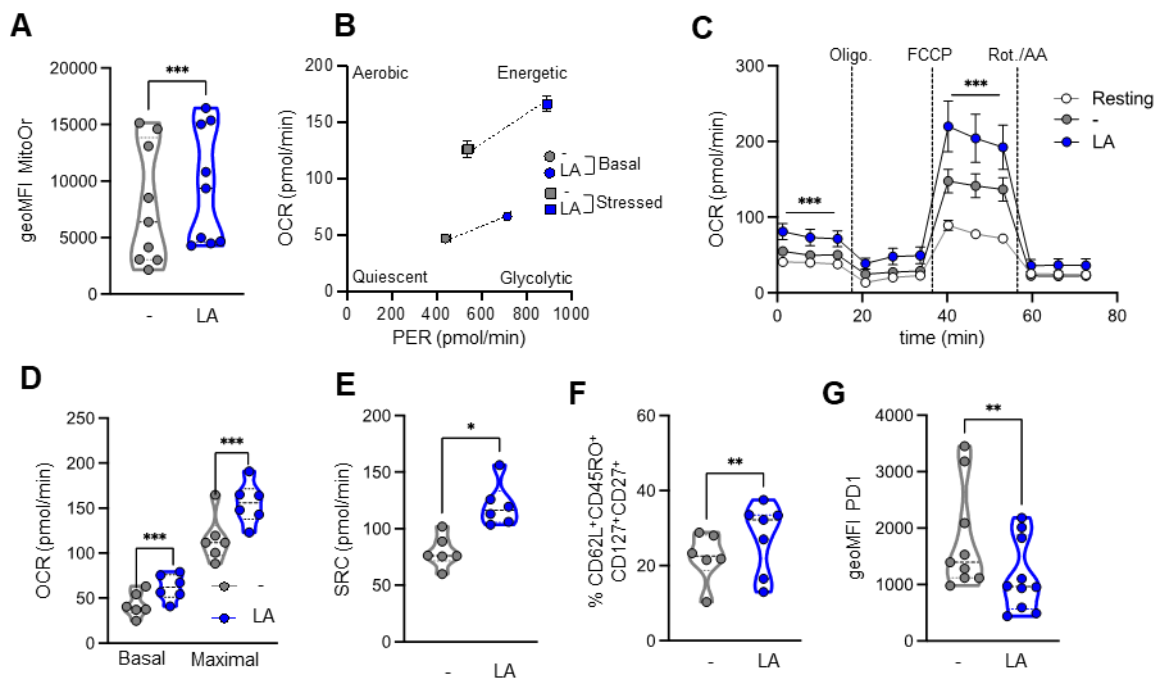
LA-CTL significantly improve ACT efficiency and tumor control.

M. Tumor growth curve and spider plots show tumor volume in all the experimental groups: No-ACT (in black, $n = 17$), ACT (CTR-CTL $n = 19$), and LA-ACT (LA-CTL; $n = 20$).

In conclusion, we have identified LA as a LCFA able to induce profound metabolic reprogramming in CTL which protects them from exhaustion and pushes them towards a memory phenotype with potent and long-lasting anti-tumor activity. Thus, LA endows CTL with hallmarks of productive tumor debulking.

13. LA promotes mitochondrial activity and memory phenotype human CTL.

Our results demonstrate that metabolic and molecular features gained by LA-treated CTL enable them to mount an efficient anti-tumor response. Thus, aiming to consolidate the therapeutic potential of these findings, first, we validate them on human CD8⁺ T cells upon polyclonal activation *in vitro*, with or without LA. Similarly to what we observed in mice, LA-treated human CTL significantly increase MitoOr. staining (Figure 13A), OXPHOS activity (Figure 13B-D), and SRC (Figure 13E), leading to an overall enhanced energetic profile. Our results also confirm an increase in T central memory population (defined as CD62L⁺CD45RO⁺CD127⁺CD27⁺ CTL Figure 13F), which is characterized by a significant decrease in PD-1 expression (Figure 13G). We hence concluded that exposure to LA drives metabolic rewiring and differentiation towards a memory phenotype with decreased PD1 expression also in human CTL.

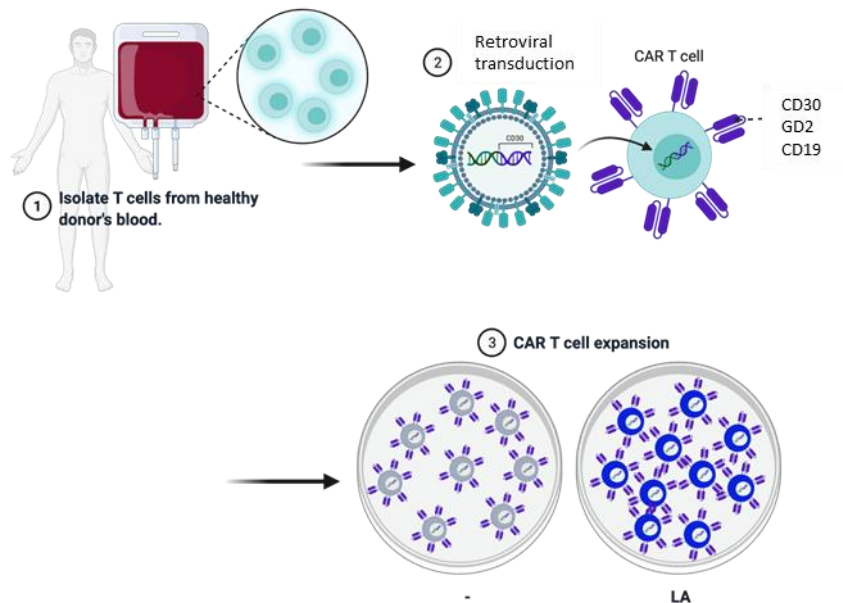


LA-induced metabolic reprogramming is recapitulated in human CD8⁺ T cells

CD8⁺ T cells were obtained from healthy donor's PBMCs by negative magnetic separation and activated using plate-bound aCD3/aCD28 in the presence or absence of LA (n=5). Phenotypic and metabolic status were assessed 48 hours post-stimulation and we show MitoOr staining (A), energetic map (B), OCR levels both in basal and stressed conditions, (C-D) and the consequent SRC (E). Memory phenotype was defined as CD62L⁺CD45RO⁺CD127⁺CD27⁺ (F) and exhaustion levels were measured as PD1 expression (G).

14. LA improves CAR-T cell therapy efficacy.

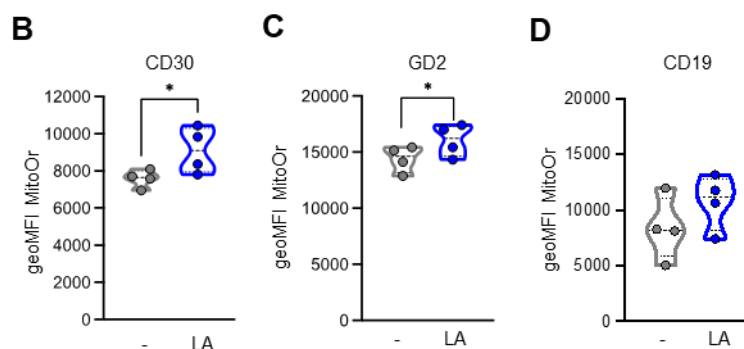
In view of the promising benefits of LA exposure for CTL fitness, we propose LA treatment as a new strategy to improve ACT clinical efficacy. Thus, we applied our *ex vivo* LA-treatment strategy on a clinically relevant system that employs chimeric antigen receptor (CAR)-modified human T cells. Although considerable clinical achievements have been made in cancer treatment via CAR T cell therapy, still many obstacles lie ahead, especially for the treatment of cancer patients suffering from a refractory disease which is often associated with a highly immunosuppressive TME²³³. Here, we have assayed the performance of three different lines of CAR T cells: CD30.CAR (for non-Hodgkin and Lymphoma)²³⁴, GD2.CAR (for neuroblastoma, some high-grade glioma, and sarcoma)²³⁵ and CD19.CAR (for acute lymphoblastic leukemia and diffuse large B cell lymphoma)²³⁶. T cells were retrovirally transduced with the corresponding constructs and expanded *ex vivo* in the presence or absence of LA (See Materials and Methods for details).



A. Schematic representation of CART cell generation and expansion in the presence or absence of LA.

First, we assessed the mitochondrial potential of CAR-T cells expanded in the presence or absence of LA to examine its influence on metabolic CAR-T cell fitness. In accordance with the data presented above, we found a significant increase in MitoTracker Orange MFI in

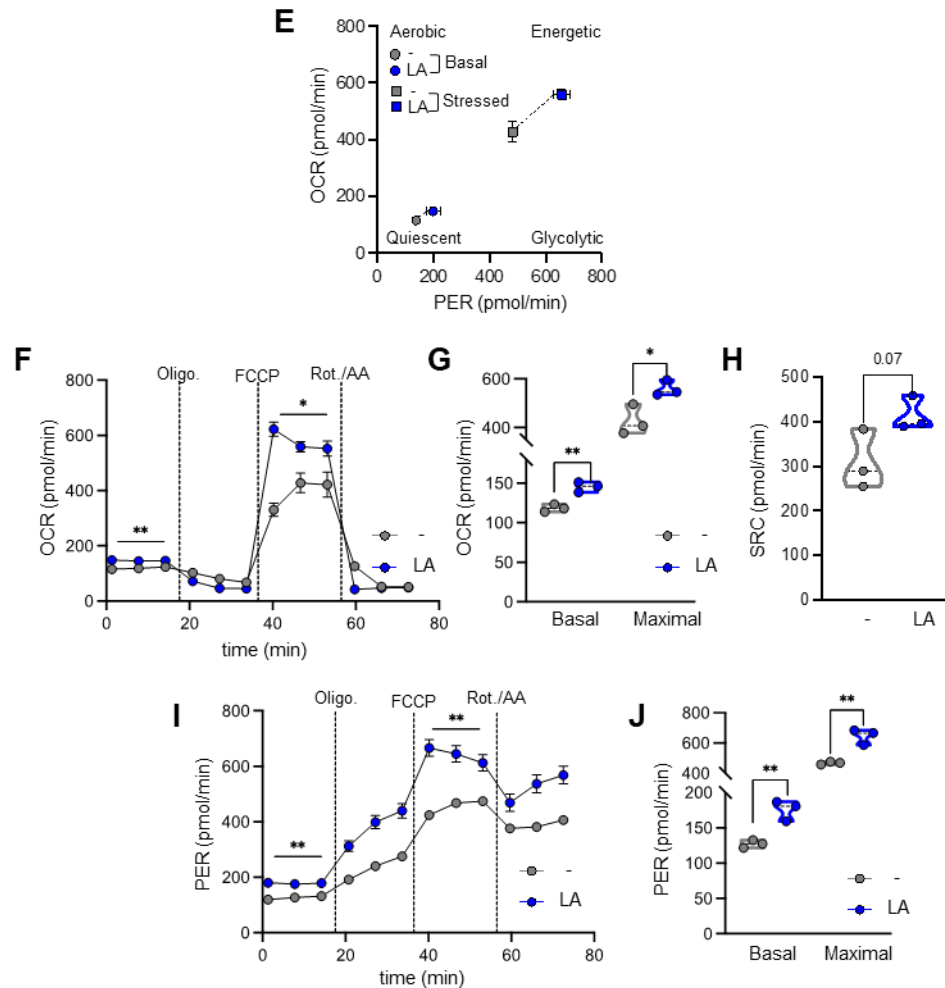
LA-treated human CTLs when compared to that observed in CTR cells, independently from the CAR construct tested (Figure 14B-D).



LA increases mitochondrial activity in CAR-T cells

Mitochondrial potential assessed by MitoOr. staining of CD30 (B), GD2 (C), and CD19 (D) CAR-T cells activated in the presence or absence of LA for 48 hours.

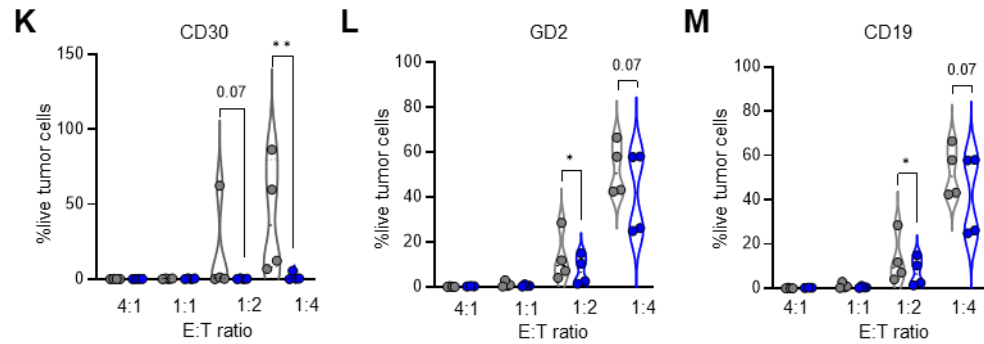
When profiled on a SeaHorse, LA-treated CD30 CAR-T cells uphold their highly energetic metabolic profile under both basal and stressed conditions (Figure 14E, G, and J) and this is driven by OXPHOS, as shown by the significant boost in OCR levels (Figure 14F-G) and the higher SRC (Figure 14H). Furthermore, the glycolytic activity shows a corresponding increase in PER levels both at basal and under stressed conditions (Figure 14I-J), again confirming what we observed in murine T cells. Hence, LA treatment promoted the establishment of a comprehensive metabolic program that resulted in higher metabolic fitness and augmented the stress response capacity of CAR-T cells.



LA treatment induces metabolic reprogramming of CAR-T cells

Metabolic profile of CD30.CAR-T cells was assessed by the Seahorse platform. We show energetic map (**E**), OCR (**F-G**), SRC (**H**), and PER (**I-J**).

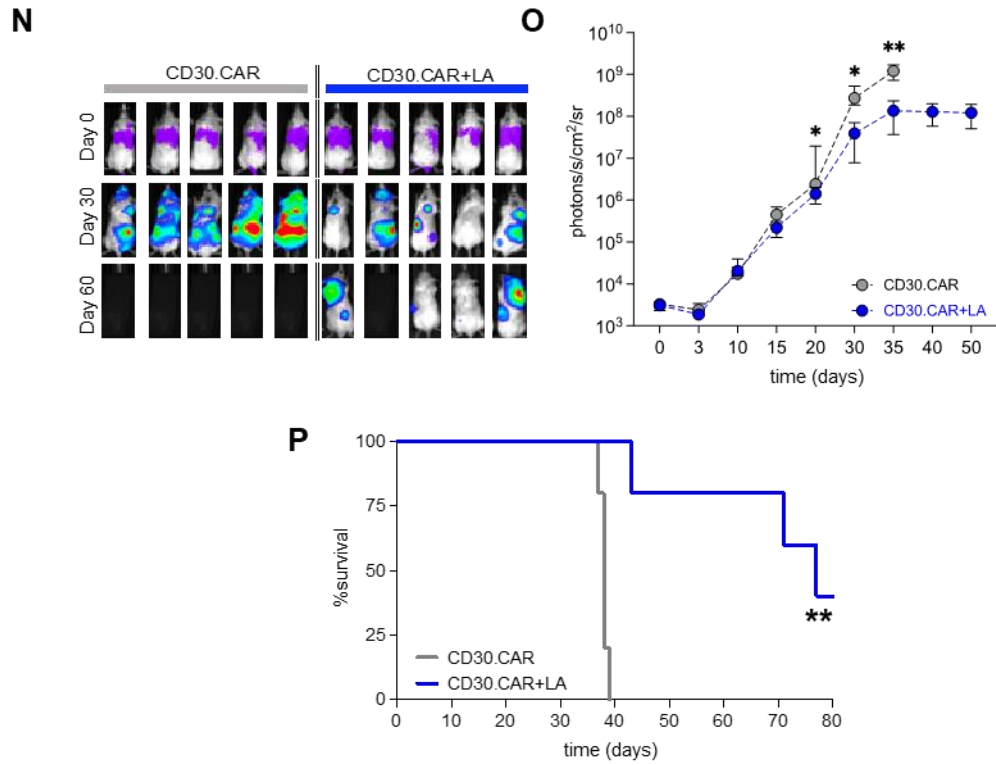
Second, we challenge our CAR T cells on a long-term co-culture assay with target tumor cells. Both LA-treated and control CAR-T cells show efficient killing when cultured at a ratio (E:T) as low as 1:1 with tumor cells (Figures 14K-M). However, only LA-treated CAR-T cells still retain efficient cytotoxic capabilities when incubated with higher proportions of target cancer cells (Figures 14K-M), suggesting that, as a consequence of their metabolic rewiring, LA enhances CAR-T cells effector function.



LA-CAR-T cells are endowed with increased anti-tumor activity *in vitro*.

Long-term killing assays were performed at the stated Effector: Target (E:T) ratio using CD30 (K), GD2 (L), and CD19 (M) CAR T cells.

Third, we tested the benefits of the *ex vivo* LA treatment on the tumor-control ability of CAR T *in vivo* by employing a mouse model of lymphoma in combination with the CD30.CAR construct (more details in Materials and Methods), which had previously demonstrated only efficacy *in vitro*. In line with our previous findings²³⁴, non-treated CD30.CAR-T cells fail to control tumor burden and all mice had to be euthanized by day 35. In contrast, LA-CAR-T cells effectively controlled tumors with a significant difference in bioluminescent tumor signal already detectable already on day 15 and day 30 post T cell infusion (Figure 14N-O). Strikingly overall survival analysis 80 days post-ACT showed improved long-term survival exclusively in mice treated with LA-CAR-T cells with four out of five mice still alive (Figure 14P). We hence conclude that LA-ACT affords long-lasting tumor control and long-term survival.



LA-CAR-T cells show increased anti-tumor capacity *in vivo* with long-term survival.

NGS mice were injected i.v. with 1×10^6 lymphoma cells and then 5×10^6 mock $\pm$ LA and CD30.CAR $\pm$ LA were given i.v. on day 3 (n=5 per each group). Tumor progression was monitored using bioluminescent imaging (**N-O**) and survival was calculated using a Kaplan-Maier curve (**P**).

In summary, our data identify LA as a LCFA able to drive the metabolic rewiring of T cells towards increased mitochondrial fitness and significant tumor-killing capabilities. Thus, LA can overcome all-at-once several obstacles encountered by CTL within the TME, and, here, we provided initial evidence of the therapeutic relevance of this approach.

DISCUSSION

The TME dampens the ability of CTL to execute anti-tumor immunity²³⁷. Nutrient availability and metabolic crosstalk have been shown to affect CTL fate in the TME^{194,238,239} with lipids emerging as key driver⁹⁸. Their accumulation is a hallmark of different types of tumors^{107–112} where they are associated with CTL dysfunction. Yet, the specific effects of different LCFAs on CTL biology have not yet been fully understood. In particular, PUFA have an immunomodulatory effect also on CTL^{240,241} but their mechanisms of action remain quite controversial. Our study reveals that LA, an essential PUFA, ameliorates CTL function and memory differentiation through mitochondrial reprogramming, ultimately preventing CTL exhaustion and granting improved anti-tumor responses.

LA was unique in operating a coordinated regulation of mitochondria and ER, which resulted in improved CTL metabolic fitness and functionality, implying that different FAs modulate CTL fate and function, each by inducing a peculiar metabolic change. Deciphering the specific mechanisms behind the different lipid-induced CTL control would be crucial to decode their influence on anti-tumor immunity. On the other hand, our data paint an even more complex scenario, which includes cell-type-specific sensing mechanisms for lipids. Indeed, LA has been previously shown to cause cell death in CD4⁺ T cells²⁴², where utilization of FAO correlates with PD1 expression²⁴³. In contrast, here we showed that LA did not affect cellular viability and it modulated the mitochondrial membrane's potential of CTL and tumor cell lines with cell-specific effects on their energetic profile. Thus, we postulated that different lipids may be instrumental to achieve cell-specific modulation of mitochondrial metabolism and, as a consequence, cell functionality.

Our data indicate that lipids can be used to coordinate the CTL-mediated anti-tumor responses through the modulation of mitochondrial function. Emphasis has been laid on mitochondrial plasticity in regulating CTL fate and function^{230,244–246}, preventing T cell exhaustion^{247–252} and promoting the generation of memory T cells^{253–255}. However, the uncertainty surrounding the molecular circuits coordinating metabolic state and cellular

functions has so far prevented an effective targeted manipulation. Here, we show that LA is able to promote memory formation while simultaneously maintaining effector and cytotoxic functions in CTLs by integrating mitochondrial respiration with glucose metabolism, which likely endows them with sustained functionality in the TME.

The elevated glycolytic capacity in LA-instructed CTL provides the intermediates necessary to fuel anabolic reactions in CTL ²²⁸, which are essential to support their superior effector functions ^{256,257}. In this regard, the use of a futile cycle coupling FA synthesis with FAO²⁵⁴ couples with LD generation, as a mechanism to store lipids and protect CTL from lipotoxicity during high lipid burden ^{258,259}. While this model may sound wasteful from an energetic perspective, on the other hand, it would ensure memory CTL and a higher overall metabolic fitness for a quicker response in the event of re-stimulation ²⁵⁶. Our results further develop this concept by highlighting the influence that LA has on the membrane's lipid composition and saturation. Indeed, the resulting increase in membrane fluidity induced by LA concurs to improve CTL sensitivity to antigens and results in superior cytotoxic activity. Further studies will be necessary to better understand how LA-mediated biophysical changes differentially affect signaling pathways in TME-exposed CTLs.

LA-induced metabolic and functional reprogramming are dependent on MERC formation, likely because favoring transmission of Ca^{2+} signals between ER and mitochondria ²⁶⁰, potentiates CTL function ²⁶¹. Interestingly, our data suggest that the impact of Ca^{2+} signaling perturbation might be influenced by T cell identity and vary across different T cell subsets. Whether changes in Ca^{2+} signaling levels activate different transcriptional and metabolic programs awaits future investigations. It would be interesting to dissect the mechanisms of calcium sensitivity in relation to CTL fate and metabolic requirements.

ACT is hampered by the acquisition of a dysfunctional state in CTL, which impairs their function and persistence within the TME. Infused T cells quickly lose their metabolic plasticity, critical to adapt to a highly immunosuppressive TME. Recent findings have underscored the importance of metabolic programs in T cell survival and effector function ^{262–265}. Considering current roadblocks, our findings offer a tangible potential for LA treatment to turn ACT into clinical success regardless of the initial fitness of T cell product.

Indeed, LA skewed CTL towards a memory-like phenotype, preventing their terminal differentiation and exhaustion, while retaining high effector activities. In this regard, the finding of Blimp1 up-regulation was of interest and raises the possibility that it might have a role in establishing effector function in LA-driven memory CTL. However, it remained to decode the mechanism by which LA could link Blimp1 and metabolic regulation for the acquisition of effector functions in memory CTL.

LA significantly improves anti-tumor responses towards several tumor types, including multiple myeloma and lymphoma, and even when targeting solid tumors such as melanoma and neuroblastoma. In this scenario, it is conceivable to believe that metabolic pathways uptaken by CTL, in relation to the nutrients they consume, direct them toward different effector profiles, and targeted manipulation of such *modus operandi* might lead to advances in T cell-based therapies. Of considerable benefit, our approach concerns *ex vivo* treatment of isolated T cells and is not limited by any need to target the lipid metabolism systemically which would otherwise risk-averse side effects ^{266–269}. Last but not least, LA treatment confers long-lasting metabolic and functional advantages to T cells, which are maintained inside the TME. In all, LA is able to ameliorate ACT efficacy and to broaden its application for the treatment of a wide range of malignancies.

Taken together, our results show that lipids play a major effect on T cell physiology engaging different metabolic, functional, and cellular programs. Based on our study, we envision that lipids will become integral to tailoring T cell-mediated immune responses in immune-based cancer therapy.

CONCLUSIONS

In this work, we provide evidence that LA acts as a potent enhancer of CTL responses through the promotion of metabolic reprogramming. The metabolic fitness induced by LA treatment grants CTL with enhanced polyfunctionality, superior tumor control, and reduced exhaustion levels with respect to conventional ACT.

LA-mediated metabolic reprogramming leads to the development of a memory-like phenotype with increased expression of canonical progenitor transcription factors that is maintained in time upon adoptive transfer. Thus providing evidence of the enormous potential of LA-induced metabolic reprogramming to increase the efficacy of current ACT approaches.

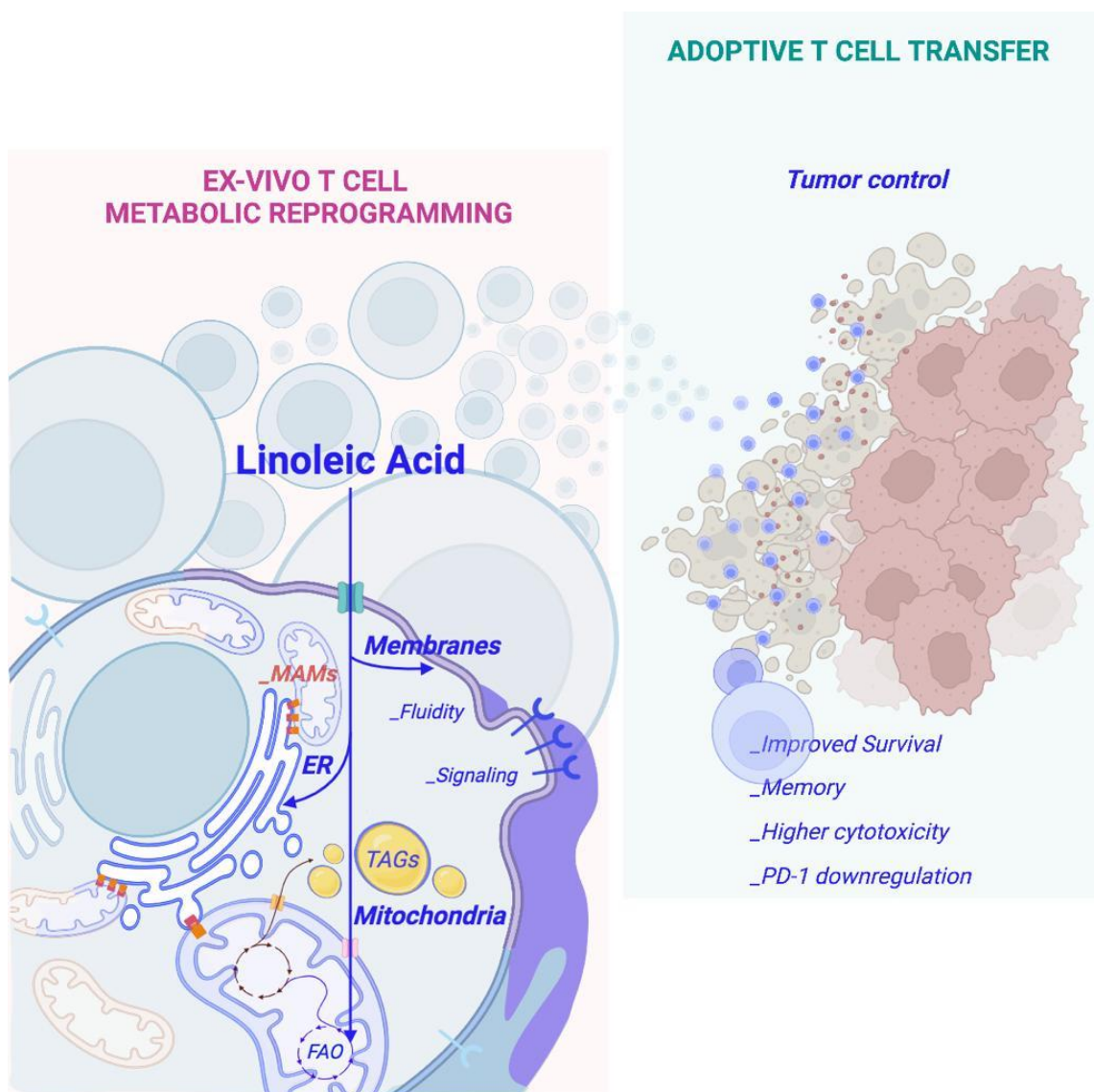
Mechanistically, the increase on MAM formation detected upon LA treatment provides the structural machinery exploited by CTL to power their effector functions. In fact, LA-CTL rely on calcium influx and mitochondrial integrity to sustain cytotoxic activity, memory phenotype and metabolic fitness.

However, several questions remain unanswered and require further studies. For instance, the molecular mechanisms behind the unique advantages provided by LA in comparison to other lipids remain unexplored. Moreover, the LA-induced metabolic reprogramming was only studied in the context of CTL; future work is required to understand its effects in other cellular contexts, such as CD4 T cells, myeloid cells, and different tumor types. These studies may inform also about the benefits and side effects related to diet intervention as an approach to improve the efficacy of cancer therapy. Linoleic acid lifts CTL metabolic fitness by improving mitochondria "quality" and "quantity".

Altogether, our data provide a glimpse of the intricate crosstalk between transcriptional, metabolic, and biophysical processes regulating T cell anti-tumor immunity and represent a small but mighty step forward in the development of new approaches to improve current anti-cancer therapies.

GRAPHICAL ABSTRACT

- Linoleic acid lifts CTL metabolic fitness by improving mitochondria "quality" and "quantity".
- Linoleic acid redirects CTL away from exhaustion and towards a memory-like phenotype with superior effector functions.
- Linoleic acid fosters MERC formation and the downstream CTL processes associated with calcium signalling.
- LA potentiates anti-tumor CTL response and improves ACT efficacy.



DISCLAIMER

The data and images presented in this PhD project are currently in press by Cell Metabolism ("Linoleic acid potentiates CD8+ T cell metabolic fitness and anti-tumour immunity"; Nava-Lauson et.al. CELL-METABOLISM-D-22-00590R1).

A patent application related to this study has been filed.

AKNOWLEDGMENTS

The development of this work wouldn't have been possible without the support and experience of Teresa Manzo. I would like to thank Prof. Doreen Cantrell and Bruno Amati for their guidance throughout the entire project. My most sincere thanks to past and present members of the labs (both Manzo's and Nezi's) for discussion and experimental support. Special thanks are addressed to Teresa-Maria Frasconi, Silvia Tiberti, and Carlotta Catozzi for their constant encouragement and dedication to this work.

I would like to thank Simona Rodighiero for her support in the acquisition and analysis of confocal microscopy images; Federica Conte, Punit Tyagi, and Ayush T Raman for bio-informatic analyses; Paola A. Corsetto for LS-MS experiments; Alessia Loffreda and Andrea Raimondi for EM acquisition and analysis; Markus Machwirth and Ignazio Caruana for CAR-T manufacturing; Stefan Ebert, Lukas Scheller, Dalia Sheta, Zeinab Mokhtari, Timo Peters, Angela M. Rizzo, Andreas Beilhack, and Johannes B. Huppa for calcium influx and migration assays; Francesco Greco, Giovanni Signore and Liam A. McDonnell for FLIM experiments; Simone Cardaci for carbon tracing experiments; Nicola Tumino and Paola Vacca for their support with *in vivo* xenograft experiments.

I'm grateful to the Flow Cytometry, Imaging Unit, and Genomic Unit at IEO for their technical assistance, ALEMBIC (Advanced Light and Electron Microscopy BioImaging Center) at IRCCS Ospedale San Raffaele for EM analyses, Giulia Casorati and Paolo Dellabona for providing B16-OVA cell line. This work was supported by Associazione Italiana per la Ricerca contro il Cancro (AIRC StartUp Grant 20464, AIRC IG 26406, and AIRC StartUp Grant 21474), Bando della Ricerca Finalizzata 2018 GR-2018-12365954, Deutsche Forschungsgemeinschaft (N° 470198222) and DKMS Stiftung Leben Spenden - John Hansen Research Grant (N° DKMS-SLS-JHRG-2021-03), and DFG TRR221 Z02 (N° 324392634). This work was partially supported by the Italian Ministry of Health with Ricerca Corrente and 5x1000 funds.

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