Mitochondria as Playmakers of CAR T-cell Fate and Longevity

Hosein Rostamian[1], Mohammad Khakpoor-Koosheh[1], Keyvan Fallah-Mehrjardi[1], Hamid R. Mirzaei[1], and Christine E. Brown[2,3]

ABSTRACT

The development of chimeric antigen receptor (CAR) T-cell therapy has led to a paradigm shift in cancer treatment. However, patients often do not benefit from CAR T-cell therapy due to poor persistence of the adoptively transferred cells. Development of strategies based on the generation and maintenance of long-lasting memory T cells may expand the therapeutic effects of CAR T cells. Mitochondrial metabolic pathways play crucial roles in regulating the fate, function, and longevity of T cells. Here, we discuss how reprogramming of mitochondrial metabolic pathways influences function, persistence, and determination of CAR T-cell fate toward a memory phenotype. Moreover, we explore how mitochondrial activity determines persistence and the clinical outcome of CAR T-cell therapy. In addition, we review some strategies for manipulating CAR T-cell mitochondria to improve the survival of CAR T cells.

Introduction

The recent success of chimeric antigen receptor (CAR) T-cell therapy in treating hematologic malignancies has heightened the hopes of promising results using this approach to treat solid tumors. However, most patients diagnosed with solid tumors have gained transient or no benefit from CAR T-cell therapy. One of the main reasons for this is the low persistence of the infused therapeutic T cells, which directly impacts clinical outcome (1).

T cells with less differentiated memory phenotypes, such as central memory T (T_{CM}) cells and T memory stem (T_{SCM}) cells, have high proliferative capacity, potential for long-term survival, and the ability to initiate more robust and long-lasting protection against tumors (2). Therefore, there has been considerable research effort put in to identifying ways to alter T-cell differentiation and guide the maintenance of long-lived memory T cells (T_{MEM}). Given that cellular metabolic pathways are key players in regulating the function and longevity of T cells, manipulating the metabolic characteristics of T cells and CAR T cells is a promising approach to improve their antitumor functions (3, 4). Each subset of T cells has its own unique way of supporting its specific functional needs using distinct energetic and biosynthetic pathways. Resting T cells, such as naïve T (T_N) cells and T_{MEM} cells, make use of mitochondrial respiration and fatty acid oxidation (FAO). Glycolysis is upregulated upon T-cell activation to meet the energy demands of mounting an immune response, and activated T cells convert glucose to lactate under aerobic conditions, a process known as the Warburg effect (5).

[1]Department of Medical Immunology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran. [2]Department of Hematology & Hematopoietic Cell Transplantation, City of Hope Medical Center, Duarte, California. [3]Department of Immuno-Oncology, City of Hope Beckman Research Institute, Duarte, California.

Corresponding Authors: Christine E. Brown, Department of Hematology & Hematopoietic Cell Transplantation, City of Hope, 1500 East Duarte Road, Duarte, CA 91010. Phone: 626-256-4673; E-mail: cbrown@coh.org; and Hamid R. Mirzaei, Department of Medical Immunology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran. Phone: 98-21-66419536; E-mail: h-mirzaei@tums.ac.ir

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Mitochondria, key regulators of T-cell metabolism, can affect T-cell function and survival. The prominent role of mitochondria and metabolism during different stages of T-cell adaptive responses was reviewed recently (6, 7). In this review, we present how manipulating the mitochondrial biogenesis of T cells could be a viable approach to improve CAR T-cell persistence and memory formation for cancer treatment (8). We discuss how the reprogramming of mitochondrial metabolic pathways affects activation, effector functions, and differentiation of T cells. Moreover, we also explore how mitochondrial activity determines the persistence and clinical outcome of CAR T cells against tumors and review some strategies for the manipulation of T-cell mitochondria to improve CAR T-cell survival.

Mitochondria Determine T-cell Fate

Mitochondria are central to cellular and molecular events involved in metabolic activity, antiviral responses, and cell death, and their function is tightly linked to inner membrane and outer membrane structure, which is continuously remodeled on the basis of cellular cues (9). Because efficient metabolism is essential for normal cellular function, it is unsurprising that a poor antitumor response is often accompanied by problems in the components of cellular energy production, such as a reduction in the number of mitochondria (10). It is not only the number of mitochondria that is important for T-cell activity, but also the shape of the mitochondria. Mitochondria have prominent roles during the metabolic alterations that occur in T cells as they differentiate. For instance, based on their mitochondrial mass and morphology, T cells can be differentially categorized into T_N cells, T effector (T_{EFF}) cells, and T_{MEM} cells (11). T_{MEM} cells are characterized by increased mitochondrial mass, with more elongated mitochondria and greater numbers of these organelles than T_{EFF} cells. Upon T-cell activation, mitochondria rapidly undergo fission leading to an increase in punctuated mitochondria with loose cristae (11). Loose cristae may result in disruption of the electron transport chain (ETC) supercomplexes, which leads to less efficient oxidative phosphorylation (OXPHOS) and alters metabolism toward aerobic glycolysis to support T-cell activation and proliferation. ETC supercomplexes are efficiently formed in the fused and elongated mitochondria and support OXPHOS, which contributes to T-cell survival and function (11). As a general cellular response to nutrient starvation and stress, such as encountered in the hostile tumor

microenvironment (TME), mitochondria undergo elongation as a protection mechanism from autophagy and to sustain cell viability (12, 13).

Furthermore, T_{MEM} cells exhibit a superior spare respiratory capacity (SRC) compared with T_{EFF} cells (14). SRC is the reserve mitochondrial capacity available in cells to produce energy in response to increased stress or work, and is an index of metabolic fitness and related to cell survival (11). One of the crucial factors that could increase SRC in T_{MEM} cells is the enhancement of FAO in the mitochondria through the expression of carnitine palmitoyltransferase 1A (CPT1a), an enzyme that is involved in transferring fatty acids from the cytosol to mitochondria for oxidation (14). Another indicator of T-cell mitochondrial fitness in the TME is mitochondrial membrane potential ($\Delta\psi m$), which is generated by pumping protons from the mitochondrial matrix into the inner membrane space. $\Delta\psi m$ is an essential part of ATP synthesis (15). Because low-$\Delta\psi m$ $CD8^+$ T cells demonstrate many metabolic characteristics of T_{MEM} cells, including increased FAO, higher expression of Cpt1a, enhanced mitochondrial SRC, and relatively low glycolysis, they exhibit superior *in vivo* persistence, enhanced capacity to expand, and heightened antitumor immune function as compared with high-$\Delta\psi m$ $CD8^+$ T cells (16). A decrease of $\Delta\psi m$ in T cells is accompanied by a reduced level of expression of exhaustion markers, reactive oxygen species (ROS), and DNA damage (16). Tumor-infiltrating lymphocytes (TIL) isolated from patients with renal cell carcinoma have small, punctate, fragmented mitochondria with poorly defined membranes and cristae and bigger inner membrane mass, indicative of limited respiratory capacity. These mitochondria are hyperpolarized, have high $\Delta\psi m$, resulting in the production of large amounts of ROS that reduce T cell–mediated antitumor immunity (17). Taken together, these observations highlight the significance of mitochondrial biogenesis and dynamics (fusion/fission) in shaping T-cell fate and response.

Impairment of Mitochondrial Function in Patient-Derived T Cells

The TME imposes a variety of challenges that hinder the antitumor responses of TILs and adoptively transferred T cells. Metabolic restrictions imposed by the TME inhibit T-cell antitumor responses by disrupting regulation of transcription and translation. Metabolic processes involved in mitochondrial activity and dynamics are, therefore, influenced by the TME, hence limiting $CD8^+$ T-cell function (18). TILs often exhibit decreased mitochondrial mass and, as a consequence, a limited respiratory capacity compared with that of peripheral blood T cells (10).

Repeated exposure of T cells to antigen, as seen in most tumors, leads to endogenous T-cell and CAR T-cell exhaustion (19–21). T cells that are chronically stimulated exhibit defects in mitochondrial oxidation of glucose and fatty acid. Furthermore, Blimp1-mediated suppression of peroxisome proliferator-activated receptor γ coactivator 1-α (PGC1α)–dependent mitochondrial reprogramming is also increased in these cells. This metabolic phenotype is characterized by an induction of mitochondrial oxidative stress, which hinders the ability of T cells to employ oxidative phosphorylation, giving rise to bioenergetic limitations. Furthermore, free radical–driven mitochondrial oxidative phosphorylation impairment is both necessary and adequate for repressing self-renewal gene expression programs, while promoting gene expression programs related to terminal T-cell exhaustion. Antioxidants can restore T-cell proliferation, effector function, memory cell–associated gene expression, and antitumor T-cell immunity in vitro and in vivo by averting the accumulation of mitochondrial ROS that is driven by repeated antigen exposure (21, 22).

Chronic lymphocytic leukemia (CLL) and acute lymphoblastic leukemia (ALL) are two blood leukemias that have been treated with CAR T cells, but with different therapeutic outcomes. CAR T-cell therapy is associated with a high remission rate among patients with relapsed and refractory ALL. In contrast, patients with CLL have lower clinical response rates following CAR T-cell therapy (23, 24). This is partly due to metabolic impairment of T cells in patients with CLL. In these patients, $CD8^+$ T cells are characterized by impaired activation, diminished proliferation, and reduced glucose uptake after stimulation. In these patients, chronic exposure of $CD8^+$ T cells to leukemic B cells, which potentially influences metabolic homeostasis, leads to aberrant metabolic reprogramming of T cells following antigenic stimulation (**Fig. 1**). Resting $CD8^+$ T cells in CLL show lower surface expression of GLUT1 after stimulation and increased $\Delta\psi m$, which is accompanied by increased mitochondrial ROS (25). PGC1α is the main regulator of mitochondrial biogenesis and scant levels of PGC1α are associated with impaired mitochondrial biogenesis in CLL-derived $CD8^+$ T cells upon stimulation (25). Gene signature analysis shows that CAR T-cell products from patients who achieve a complete response (CR) have a gene expression profile enriched in markers associated with an early memory T-cell phenotype. In comparison, CAR T cells from patients who have a partial response (PR) or no response (NR) exhibit higher expression of genes associated with late memory and T_{EFF}-cell differentiation, aerobic glycolysis, and apoptosis. CAR T cells from NR patients also show a higher rate of glucose analog uptake compared with CAR T cells from CR patients, confirming the gene signature analysis (26). Altogether, acquired T-cell dysfunction that evolves in certain cancer settings, such as CLL, could be a factor in limiting response following CAR T-cell therapy. Elucidating the mechanistic basis of the underlying metabolic impairments is vital for establishing effective treatments.

Mitochondrial Biomass Has a Key Role in Determining CAR T-cell Fate

Mitochondrial biomass also has a key role in the outcome of CAR T-cell therapy. For instance, in patients with CLL who have a CR, $CD8^+$ CAR T cells have increased mitochondrial mass compared with the same cells in NR patients (**Fig. 1**; ref. 25). Mitochondrial biomass in CAR T cells *in vitro* is also positively correlated with the expansion and persistence of CAR T cells *in vivo* (25). Therefore, it may be possible to achieve more durable clinical responses following CAR T-cell therapy by enhancing mitochondrial biogenesis during CAR T-cell production. Mitochondrial biomass can, therefore, be used as a biomarker of metabolic fitness of CAR T cells (25). Loss of mitochondrial mass in CAR T cells and TILs may stem from constitutive activation of Akt, resulting in progressively decreased expression of PGC1α, which augments mitochondrial biogenesis and FAO (10). In addition, overexpression of PGC1α can lead to an increase in *in vitro* metabolic competence of CAR T cells without opposing effects on their killing capacity or other functional qualities, and it may be possible to harness this knowledge to boost the metabolic capability of CAR T cells (27). Like CLL, decreased rates of mitochondrial biogenesis might be one of the reasons why the therapeutic efficacy of CAR T cells is generally poor in solid tumors. Modulation of mitochondrial biogenesis through manipulation of a wide range of upstream energy-sensing pathways, including AMPK and sirtuins, as well as through direct pharmacologic

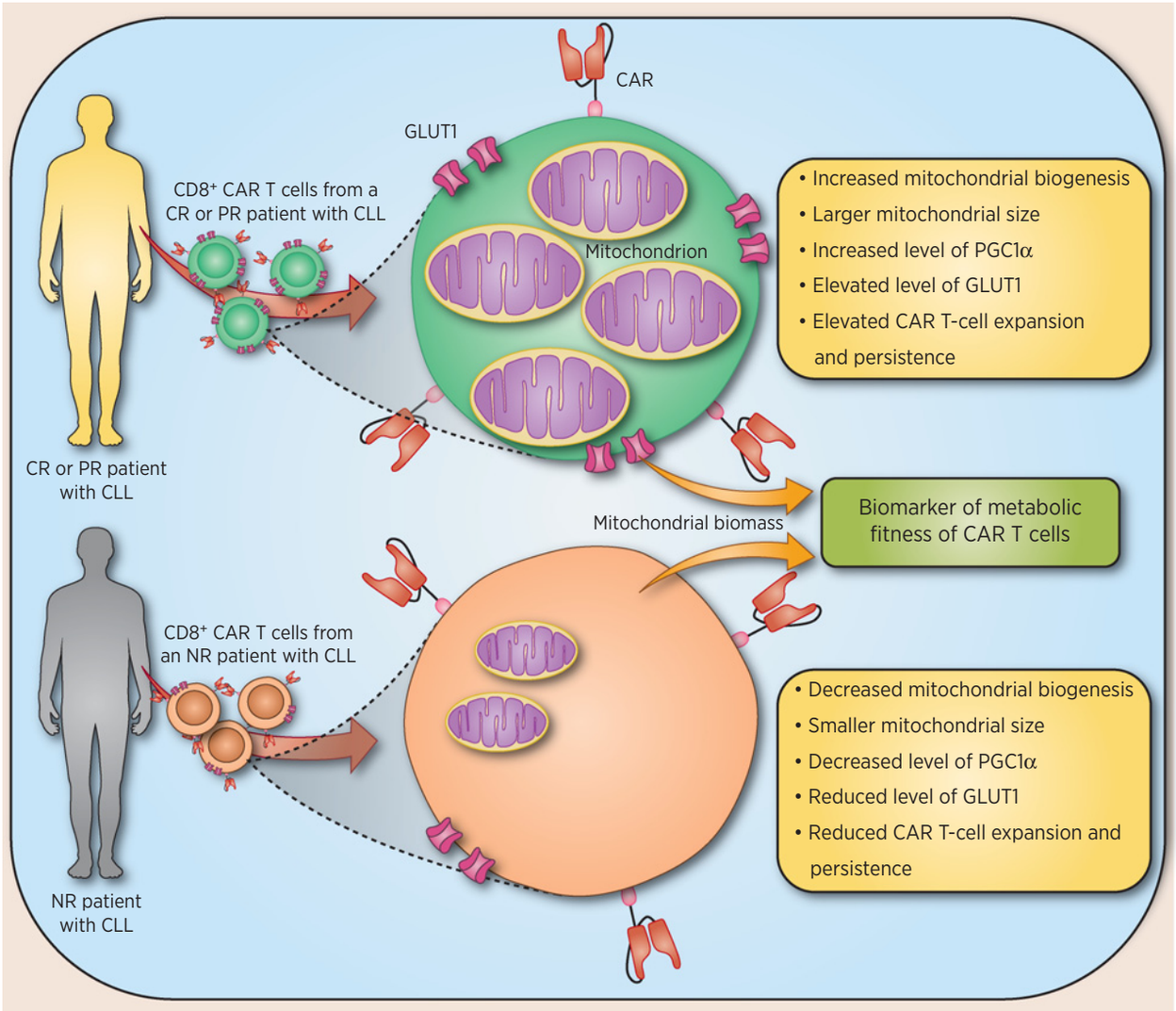

Figure 1.

Mitochondrial biogenesis in CAR T cells following infusion into patients with CLL. Comparison of mitochondrial size and features between $CD8^+$ CAR T cells from patients with CLL who have a CR, PR, or NR, as identified in a previous research article (25). Mitochondrial size is considerably larger in patients who have a CR versus NR. This enhanced mitochondrial size is related to some CAR T-cell persistence markers such as CAR T-cell expansion. Mitochondrial biomass is linked to mitochondrial biogenesis, as T cells have been activated during CAR T-cell manufacture and it is hypothesized that it can be used as a biomarker of metabolic fitness of CAR T cells.

activation of downstream effectors, including PPARs (28), may be a promising approach for generating more proliferative and persistent CAR T cells in the TME. Together, these findings support the hypothesis that enhanced mitochondrial biogenesis and fitness improves the antitumor function of T cells and the idea that mitochondrial modulation is a rational approach for developing novel and more effective immunotherapeutic strategies against cancer.

Modulation of CAR T-cell Mitochondria by Cell Signaling

4-1BB signaling

Optimal CAR T-cell function, persistence, and antitumor activity requires activation signals from CD3 and costimulatory molecules. To provide the costimulatory signals, intracellular signaling domains from one or more T-cell costimulatory molecules, such as CD28 or 4-1BB, are integrated into the CAR. Low mitochondrial mass, high glucose metabolism, and limited T-cell persistence are some of the characteristics of CAR T cells with an integrated CD28 signaling domain (29, 30). In contrast, CARs that include 4-1BB signaling domains display enhanced catabolic activity, FAO, and improved mitochondrial biogenesis, which supports heightened T-cell persistence and maintenance of a $CD45RO^+CCR7^+$ T_{CM} cell phenotype (30). 4-1BB–based CARs have a higher mitochondrial mass and greater antigen-dependent proliferative and survival potential *ex vivo* (29, 30). It has been suggested that the increased persistence of such CAR T cells could be due to reserved bioenergetic potential (also known as SRC) accompanied by the capability to meet the

metabolic demand of activated T cells (29). As shown by Menk and colleagues (31), stimulating the p38–MAPK pathway via 4-1BB agonistic antibody administration triggers PGC1α-dependent mitochondrial fusion and biogenesis in T cells, significantly increasing SRC. 4-1BB ligation also increases T-cell metabolic capacity *in vivo*, improving intratumoral metabolic sufficiency and enhancing responses generated with adoptive T-cell therapy and anti-PD1 immune checkpoint inhibitor (ICI) therapy in mice. In this study, pretreatment with 4-1BB agonists provided metabolic support for a complete anti-PD1 response, implying that 4-1BB agonists could be used in combination with ICIs to improve the efficiency of cancer immunotherapy (31).

Notch signaling

T_{SCM} cells represent a less differentiated and less abundant subset of T_{MEM} cells than T_{CM} and effector memory (T_{EM}) cells. They are characterized by high homeostatic self-renewal, enhanced proliferative capacity, and a multipotent developmental potential. T_{SCM} cells can undergo lineage specific differentiation to produce more differentiated T_{MEM}-cell subsets, such as T_{CM} and T_{EM} (32). T_{SCM} cells are an attractive option for CAR T-cell therapy, as they provide a long-term antitumor response (26, 33). Induced T_{SCM}-like (iT_{SCM}) cells can be generated from activated or more differentiated T_{MEM} cells by co-culturing the T_{MEM} cells with NOTCH ligand–expressing feeder cells, and similar to T_{SCM}, iT_{SCM} exhibit favorable therapeutic properties, including higher proliferative capacity, superior longevity, and more potent antitumor function as compared with other T-cell subsets (34). Activation and effector functions of $CD4^+$ and $CD8^+$ T cells depend on the NOTCH receptor family, and NOTCH signaling is needed for optimal T cell–mediated antitumor immunity (35). Mitochondrial quantities, PGC1A expression, ATP content, and the NADH/NAD ratio are all higher in iT_{SCM} cells generated by NOTCH signaling pathway induction than in T_N, T_{EM}, and T_{CM} cells. Also, iT_{SCM}s higher proliferative capacity relies on OXPHOS rather than glycolysis (36). NOTCH and its downstream gene, forkhead box M1 (FOXM1), assist mitochondrial biogenesis, fatty acid synthesis, and OXPHOS, leading to iT_{SCM} formation. Overexpressing FOXM1 in CAR T cells can induce CAR-iT_{SCM}s that have superior antitumor effects in comparison with conventional CAR T cells *in vivo* (36).

PI3K signaling

The PI3K–Akt–mTOR pathway is induced by T-cell activation. Activation of this pathway reprograms the metabolism of T cells toward glycolysis. Blocking the PI3K–Akt–mTOR pathway improves CAR T-cell lifespan and T_{MEM} generation in some studies (4). Treating CAR T cells with a PI3K inhibitor (PI3Ki) during *ex vivo* expansion preserves less differentiated T_N, T_{SCM}, and T_{CM} populations while decreasing accumulation of T_{EFF} populations, resulting in better *in vivo* persistence, function, and cytokine production (37). One explanation for these results is that the PI3Ki decreases the negative effects of antigen-independent tonic signaling of CARs (37). Tonic signaling, which is linked to spontaneous clustering of CAR molecules, decreases the lifespan and function of CAR T cells (38). Activation of tonic signaling stimulates PI3K–Akt signaling, which could influence glucose metabolism and promote differentiation toward an effector phenotype (37). However, establishing whether PI3K inhibition changes the fate of CAR T cells by constraining tonic signaling needs further research.

Treating T cells from patients with CLL with a PI3Ki during the CAR T-cell manufacturing process, increases the population of T_{SCM} and T_{CM} CAR T cells. Gene expression profiles of CAR T cells are also altered by PI3Ki treatment. Genes encoding proteins involved in the glycolysis pathway and glucose transporters are downregulated in PI3Ki-treated CAR T cells, whereas genes encoding glutamine transporters are upregulated. In addition, PGC1α protein expression is enhanced, and this is related to increased mitochondrial mass. There is also an increase in mitochondrial volume, as assessed using transmission electron microscopy. PI3Ki-treated CAR T cells show higher *in vivo* expansion, more rapid CLL elimination, and improved mouse survival in human xenograft models of CLL (39). Therefore, blocking the PI3K signaling pathway can be a helpful strategy to manipulate mitochondria, leading to long-lived CAR T cells with high antitumor function.

Modulation of CAR T-cell Mitochondria by Cytokines

Many of the key regulators of T-cell metabolism are cytokines (40). IL2 is the most common cytokine utilized for the outgrowth of CAR T cells administered to patients (41, 42). Cytokines, such as IL2, and ligation of costimulatory molecules with their cognate ligands stimulate the switch to glycolysis by increasing Glut1 expression and stimulating the key metabolic regulator mTOR (40, 43). Chronic stimulation of T cells with IL2 during *ex vivo* expansion can, therefore, result in T-cell exhaustion and reduced T-cell longevity (44). Treatment of T cells and CAR T cells with IL15 results in an enhanced oxygen consumption rate and SRC through stimulation of mitochondrial biogenesis and expression of CPT1A and FAO, which confer a metabolic advantage for survival and memory formation. These features are observed for both CD28- and 4-1BB–based CAR T cells (14, 45).

Treating CAR T cells with IL15 leads to decreased activity of mTORC1, a glycolysis regulator, and increased frequencies of cells with a T_{SCM} phenotype. Transferring IL15-expanded CAR T cells into mice harboring tumor cells results in the cells having a less exhausted phenotype, fewer apoptotic characteristics, less active caspase-3, enhanced proliferative potential, and greater antitumor activity. These CAR T cells have lower expression of glycolytic enzymes and GLUT1, but higher expression of enzymes associated with FAO compared with CAR T cells expanded in IL2 (45).

Coexpression of a second-generation anti-CD19 CAR with a membrane-tethered chimeric IL15 improves T-cell persistence independent of CAR signaling, without obvious cell-autonomous growth or transformation, and yields a potent antileukemic effect. These long-lived CAR T cells are phenotypically similar to T_{SCM} cells and have a memory-like transcriptional signature (46). These effects could be due to mitochondrial metabolic reprogramming as other research shows that membrane-bound IL15 can increase mitochondrial biomass in CAR T cells (29). In summary, these data indicate that IL15 may outperform IL2 in CAR T-cell manufacturing and become a seminal component of the *ex vivo* culture of CAR T cells.

The IL7 and IL7R signaling axis is required for the formation and homeostasis of T_{MEM} cells (47, 48). IL7 and IL15 treatment of T cells promotes a shift in lipid metabolism toward FAO, favoring a T_{MEM} cell phenotype (14, 49). IL7 promotes memory $CD8^+$ T-cell survival by increasing triglyceride synthesis and storage. Triglycerides are tri-esters made up of three fatty acid molecules bound to a glycerol (49). IL15 increases hydrolysis of stored triglycerides in T_{CM} cells with the enzyme lysosomal acid lipase to support FAO (50). A possible "store-and-burn" model has been proposed, in which IL7 and IL15 work together to trigger both triglyceride synthesis and lipolysis in memory $CD8^+$ T cells to maintain lipid supplies and FAO (49). However,

further *in vivo* research is required to better elucidate the role of IL7 and IL15 signaling in triglyceride metabolism by memory $CD8^+$ T cells and the metabolic regulation of memory $CD8^+$ T-cell survival and self-renewal (50). CAR T cells treated with IL7 and IL15 have a more T_N, T_{SCM}, and T_{CM} phenotype with superior proliferation capacity in comparison with IL2-expanded CAR T cells (33, 51). In addition, these cells have better *in vivo* persistence, which can provide greater antitumor immunity (33, 51). More studies are required to further clarify the relationship between IL7 and CAR T-cell metabolism.

Modulation of CAR T-cell Mitochondria by ICIs

Signaling from immune checkpoints such as PD1 suppresses immune activation of CAR T cells and there seems to be a close link between immune checkpoint pathways and cellular metabolism (52), although this interaction appears to be complex. For instance, PD1 signaling in $CD8^+$ T cells results in impaired functional and structural integrity in mitochondria, including a reduction in the number and length of mitochondrial cristae (53). Conversely, other studies show that $CD8^+$ T-cell metabolism is reprogrammed by PD1 signals for efficient use of FAO-dependent mitochondrial OXPHOS, which is partially similar to the metabolism of long-lived T_{MEM} cells. These latter data suggest a mechanistic explanation for the improved survival of TILs in a metabolically deficient TME (52, 53). Because PD1 signaling can inhibit glycolytic activity yet promote OXPHOS (52), its activation may increase mitochondrial ROS synthesis and mitochondrial damage by restraining glucose consumption in the hypoxic TME (18). Indeed, increased NFAT transcription factor activity in response to mitochondrial ROS is associated with T-cell exhaustion (21). Overexpression of catalase, which metabolizes H_2O_2, in CAR T cells, to counteract elevated levels of ROS, yields CAR T cells that exhibit less oxidative stress after activation and have greater cytotoxic function under high level of H_2O_2 *in vitro*. Furthermore, these cells protect nearby T and natural killer cells from oxidative stress–dependent inhibition (54).

T-cell metabolism can be directly affected by ICI therapy. For example, mitochondrial activities can be increased by blocking PD1 signaling (55). Treatment of tumor-bearing mice with anti-PD1 combined with the pan-PPAR–PGC1α agonist, bezafibrate, increases mitochondrial biogenesis and OXPHOS in $CD8^+$ T cells isolated from tumor-draining lymph nodes. This correlates with increased abundance and antitumor activity of T cells with memory characteristics (56). Other studies show anti-PD1 treatment can enhance CAR T-cell activity, survival, and even restore the effector function of exhausted CAR T cells after persistent antigen stimulation with PD-L1–positive tumor cells (19, 57). Therefore, combining drug therapies inducing mitochondrial biogenesis with ICIs and CAR T cells, are intriguing approaches that could lead to enhanced therapeutic results. With the help of combination therapies, we may be able to improve the function and lifespan of CAR T cells *in vivo*, although more work is needed to test this hypothesis.

Concluding Remarks

Promising outcomes with CAR T-cell therapy are generally associated with high levels of homing of CAR T cells to cancerous tissue and their long-term persistence. Accordingly, some cell types with the ability to survive for long periods of time, such as certain T_{MEM} cell subsets, exhibit the highest therapeutic potential. Thus, developing strategies that promote the generation and preservation of such cells may provide invaluable therapeutic opportunities for treating cancer using CAR T-cell therapy. Over the last two decades, studies have shed light on the role of metabolic pathways and activation signaling cascades in regulating immune-cell differentiation, proliferation, survival, and function. In this review, we have discussed how mitochondrial dynamics can control T-cell and CAR T-cell fate and function and how we can target metabolism of mitochondria to achieve a better response in immunotherapy. However, further study is required to comprehend the detailed regulation and contribution of mitochondrial dynamics to T cell and CAR T cells. Understanding these underlying immunometabolic forces will provide the essence for developing novel immunotherapies that can selectively adjust the immune responses of dysregulated immune cells in cancer.

Authors' Disclosures

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