Fatty acid metabolism in CD8$^+$ T cell memory: challenging current concepts

Brenda Raud[1], Peter J. McGuire[2], Russell G. Jones[3], Tim Sparwasser[1], Luciana Berod[1]

[1]Institute of Infection Immunology, TWINCORE, Centre for Experimental and Clinical Infection Research, a joint venture between the Medical School Hannover (MHH) and the Helmholtz Centre for Infection Research (HZI), Hannover, Niedersachsen, Germany.

[2]Metabolism, Infection, and Immunity Section, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD, USA.

[3]Goodman Cancer Research Centre, Department of Physiology, McGill University, Montreal, QC, Canada.

Correspondence to:

Luciana Berod

Institute of Infection Immunology

TWINCORE - Centre for Experimental and Clinical Infection Research

Feodor-Lynen-Str. 7-9

30625 Hannover

Tel. (0511) 220027-201

Fax (0511) 220027-203

luciana.berod@twincore.de

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$CD8^+$ T cells are key members of the adaptive immune response against infections and cancer. As we discuss in this review, these cells can present diverse metabolic requirements, which have been intensely studied during the past few years. Our current understanding suggests that aerobic glycolysis is a hallmark of activated $CD8^+$ T cells, while naïve and memory (T_{mem}) cells often rely on oxidative phosphorylation, and thus mitochondrial metabolism is a crucial determinant of $CD8^+$ T_{mem} cell development. Moreover, it has been proposed that $CD8^+$ T_{mem} cells have a specific requirement for the oxidation of long-chain fatty acids (LC-FAO), a process modulated in lymphocytes by the enzyme CPT1A. However, this notion relies heavily on the metabolic analysis of *in vitro* cultures and on chemical inhibition of CPT1A. Therefore, we introduce more recent studies using genetic models to demonstrate that CPT1A-mediated LC-FAO is dispensable for the development of $CD8^+$ T cell memory and protective immunity, and question the use of chemical inhibitors to target this enzyme. We discuss insights obtained from those and other studies analysing the metabolic characteristics of $CD8^+$ T_{mem} cells, and emphasise how T cells exhibit flexibility in their choice of metabolic fuel.

Keywords: fatty acid oxidation, memory, CPT, etomoxir, T cells, carnitine palmitoyltransferase.

1. INTRODUCTION

Adaptive immunity and immunological memory mediated by $CD8^+$ T lymphocytes are a key part of the protective response against intracellular pathogens such as viruses and intracellular bacteria. A typical immune response against an infection is initiated by the activation of antigen-specific T cells which rapidly proliferate to generate the necessary number of effector cells required for pathogen clearance. A contraction phase follows the resolution of the infection, leaving a population of memory T cells that persist for long periods of time without antigen stimulation. During these stages, T cells are not only characterized by defined immunological properties, but also by specific metabolic programs that support their biosynthetic and energetic needs. While naïve $CD8^+$ T cells (T_n) have relatively low energetic requirements, effector T cells (T_{eff}) present an increased demand for energy and biosynthetic precursors to support proliferation and effector function. Furthermore, memory $CD8^+$ T (T_{mem}) cells need to survive for long periods of time while sustaining homeostatic proliferation and the capacity to rapidly respond to secondary antigenic stimulation. The adoption of a particular metabolic program is coordinated by the expression and regulation of a network of enzymes and transcription factors that are linked to internal and external cues such as receptor and cytokine signalling or nutrient availability, and can be a determinant factor in the success of the immune response.

The study of these metabolic features in immune cells is the basis of the rapidly evolving field of immunometabolism, which has benefited from the development and widespread use of modern techniques including metabolomics and real time extracellular flux analysers that facilitate the characterization and quantification of the metabolic changes affecting immune cells under experimentally defined conditions. One of the main questions in the field of immunometabolism concerns the exact nature of the relationship between metabolism and immune function, and to which degree immune cells are (in)flexible in their choice of substrate to produce energy or synthesize macromolecules. The answers to this and other related questions can have far reaching implications for promoting adaptive immunity and immune memory formation following vaccination, and also for the treatment of cancer, against which cytotoxic $CD8^+$ T cell responses represent the main line of defence. In this context, this knowledge can prove invaluable for the rational design of treatments involving adoptive transfer therapies such as chimeric antigen receptor (CAR) T cells, by taking advantage of the right metabolic conditions to balance effector function and longevity of adoptive cells.

In this review, we will explore the latest advancements in the understanding of the metabolic requirements of $CD8^+$ T cells, with a particular focus on the metabolism of fatty acids. The increasing availability of mouse models targeting metabolic enzymes and transcription factors has provided new tools to reliably establish the role of metabolic checkpoints in the function of immune cells *in vivo,* complementing - and sometimes challenging - the knowledge previously obtained from the use of chemical inhibitors. We will critically examine the work that has led to the current understanding of $CD8^+$ T_{mem} cell metabolism, and discuss recent studies that describe how cellular metabolism correlates with or influences the establishment of immune memory, while emphasizing the functional and metabolic diversity found among different types of memory cells and their potential for therapy and human medicine.

2. $CD8^+$ T CELL IMMUNITY

The development of the immune response mediated by $CD8^+$ T cells can be characterized by three main stages (1, 2). During the initial phase, naïve T cells that circulate through secondary lymphoid organs are met and activated by antigen presenting cells (APCs) displaying antigens from a pathogen in the context of MHC molecules, and this is accompanied by a remarkable increase in cell size and metabolic activity. An extensive clonal expansion follows, with cell divisions occurring as often as every 4 hours (1), generating cells that differentiate into cytotoxic T lymphocytes (CTLs). CTLs migrate to the sites of infection to fight the pathogen through production and targeted release of granzymes and perforins to infected cells, and the secretion of cytokines such as TNF or IFN-γ that stimulate the immune system and activate macrophages to phagocytose infected and dying cells. At the peak of the response, most cells exhibit an activated phenotype characterized by the production of cytokines, high KLRG1 expression (as a marker of terminal effector differentiation) and a short lifespan. Once the pathogen is cleared, TCR and cytokine stimulation are interrupted and most activated cells die by apoptosis, resulting in a massive reduction in their number. Only a small percentage (about 5-10%) persist as long-lived memory cells (1, 3), with the potential to quickly proliferate in response to a new infection by the same pathogen.

The population of $CD8^+$ T_{mem} cells that survive after the initial immune response is heterogeneous, consisting of different types of cells that differ in their function, longevity and location (4, 5). Long lived "central" memory T cells (T_{CM}) present increased expression of the IL-7 receptor alpha (IL7ra also known as CD127) (6), the lymph-node homing selectin CD62L, the chemokine receptor CCR7, and have reduced immediate production of effector cytokines such as IFN-γ. The longevity of the T_{CM} cell population is partly due to the expression of the antiapoptotic protein Bcl-2, telomerase activity and sustained homeostatic proliferation (7). Importantly, these cells have the capacity to produce IL-2 and robustly proliferate upon secondary activation. In contrast, effector memory $CD8^+$ T cells (T_{EM}), comprising another population that also survives the primary immune response, exhibit low expression of CD62L and CCR7, high expression of KLRG1, and display effector activity (e.g. cytotoxic activity and the production of cytokines). T_{EM} are usually present in non-lymphoid tissues and, unlike T_{CM}, have a low proliferative potential upon secondary activation (7, 8). More recently, a third group of $CD8^+$ T_{mem} cells located in the epithelial barrier, as well as in mucosal and adipose tissues, has been described. These tissue resident memory cells (T_{RM}) can respond to pathogens quickly and independently of cells recruited from the circulation, present a characteristic $CCR7^{low}CD69^{hi}CD27^{low}$ phenotype and settle in tissues due to their expression of tissue-homing chemokine receptors and adhesion molecules (9-11). This classification of $CD8^+$ T_{mem} cell subsets is not absolute, and there are cells that are found after the resolution of the immune response that do not belong to any group. Rather, differentiation of cells after infection results in a spectrum of phenotypic characteristics, from cells with greater longevity and memory potential, to short-lived effector cells that are terminally differentiated - with variants in between (4).

The cues that guide development of $CD8^+$ T_{mem} cells are not completely understood but models of T cell diversification indicate that the potential of activated cells to give rise to $CD8^+$ T_{mem} populations is most probably not pre-determined in naïve cells, but it is likely to result from a combination of intrinsic and extrinsic signals that respond to the time, the intensity and the specific conditions during the activation of a $CD8^+$ T cell (2, 4). Cytokines such as IL-15 and IL-7, which provide indispensable survival signals to naïve T cells (12-14), also promote the survival of $CD8^+$ T_{mem} cells. On the one hand, expression of the IL-7 receptor subunit CD127 correlates with survival and expression of Bcl-2 (12-15). On the

other hand, IL-15 favours $CD8^+$ T_{mem} homeostatic proliferation and mice lacking IL-15 or the IL-15 receptor alpha chain (IL-15rα) develop fewer polyclonal $CD8^+$ T_{mem} cells (16, 17). In the absence of both cytokines, essentially no $CD8^+$ T_{mem} cells are formed after an infection (1). As we will discuss in this review, it has been lately proposed that these cytokines can influence the development of the different memory subsets by promoting specific metabolic changes, particularly during the transition between the peak of the effector phase and the establishment a of long-lived memory population. The mechanisms behind this metabolic modulation have not been fully characterized yet, and are thus a subject of intense study.

3. METABOLIC CHANGES DEFINING $CD8^+$ T CELL IMMUNITY

3.1. Naïve T cell metabolism

The metabolic demands of T_n cells are relatively low and focused on maintaining homeostatic proliferation and supporting cellular migration (18-20). A preferred source of energy for T_n cells is glucose that through glycolysis is first processed into pyruvate, which is transported into the mitochondria and decarboxylated into acetyl-CoA (Figure 1). Acetyl-CoA can then be incorporated into the tricarboxylic acid (TCA) cycle, where it is completely oxidized into CO_2. This process generates NADH and $FADH_2$, reduced molecules that transfer electrons to the mitochondrial electron transport chain (ETC), resulting in the synthesis of ATP and consumption of O_2 during oxidative phosphorylation (OxPhos). Through this pathway, cells can maximize the energy output of glucose, calculated as up to 36 molecules of ATP for each molecule of substrate, while maintaining cellular redox balance by regenerating the necessary NAD^+ and FAD through oxidation in the mitochondria (20). Tonic signals from IL-7 and the TCR are crucial for the survival of T_n cells partly by sustaining the uptake of glucose used in mitochondrial respiration. Therefore, cytokine withdrawal that results in T_n cell atrophy and death also causes a loss of mitochondrial membrane potential and cellular ATP even in the presence of high levels of extracellular nutrients (13, 18, 21, 22). Glucose is not the only nutrient used by T_n cells, which can also oxidize external lipids like oleate and palmitate in their mitochondria (23, 24). To this end, T_n cells express enzymes that support the oxidation of long-chain fatty acids (LCFAs) (25), and metabolomic analysis of these cells found abundant acylcarnitine molecules, which correlate with this process (24, 26).

3.2. Upregulation of aerobic glycolysis upon T cell activation

Signalling through the TCR receptor and co-stimulatory molecules (such as CD28 and cytokine receptors) in T cells leads to the activation of immunological pathways which are accompanied by a profound alteration in the cellular metabolism to support their proliferation and effector function (27-32). One crucial step taken by T cells to sustain this enhanced metabolism is to augment their uptake of glucose through the increased expression of members of the solute carrier family 2 (Slc2, also known as Glut). Glut1 (Slc2a1) was the first glucose transporter described to be upregulated by co-stimulatory signals from CD28 (30) and to be essential for the activation of $CD4^+$ T_{eff} cells (33). $CD8^+$ T cells also increase the expression of the transporter Glut3 and are thus less reliant on Glut1 for their activation (33, 34). Glucose is taken up and metabolized into two molecules of pyruvate through the process of glycolysis (Figure 1), which in addition yields 2 molecules of ATP and reduced NADH. Yet, in contrast to resting cells, most of the produced pyruvate and NADH in activated T cells is not oxidized in the mitochondria, but is reduced into lactate by the enzyme lactate dehydrogenase (Ldha) and excreted from the cell. This process is named aerobic glycolysis, or Warburg effect after the German Nobel laureate Otto Warburg who described this

behaviour in activated leukocytes 60 years ago (35), and has been extensively described in T cells ever since (28, 36-38). It should be pointed out, however, that activation of T cells does not lead to a complete switch from mitochondrial respiration towards aerobic glycolysis. Instead, OxPhos is upregulated but to a lesser extent than aerobic glycolysis. Indeed, mitochondrial oxidation plays an important role in T cell activation, as evidenced by the deleterious effects that inhibiting mitochondrial function has on T_{eff} differentiation (39-41).

Although inherently inefficient to produce ATP, aerobic glycolysis is required for T cells to engage in productive growth and cytokine production. Part of the pyruvate and glycolytic intermediaries are shuttled towards the synthesis of biomolecules, and the reduction of pyruvate to lactate allows the regeneration of oxidized NAD^+, indispensable to continue with the glycolytic process. Moreover, it has been described that, in the absence of substrate, the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) can repress the translation of IFN-γ (37). Therefore, an active glycolytic function, which would signal favourable nutrient availability, engages GAPDH and thus positively regulates the acquisition of an effector phenotype. This alternative function regulating gene expression (referred to as "moonlighting") is observed for several metabolic enzymes, and provides an intriguing mechanism by which cells can respond to specific metabolic conditions (31, 42). As we will describe, the glycolytic pathway is actively coordinated by several molecules that are of great importance for the successful activation and proliferation of T_{eff} cells.

3.3. Molecular regulation of metabolism during T cell activation

One of the most studied orchestrators of the cellular metabolism that characterizes activated T cells is the mechanistic (formerly *mammalian*) target of rapamycin (mTOR), a serine/threonine kinase that plays a key role in regulating cellular growth, proliferation and survival (reviewed in (43)). mTOR acts as the catalytic subunit of two complexes: mTOR complex 1 (mTORC1) and mTORC2, that have different cellular functions. mTORC1 is composed of three core components: mTOR, Raptor (regulatory protein associated with mTOR) and mLST8 (mammalian lethal with Sec13 protein 8) while mTORC2 contains mTOR, Rictor (rapamycin insensitive companion of mTOR) and mLST8.

Activation of mTORC1 follows TCR and CD28 stimulation through PI3K and Akt signalling (Figure 1) (44, 45). Activated Akt inhibits the key negative regulator of mTORC1 known as tuberous sclerosis complex (TSC) by phosphorylating one its members, TSC2. This prevents TSC from functioning as a GTPase-activating protein (GAP) for the RAS homolog enriched in brain (Rheb), which is a small GTPase with a crucial function in activating mTORC1. Therefore, deletion of TSC2 results in $CD8^+$ T cells that have constitutive activity of mTORC1, even in unstimulated cells (46). mTORC1 is particularly critical to $CD8^+$ T cell activation, demonstrated by the fact that TSC2-deficient cells present enhanced proliferation and improved production of cytokines, while inhibition of mTORC1 by deletion of Rheb reduces $CD8^+$ T cell activation and effector function. Similarly, inhibition of this complex with the bacterial compound rapamycin reduces T_{eff} cell differentiation (44, 46-51). Due to this effect, rapamycin has been approved as an immunosuppressive treatment for kidney transplant since 1999. Rapamycin affects mTORC1 activity acutely but does not bind to or inhibit mTORC2, although reduced mTORC2 signalling has been observed during prolonged treatment, presumably due to an exclusion of mTOR bound to rapamycin from integrating into the mTORC2 complex (43, 52).

The activation of mTORC1 strongly correlates with the increased expression of enzymes and substrate transporters (notably Glut1 and Glut3) mediating glycolytic and specific

biosynthetic pathways in $CD8^+$ T cells (34, 53). Mechanistically, the main targets of mTORC1 are the phosphorylation of p70S6 Kinase 1 (S6K1) and eIF4E binding protein (4EBP) that prevents assembly of the eIF4F complex, necessary for the initiation of translation in eukaryotic cells. Thus, mTORC1 promotes protein biosynthesis, including that of transcription factors such as HIF1α, which together with the transcription factor Myc are indispensable mediators of the metabolism of activated T cells (24, 34, 54, 55).

Expression of Myc (also known as c-Myc) in T cells is upregulated within 3 hours after TCR stimulation (24). Myc promotes the expression of Glut1, Glut3, hexokinase 2 (Hk2), and 6-phosphofructokinase (Pfkl) among others, which are all enzymes essential for glycolysis, as well for the pentose phosphate pathway (PPP) that mediates the synthesis of nucleotides and NADPH from glucose (24). Myc can also support glutamine transport and metabolism, critical for activated T cells, through inducing the expression of glutaminase 2 (Gls2) and the glutamine transporter CD98 (24). Compared to wild-type cells, Myc-deficient $CD8^+$ T cells present an impaired glycolytic flux and reduced accumulation of lipids, amino acids and nucleotides, thus supporting the idea that this transcription factor is crucial for early metabolic reprogramming upon T cell activation (56).

Hypoxia-inducible factor 1 (HIF1) is a transcription factor composed of the subunits HIF1α and HIF1β (the latter also known as aryl hydrocarbon receptor (AHR) nuclear translocator, ARNT). HIF1α is stabilized under hypoxic conditions and coordinates a switch from mitochondrial respiration to glycolysis to adapt to low oxygen availability (55, 57). HIF1α is not required for the initial activation of T cells (53) but is highly expressed in T_{eff} even in the presence of oxygen and upregulates aerobic glycolysis by promoting the transcription of the enzymes pyruvate dehydrogenase kinase 1 (Pdk1) and lactate dehydrogenase A (Ldha). Pdk1 inhibits the enzyme pyruvate dehydrogenase, thus preventing the transport of pyruvate into the mitochondria, and Ldha reduces pyruvate into lactate while regenerating NAD^+, therefore inducing the Warburg effect. Constitutive HIF1α activity increases glycolytic metabolism in $CD8^+$ T cells and improves their effector function (54).

3.4. Engagement of the glycolytic-lipogenic pathway during T cell activation

Activation of T cells also results in a considerable proportion of carbons obtained from glucose being shuttled towards *de novo* fatty acid synthesis (FAS). The upregulation of FAS is initiated by mTORC1 by inducing the activity of sterol responsive element binding proteins (SREBPs) (24, 34, 55, 58), a family of transcription factors that modulate the metabolism of fatty acids (FAs) and cholesterol and the flux through the oxidative arm of the PPP, which generates NADPH required for the synthesis of lipids from glucose (55, 59, 60). SREBP in T_{eff} cells induces the expression of the enzymes acetyl-CoA carboxylase (ACC), fatty acid synthetase (FASN) and the hydroxy-methyl-glutaryl-CoA reductase (HMGCR), which are rate limiting in the synthesis of FAs and cholesterol, respectively (61). Recent studies have highlighted the importance of FAS in T cell activation and have proposed that inhibiting this pathway could potentially be used to modulate the differentiation of T_{eff} cells (61-65).

De novo FAS is initiated with the carboxylation of acetyl-CoA to malonyl-CoA catalyzed by ACC1, located in the cytoplasm (Figure 1)(66, 67). After the synthesis of malonyl-CoA, the FAS reaction proceeds by progressive elongation of the acyl-chain by the FASN complex in a process that requires NADPH and usually results in the production of LCFAs such as palmitate which can be then elongated or desaturated to form more complex lipids. As the carboxylation of acetyl-CoA is the first committed step of *de novo* FAS, regulation of this reaction can determine the flux through the whole pathway. Therefore, the enzymatic activity

of ACC1 is modulated by the cell to favour FAS under the right metabolic conditions. ACC1 can be found as a phosphorylated, enzymatically inactive homodimer or as a dephosphorylated, active polymer. The active form is allosterically stimulated by citrate, indicating ample supply of precursors (as cytoplasmatic acetyl-CoA used by ACC1 is derived from citrate exported from the mitochondria), and constrained by palmitoyl-CoA (a product from FAS) as a feedback mechanism. In activated T cells, the endogenous synthesis of LCFAs and cholesterol relies heavily on acetyl-CoA obtained in the mitochondria after glycolysis which is exported to the cytoplasm and used to initiate lipid synthesis through a brief conjugation to mitochondrial oxaloacetate to form citrate (Figure 1). Endogenous FAS is necessary to sustain the expansion of activated T cells, as demonstrated by the impaired proliferation and effector response observed after genetic deletion of the SREBP cleavage-activating protein (SCAP) (61) or ACC1 (62, 63, 65, 68). This connection between the glycolytic and lipogenic pathways provides another reason why activated T cells and other cells that vigorously proliferate, such as tumour cells, depend to such degree on an upregulation of the glycolytic pathway, not only to produce ATP, but also to sustain anabolic reactions.

In summary, by promoting the translation and activity of transcription factors and other molecules involved in the glycolytic-lipogenic pathway, mTOR coordinates the metabolic profile required by activated T cells, namely one characterized by an increased uptake of glucose and glutamine that feeds the synthesis of biomolecules such as lipids or proteins to support proliferation and effector function in addition to generating ATP. This active modulation of the metabolism of T cells by transcription factors and kinases that are activated by specific immune signalling from CD28 or the TCR illustrate that the establishment of certain metabolic profiles plays an important role in the adaptive T cell response.

4. METABOLIC COORDINATION OF $CD8^+$ T_{MEM} DEVELOPMENT

4.1. Adaptation at the T_{eff} to T_{mem} transition

$CD8^+$ T_{mem} cells possess specific metabolic demands due to their role in the immune response, that requires them to persist for a long time through homeostatic proliferation and unlike naïve cells do so without TCR stimulus (2). The evidence so far suggests that upregulation of mitochondrial OxPhos is used to support ATP production and preserve redox balance, and is characteristic of at least the T_{CM} subset of $CD8^+$ T_{mem} cells (69, 70) Moreover, $CD8^+$ T_{mem} cells have the ability to swiftly and vigorously become activated upon re-stimulation, and to adopt an effector phenotype faster than naïve cells. This has been attributed to a metabolic advantage that allows them to quickly react to increased energy demands, usually quantified as an augmented spare respiratory capacity (SRC) (71). SRC is a metabolic readout experimentally measured by uncoupling oxygen consumption from ATP synthesis, simulating a sudden increment in ATP demands like found upon TCR stimulation. In other words, the SRC tests the maximum capacity of the cell to rapidly oxidize substrates in the mitochondria to meet increased demand. Accordingly, it was described that $CD8^+$ T_{mem} cells obtained after infection have higher SRC and reduced extracellular acidification rate (ECAR, an indirect measurement of aerobic glycolysis) when compared to effector cells during the active immune response (69, 70).

Several studies have shown that mTORC1 activity is negatively related to the development of $CD8^+$ T cell memory (46, 47, 72-74). mTORC1 inhibition at the end of the effector phase

favours the switch from aerobic glycolysis, characteristic of T_{eff} cells, towards mitochondrial oxidation that supplies energy and protects cells from apoptosis after glucose or cytokine stimulation withdrawal (46, 74). *In vivo*, reduced mTORC1 activity thus allows the generation of T cells that are better suited to survive the conclusion of the primary immune response, which is marked by a decrease of cytokine (e.g. IL-2) and TCR survival signals including a reduction of glucose and amino acid uptake (46). Accordingly, uncurbed activation of mTORC1 due to TSC2 deletion precludes the generation of a memory population after the primary response, while promoting the development of terminally differentiated effector cells with high expression of KLRG1 and the transcription factor T-bet, which directs differentiation into a CTL phenotype (46). This defect can be reverted by rapamycin treatment during the contraction phase, demonstrating that a downregulation of mTORC1 activity after the peak of the immune response improves the survival of antigen specific cells (46, 72, 75). Nevertheless, mTORC1 is also necessary for the reactivation of $CD8^+$ T_{mem} cells. Deletion of the mTORC1 activator Rheb favours the survival after viral infection of antigen specific T cells, which however fail to respond to re-stimulation and thus would not provide protective immunity (46).

Examination of asymmetric cell division during $CD8^+$ T cell activation has provided more insights into the relationship between mTORC1 activity and memory development. Asymmetric division has been proposed to occur due to an uneven distribution of cytoplasmic components after the T cell division that follows the formation of the immune synapse between an APC and a $CD8^+$ T cell. This can lead to a daughter cell proximal to the APC which is more prone to differentiate into a $CD8^+$ T_{eff} cell, and a daughter cell distal to the APC with higher potential to develop into a $CD8^+$ T_{mem} cell (76). Uneven distribution of mTORC1 activity between daughter cells after this first division was proposed to determine their effector *versus* memory fate (77), with higher mTORC1 activity in the proximal cell that also presents a more glycolytic metabolism, along with enhanced expression of the Myc, Glut1 and the amino acid transporter CD98 (77, 78).

In contrast to mTORC1, mTORC2 appears to be dispensable for the generation of $CD8^+$ T_{eff} cells, but can interfere with the development of memory by phosphorylating and inhibiting the nuclear translocation of the transcription factor Foxo1 (46, 79). Foxo1 promotes the expression of CD127, CD62L, SIP_1 and CCR7 and thus mediates the survival and migration of T_{mem} cells (73, 80-82). Hence, $CD8^+$ T cells with a deletion of the mTORC2 component Rictor have higher expression of T_{mem} surface markers after primary infection *in vivo* (46, 79). Moreover, mTORC2 was found to influence T_{mem} metabolism and inhibit certain catabolic pathways. Because mTORC2 deletion does not impair $CD8^+$ T cell effector function, selective inhibition of mTORC2 could be a strategy to improve $CD8^+$ memory generation, without compromising the primary immune response (79).

4.2. AMPK controls metabolic adaptation

The studies mentioned above propose that, in contrast to their role in T cell activation, mTORC1 and mTORC2 have a negative influence over the transition from the effector to the memory phase of the immune response, through mechanisms that involve immune signalling and metabolic adaptation. Yet, how $CD8^+$ T cells can actively coordinate mTOR activity and thus memory development at the molecular level is still not fully understood. One important negative regulator of mTORC1 that has been associated with T_{mem} cell development is the AMP-activated protein kinase (AMPK) (Figure 1) (recently reviewed in (83, 84)). AMPK is a heterotrimeric serine/threonine protein kinase composed of one catalytic α subunit, and regulatory β and γ subunits that is typically activated under metabolic stress (translated in

high AMP and ADP concentrations) and modulates cellular metabolism by inhibiting anabolic and promoting catabolic metabolism (85-89). Phosphorylation of the Thr172 in the AMPKα subunit by the kinase LKB1, together with the binding of ADP or AMP to the nucleotide-binding sites of the γ subunit activates AMPK in response to metabolic stress in T cells (90-92). Deletion in T cells of AMPKα1 (the catalytic subunit of AMPK found in lymphocytes) does not prevent the development or homeostasis of naïve cells, but results in an increased basal mTORC1 activity, supporting the notion that AMPK antagonizes mTORC1 (90, 93-95). AMPK can restrain mTORC1 function through phosphorylation and activation of the negative regulator TSC2 (96) as well as the mTORC1 member Raptor (97), thus reducing protein translation and lipid synthesis and therefore energy expenditure. Moreover, AMPK inhibits *de novo* FAS by directly phosphorylating ACC1, and can also promote long chain fatty acid oxidation (LC-FAO) through phosphorylation of ACC2, an isoform of ACC present in the outer mitochondrial membrane (67, 98, 99). In addition, AMPK phosphorylates the kinase ULK1 (100), and hence stimulates the degradation of cellular components to provide nutrients by initiating autophagy, which is important for the development $CD8^+$ T_{mem} cells *in vivo* (101), and promotes mitochondrial biogenesis through the activity of PPARγ coactivator-1α (PGC1α) (84, 102, 103).

To study the role of AMPK on $CD8^+$ T_{mem} differentiation, Cantrell and colleagues evaluated the immune response to an infection with *Listeria monocytogenes* expressing ovalbumin (LmOVA) in mice with a T cell-specific deletion of AMPKα1. The authors observed a normal proliferation of OVA-specific cells during the primary response, but a reduction in their number after secondary challenge (95). This suggests a relevant role of AMPK in the survival of memory cells *in vivo,* although it is not clear if T cells actively modulate the function of this enzyme to favour $CD8^+$ T_{mem} survival. Because of the established function of this kinase in modulating cellular metabolism, as well as its ability to induce LC-FAO in several tissues (104, 105), AMPK has been proposed to function as a key metabolic switch in T_{mem} cells by promoting the oxidation of LCFAs (32, 83, 106-108). However, more research is needed to clearly establish if induction of AMPK activity can be used to improve memory development *in vivo*.

5. THE ROLE OF LC-FAO IN $CD8^+$ T_{MEM} CELLS

5.1. TRAF6-mediated signalling in $CD8^+$ T_{mem} development

The first data linking LC-FAO to $CD8^+$ T_{mem} cell development came from a pioneering study by Erika Pearce using mice with a T cell-specific deletion of the TNF-associated factor 6 (TRAF6)(106). TRAF6 is an adapter protein that is involved in mediating several protein-protein interactions and was originally identified as a mediator of interleukin-1 receptor-dependent activation of NF-□B (109). TRAF6 expression is broad and important in several tissues, as witnessed by the fact that TRAF6 complete knockout mice do not survive more than 2 weeks after birth (110). Studies using bone marrow chimeras showed that TRAF6 participates in many signalling pathways in immune cells, including those from members of the TNFR superfamily, tumour-growth factor-β receptors (TGFβR) and the TCR (109). T cells strongly upregulate TRAF6 expression after TCR engagement and mice with a deletion of TRAF6 specifically in T cells generate $CD4^+$ and $CD8^+$ naïve T cells with hypersensitivity to TCR stimulation and enhanced PI3K-Akt activation (111). Moreover, these mice have a progressive autoimmune disease and by 10-12 weeks of age present splenomegaly and lymphadenopathy caused by hyperactivation and proliferation of B cells and activated $CD4^+$ T cells with reduced susceptibility to suppression by $CD4^+CD25^+$ regulatory T (T_{reg}) cells.

Additionally, T cell-specific TRAF6-deficient mice exhibit excessive cytokine production that suggests an abnormal T helper type 2 effector response (109, 111). In contrast, naïve $CD8^+$ T cells have defects in their homeostatic or lymphopenia-induced proliferation and thus naïve T cell-specific TRAF6-deficient mice have reduced numbers of $CD8^+$ T cells in the periphery (106, 111, 112). Despite this strong immune dysregulation that resembles a delayed *scurfy* autoimmune phenotype, it was reported that T cell-specific TRAF6-deficient mice can mount a normal primary immune response against an attenuated strain of LmOVA, but had a reduced $CD8^+$ T_{mem} population 60 days after infection (106). However, in this model, an influence of the hyperactive $CD4^+$ T cell compartment on the development of $CD8^+$ T_{mem} cells cannot be excluded.

To assess the intrinsic effects of TRAF6-deficiency in $CD8^+$ T cells without the influence of the $CD4^+$ T cell-driven autoimmunity, Pearce and colleagues then transferred TRAF6-deficient OT-I cells into a congenic host and followed their fate during infection with LmOVA. They observed that these T cells could be activated normally and proliferate during the primary infectious challenge but failed to further proliferate and survive upon re-infection (106). Moreover, at the end of the primary response, $CD8^+$ T cells displayed increased KLRG1 expression, a marker of terminally differentiated T_{eff} cells, suggesting that TRAF6-deficient T cells had an intrinsic defect in adopting a memory phenotype. Surprisingly, despite TRAF6 modulating various signalling pathways, the authors identified specific genes related to FA metabolism differentially regulated between TRAF6-deficient and WT cells 10 days after infection, coinciding with the transition between the primary response and memory development. *In vitro*, WT OT-I activated cells subjected to IL-2 withdrawal increased the oxidation of palmitate, but TRAF6-deficient cells did not. It was interpreted from these data that the transition of $CD8^+$ T cells from T_{eff} to T_{mem} state was dependent on a shift to catabolic metabolism, mediated by TRAF6 and LC-FAO, to promote survival in the face of diminishing growth signals and glucose metabolism.

To date it is still unclear how TRAF6 regulates LC-FAO in $CD8^+$ T cells (109). Pearce *et al.* proposed that this effect was mediated by AMPK because, in contrast to WT cells, TRAF6-deficient OT-I T cells transferred *in vivo* presented lower AMPK activation at the peak of the primary response (106). *In vitro,* TRAF6-deficient cells failed to activate AMPK in response to IL-2 withdrawal, but treatment with the biguanidine anti-diabetic drug metformin restored their AMPK activity and enhanced their oxidation of palmitate (as it is the case for other tissues (113)) while improving T_{mem} survival *in vivo*. However, metformin activates AMPK indirectly through the induction of metabolic stress by inhibiting complex I of the ETC, and thus mitochondrial respiration (113-115). Metformin can also reduce mTORC1 signalling in T cells independently of AMPK (115). Accordingly, treatment with rapamycin after the effector phase could rescue T_{mem} survival, suggesting that TRAF6 deletion acted upstream of mTORC1. Therefore, metformin may exert FAO- and AMPK-independent effects on cellular metabolism to affect T_{mem} cell development.

5.2. Control of LC-FAO

Mitochondrial fatty acid oxidation (FAO) is the process through which acyl molecules are degraded by sequential removal of 2-carbon fragments inside the mitochondrial matrix. As this process is initiated by the oxidation of the □-carbon atom of the acyl molecule it is therefore referred to as fatty acid □-oxidation. To be oxidized, FAs first need to enter the mitochondrial matrix. While short- and medium-chain FAs (SCFA and MCFA, chain length < C_{12}) do so freely, the transport of long-chain FAs (LCFA, chain length C_{14} to C_{18}) is regulated by the activity of the carnitine shuttle system that is composed by carnitine O-

palmitoyltransferases 1 and 2 (CPT1 and CPT2) and the carnitine-acylcarnitine translocase (CACT) (Figure 1) (116, 117). The first step of the transport is catalysed by CPT1, an enzyme that can be found in 3 isoforms in different tissues (117). CPT1A is the isoform found in liver, kidney, intestine, pancreas, and the only one described so far in primary T cells, according to publicly available data (*immgen.org*). CPT1B is expressed in heart, skeletal muscle and testis; and CPT1C, the most recently characterized, is found primarily in brain, but has also been reported in tumour cell lines (118-121). The reaction catalysed by CPT1 is rate-controlling for the oxidation of LCFAs and involves the conjugation of the long chain acyl group to carnitine. The resulting long chain acylcarnitines can then be transported through the mitochondrial inner membrane into the matrix by CACT and restored by CPT2 to acyl-CoAs, which can then be oxidized. The final products of FAO include acetyl-CoA which is combined with oxaloacetate to generate citrate that enters the mitochondrial TCA cycle, and reduced molecules that directly feed the ETC via the electron transport flavoprotein (ETF). The complete β-oxidation of one molecule of palmitate (C_{16}) theoretically yields 8 acetyl-CoA, 15 $FADH_2$, and 31 NADH molecules, producing a net of 129 ATP, which reinforces the energetic value of LCFAs.

Since CPT1 catalyses the rate-limiting step in the oxidation of LCFAs (but not of SCFAs or MCFAs), it is largely considered as the main target to regulate LC-FAO in response to intrinsic cues (such as energy availability) or extrinsic signals (insulin, receptor signalling and growth factor stimulation) (122-124). CPT1 can be inhibited by malonyl-CoA produced by ACC2, with whom CPT1 shares the location at the outer mitochondrial membrane (98, 99, 104). Activated AMPK can thus directly induce LC-FAO through phosphorylation and inhibition of ACC2, a mechanism known to be used by certain cell types, such as muscle or liver, to increase LC-FAO in response to fasting or increased energy demands. Accordingly, complete deletion of ACC2 in mice increases basal oxidation of LCFAs in muscle and reduces accumulation of fat (98, 99, 125), which can be attributed to increased CPT1 activity and transport of LCFAs into the mitochondria for oxidation. It is however not clear if T cells also employ this mechanism to regulate their rates of LC-FAO, given that ACC2 deletion in these cells has only very modest effects on FAO while not influencing their differentiation, effector function or memory development (25, 62, 65, 126). Instead, transcriptional regulation of *Cpt1a* expression could be more relevant in T cells. Indeed, it has been reported that PI3K-Akt-mTORC2 signalling initiated by growth factor or TCR/CD28 stimulation induces a downregulation of *Cpt1a* expression (25, 46, 127, 128), which in activated T cells is accompanied by a reduction of LC-FAO (24).

5.3. LC-FAO and the development of $CD8^+$ T_{mem}

Previous studies have suggested that, during the T_{eff} to T_{mem} transition, $CD8^+$ T cells can specifically increase the expression of *Cpt1a* to adopt an oxidative metabolic program after the primary immune response, and that this expression correlates with T cell longevity and metabolic fitness upon re-stimulation (46, 70). To convey this idea, it was shown that OT-I T cells transduced *in vitro* to overexpress *Cpt1a* and transferred into congenic hosts present an improved survival after LmOVA infection, even when transferred into IL-15-deficient mice, which suggests that CPT1A mediates longevity downstream of IL-15 signalling (70). However, because of the difficulties in studying the T_{eff} to T_{mem} transition *in vivo*, much of the analysis done to further investigate this connection relies on the *in vitro* generation of T_{mem}-like cells (70, 71, 129-132). This method involves the short-term activation of OT-I splenocytes with antigen, followed by antigen removal and incubation with IL-15 (or IL-7), and gives rise to $CD8^+$ T cells with phenotypic characteristics that resemble *ex vivo* T_{CM} cells

(129). In comparison with cells kept in IL-2 after antigen removal ("IL-2 T_{eff}" cells), these *in vitro* generated "IL-15 T_{mem}-like cells" are more quiescent (with most cells in the G0/G1 phase of the cell cycle), express surface molecules found in T_{CM} cells (such as CD44 and CD62L), downregulate CD69, CD25 and the production of IFN-γ, and can be reactivated upon re-stimulation (129). Their metabolism is also more oxidative than that of IL-2 T_{eff} cells, as they present more mitochondrial mass, lower ECAR and higher SRC, similar to what has been observed in *bona fide* $CD8^+$ T_{mem} cells compared to *ex-vivo* $CD8^+$ T_{eff} cells (70). IL-15 T_{mem}-like cells have also higher expression of *Cpt1a*, and their SRC is reduced after downregulation of *Cpt1a* mRNA levels using shRNA (70). Moreover, overexpression of *Cpt1a* in activated CTLs was reported to increase their survival after transfer into a naïve congenic recipient (70, 71).

Taken together, these observations may support the idea that, by upregulating the expression of *Cpt1a*, $CD8^+$ T_{mem} cells enhance mitochondrial oxidation by increasing the provision of LCFAs to the mitochondrial matrix, thus assuring a crucial supply of energy after the removal of TCR and cytokine stimulus (32, 108, 133). However, it needs to be considered that although IL-15 T_{mem}-like cells provide a simple *in vitro* system to study the influence of cytokines on the survival of activated cells after the removal of antigenic stimulation, this model does not completely reflect the *in vivo* conditions at the end of the effector and start of the memory phase. Thus, the cells generated with this method are not identical to the long-lived $CD8^+$ T_{mem} found after the peak of the primary immune response (129), and may not reflect the metabolism of $CD8^+$ T_{mem} cells in an *in vivo* setting.

5.4. CPT1A is dispensable for $CD8^+$ T_{mem}

To answer the question of whether LC-FAO mediated by CPT1A is critical for the development of $CD8^+$ T_{mem} cells , we generated T-cell specific *Cpt1a* conditional knockout mice (25). In contrast to the phenotype of TRAF6-deficient mice, deletion of *Cpt1a* in T cells did not affect the development or activation status of the $CD4^+$ and $CD8^+$ T cell compartment under homeostatic conditions, despite reduced synthesis of long-chain acylcarnitines and rates of LC-FAO. Likewise, T-cell specific *Cpt1a* knockout mice were able to mount a typical primary immune response after infection with LmOVA, with normal numbers of OVA-specific T cells and inflammatory cytokine production. Most importantly, these mice generated a $CD8^+$ T_{mem} population that survived for weeks after the primary response, and robustly proliferated and produced IFN-γ after re-challenge (25). These data demonstrate that CPT1A-mediated transport of LCFAs into the mitochondria is not required for the development of immunological memory. Furthermore, the differentiation of $CD4^+$ T_{reg} cells, another T cell subset that has been described to depend on LC-FAO (19, 134) was unaffected by the absence of *Cpt1a* (25). We also evaluated the memory response in mice deficient in other enzymes involved in LC-FAO, namely the long-chain acyl-CoA dehydrogenase (LCAD) and the very-long chain acyl-CoA dehydrogenase (VLCAD), which catalyse the first step in the process of mitochondrial β-oxidation downstream of CPT1A (124, 135, 136). These mice are models of human VLCAD-deficiency, which is the most common FAO disorder in patients (137, 138) and present an array of cardiac and hepatic disorders due to reduced FAO capacity that are acutely manifest upon fasting or cold exposure (138). LCAD- and VLCAD-deficient mice vaccinated with mouse-adapted H3N2 influenza could generate a protective response against H1N1 influenza, due to a normal T cell memory response (25). These results obtained from genetic mouse models of LC-FAO deficiency challenge previous reports using IL-15 T_{mem}-like cells suggesting that inhibition of CPT1 with the oxirane carboxylic acid etomoxir reduces the survival of $CD8^+$ T_{mem} cells (70)

and impairs differentiation of T_{reg} cells (134). This discrepancy thus incited a critical analysis of the use of chemical inhibitors to regulate LC-FAO and, as we will describe, a careful examination found unexpected effects of etomoxir on T cell differentiation.

5.5. Off target effects of etomoxir in immune cell differentiation

Since germline deletion of *Cpt1a* is embryonically lethal (139), to date much of the evidence that supports its significance in regulating the metabolic program of immune cells has been obtained through the pharmacological inhibition of CPT1 with etomoxir. With a half-maximal inhibitory concentration in the nanomolar range (IC_{50}= 10-700 nM), etomoxir is a potent inhibitor of CPT1 (140-143). Nonetheless, there are important caveats to consider when using this compound. One point is that the concentrations of etomoxir used in most published studies analysing LC-FAO in immune cells are considerably higher, usually around 200 µM (70, 71, 130, 131, 134, 144, 145). The possibility of off-target effects in immune cells was first raised in response to publications that reported that etomoxir could interfere with anti-inflammatory (M2) macrophage polarization with IL-4, an effect that was attributed to a reduction of LC-FAO (145). However, Finkel and colleagues observed that macrophages lacking CPT2, and thus exhibiting reduced LC-FAO, presented no defects in M2 polarization. Furthermore, CPT2-deficient macrophages responded to etomoxir treatment in a similar fashion than their WT counterparts (146). These data suggested that the effects of this compound were mediated not by inhibition of LC-FAO, but rather some other effect of etomoxir on cellular function.

To identify off-target effects of etomoxir in cell differentiation, a recent study by Divakaruni and colleagues used permeabilized cells to measure the effect of etomoxir on substrate specific oxygen consumption, and thus better characterize its influence on mitochondrial metabolism (143). In accordance with previous findings (140, 141, 147), the authors reported that etomoxir is a very efficient inhibitor of CPT1 activity in multiple tested cell lines, primary bone marrow-derived macrophages (BMDM) and T cells, even at concentrations below 1 µM (25, 143). Therefore, concentrations in the low micromolar range should suffice to specifically inhibit CPT1 activity, calling into question the use of concentrations as high as 200 µM to meaningfully influence cellular metabolism. Concentrations of etomoxir above 100 µM were discovered to reduce mitochondrial OxPhos independently from LC-FAO by inhibiting the adenine nucleotide transporter (ANT) located in the internal mitochondrial membrane and to a lesser extent the mitochondrial complex I of the ETC. During OxPhos, inhibition of the ANT prevents ADP import and ATP export from the mitochondrial matrix, with the consequent inhibition of ATP synthase activity. This in turn slows the rate of ETC activity and oxygen consumption regardless of the oxidizable substrate (25, 143) (Figure 2). The secondary inhibitory effect on complex I slows oxidation of all substrates providing NADH to the ETC. In intact T cells, 200 µM of etomoxir profoundly constrained mitochondrial respiration, which negatively affected activation, proliferation and cytokine production of stimulated lymphocytes. These effects were equally observed in WT and CPT1A-deficient cells, and comparable to those of oligomycin, an inhibitor of the mitochondrial ATPase (ETC member complex V) that blocks mitochondrial ATP production (25). Moreover, it has been described that, in the cytoplasm, etomoxir can readily conjugate to coenzyme A (CoA) to form etomoxiryl-CoA, which is the active form that inhibits CPT1 (141, 148). At high etomoxir concentrations this conjugation can have the consequence of effectively reducing the amount of cellular CoA available as a cofactor for various enzymatic reactions. These data provide support for an alternate mechanism of etomoxir action – altering cellular CoA pools rather than inhibiting LC-FAO – that interferes with the polarization of anti-inflammatory

macrophages (143). Thus, high concentrations of this reactive compound can have a variety of off-target effects with differing consequences depending upon the cellular context.

Taken together, these new studies illustrate the importance of using genetic models to validate data obtained from the use of chemical inhibitors. The normal differentiation of $CD8^+$ T_{mem} cells in CPT1A-deficient mice, together with the likelihood of multiple unintended metabolic consequences of high concentrations of etomoxir encourage a more cautious consideration of the role that LC-FAO plays in the development of certain immune phenotypes.

5.6. Mitochondrial oxidation and T_{mem} cell development.

Although the aforementioned studies demonstrate that LC-FAO is not crucial for supporting the metabolism of $CD8^+$ T_{mem} cells, ample evidence indicates that sustained mitochondrial oxidation is a metabolic characteristic of the T_{CM} subset of $CD8^+$ T_{mem} cells (70, 149). $CD8^+$ T_{mem} cells obtained after LmOVA infection were described to have higher SRC and reduced ECAR when compared to T_{eff} cells during the active immune response and thus to rely on OxPhos for ATP production and survival (69, 70). To support their increased SRC, $CD8^+$ T_{mem} have higher mtDNA/nDNA ratio, mitochondrial mass and expression of key mitochondrial proteins (70). Moreover, it was suggested that besides providing an efficient energy supply during the periods of quiescence, mitochondrial OxPhos is crucial for the reactivation of $CD8^+$ T_{mem} cells after secondary challenge because it provides the initial ATP required for the function of hexokinase, an enzyme that phosphorylates glucose and thus catalyses the first step of glycolysis (71). Hexokinase activity facilitates the rapid adoption of a glycolytic metabolism and T_{eff} phenotype after TCR stimulation that would contribute to the observed ability of $CD8^+$ T_{mem} to activate and proliferate faster than T_n upon stimulation. Correspondingly, inhibition of mitochondrial respiration with low concentrations of oligomycin during re-stimulation of $CD8^+$ T_{mem} cells was found to be detrimental to their activation and proliferation, highlighting the importance of mitochondrial ATP to mediate this process (71).

Recent studies have taken a closer look at mitochondrial morphology during the development of $CD8^+$ T_{eff} and T_{mem} cell responses. It has been demonstrated that in $CD8^+$ T_{mem} cells mitochondria tend to fuse and develop linear or tubular networks and present tight cristae structure (130, 131, 144). This morphology has been associated with improved OxPhos due to the arrangement of the proteins of the mitochondrial ETC into tightly packed supercomplexes, which improves electron flow and thus their function (150, 151). In contrast, mitochondria in $CD8^+$ T_{eff} were described to be diffusely distributed in the cytoplasm and to have a small and punctuate configuration (70, 144). Pharmacological induction of mitochondrial fusion (using a combination of the fusion-inducer M1 and fission-inhibitor Mdivi-1) increased the formation of mitochondrial networks in T_{eff} cells activated *in vitro*, and thus their OCR and SRC. Conversely, deletion in transferred OT-I T_{mem} of the enzyme Optic atrophy 1 (Opa1), which mediates mitochondrial fusion and cristae structure, did not affect expansion and effector activity after LmOVA infection but resulted in impaired $CD8^+$ T_{mem} survival and secondary activation (144). These results suggest that the generation of mitochondrial networks that regulate the level of mitochondrial oxidative capacity characterizes $CD8^+$ T_{mem} metabolism. In addition, and with interesting prospects for adoptive cellular immunotherapy, OT-I effector cells differentiated *in vitro* with mitochondrial fusion inducers and transferred into a congenic host had improved *in vivo* survival and antitumor function and could better develop a memory population capable of mounting a response against subsequent LmOVA infection (144). Moreover, treatment with M1 and Mdivi-1 also

improved mitochondrial metabolism in activated human $CD8^+$ T cells and increased the expression of T_{CM} markers CD62L and CCR7 (144).

These studies provide strong evidence that mitochondrial oxidation is important for the long-term survival and secondary activation of at least a subset of $CD8^+$ T_{mem} cells. However, a reliance on mitochondrial metabolism is not a universal requirement for the establishment of protective memory *in vivo*. This is illustrated by the deletion of the von Hippel Lindau tumour suppressor protein (VHL), an E3 ubiquitin ligase that induces degradation of HIF1α in normoxic conditions, and whose absence in T cells results in constitutive glycolytic metabolism (54). Mice with a T cell-specific deletion of VHL have an unaffected capacity to develop protective immune memory after viral infection, despite their prominent glycolytic profile and their inability to upregulate mitochondrial metabolism (149). Interestingly, VHL-deficient $CD8^+$ T_{mem} cells develop preferentially into a T_{EM} phenotype, further hinting at a relationship between the phenotypical and metabolic heterogeneity of T_{mem} populations. In fact, in mice immunized with LCMV, $CD62L^{hi}$ T_{CM} cells presented a higher SRC than $CD62^{lo}$ T_{EM}, which underpins the notion that mitochondrial capacity may not be universally related to memory formation, but rather to central memory (149, 152). Together these data hint at metabolic plasticity and differential fuel usage as key determinants of $CD8^+$ T_{mem} cell development.

5.7. A role for short- and medium-chain fatty acids in T cell metabolism

Mitochondrial respiration can be sustained by different carbon sources, such as pyruvate from glucose, glutamine and other amino acids and lipids, including not only long-, but also medium- and short-chain fatty acids. The complete oxidation of these substrates through the TCA cycle maximizes energy output, and allows quiescent cells to sufficiently satisfy their energy needs with lower amounts of nutrients. Despite this variety, virtually all of the studies analysing lipid oxidation in T cells have focused on the metabolism of LCFAs such as palmitate or oleate, which require conjugation to carnitine to enter the mitochondria, while ignoring the potential contribution to mitochondrial oxidation of medium- and short-chain acyl groups, which also become substrates in β-oxidation (153). Unlike LCFAs, there are no known key regulators of the oxidation of MCFAs and SCFAs, whose metabolism is not influenced by CPT1 because they can freely diffuse through the mitochondrial membrane (153). SCFAs in humans are mostly produced by colonic bacteria that ferment dietary fibre and sugars in the gut, and acetate (C_2) is also produced by the liver during fasting. SCFAs are readily used for energy by enterocytes in the gut (154) and it has been described that the SCFAs butyrate (C_4) and propionate (C_3) can inhibit histone deacetylases (HDAC) in colonic T cells (155). Although oxidation of SCFAs was not evaluated, the study shows that they can enter T cells, and therefore be potentially metabolized. Work by Balmer and colleagues demonstrated that IL-15-derived $CD8^+$ T_{mem} cells actively take up and metabolize acetate in the TCA cycle (156). Acetate levels in serum increase following pathogen infection, suggesting that acetate may be a usable fuel during immune responses (156). MCFAs are abundant in milk and can be produced by the liver through peroxisomal oxidation. MCFAs are usually not stored in the adipose tissue, and they are readily available from the circulation in their free form, usually bound to serum albumin, like LCFAs (153). Unlike LCFAs, they do not require active transport or cytosolic fatty acid-binding proteins (FABP) to enter the cells, and can therefore be oxidized faster than LCFAs (153). To date, the potential of SCFAs and MCFAs to be oxidized by T cells has not been thoroughly evaluated, though there is clear evidence showing that activated T cells can use certain MCFAs (octanoic and decanoic acid but not dodecanoic acid) to sustain proliferation (26).

5.8. AMPK and FAO in T_{eff}

Apart from the role of AMPK and oxidative metabolism in promoting metabolic resilience in T_{mem} populations, this kinase is also active and can play a role in T_{eff} cells. Upon TCR stimulation, a transient activation of AMPK in T cells has been reported, which is mediated by phosphorylation by the Ca^{2+}-calmodulin-dependent protein kinase kinase (CaMKK), triggered in response to the elevated concentration of cytosolic Ca^{2+} that results from TCR signalling (157). However, the relevance of this process in the activation or function of T cells is not yet fully understood. Importantly, AMPK has a critical function in regulating the phenotype and metabolism of activated cells, in particular upon nutrient deficiency. Deletion of AMPKα1 results in $CD8^+$ T_{eff} cells that have a more activated phenotype and increased production of IFN-γ *in vivo* and *in vitro* due to their increased mTORC1 activity (93, 95). *In vitro,* AMPK-deficient activated T cells deprived of glucose showed reduced cellular ATP concentration and increased mortality. This is attributed partly to their inability to reduce energy expenditure through inhibiting mTORC1 activity and their failure to upregulate glutamine transport and metabolism to increase mitochondrial energy output (93, 95). *In vivo*, mice with T cell-specific deletion of AMPK cells had markedly reduced expansion of antigen specific $CD8^+$ T cells in response to bacterial (*L. monocytogenes*) or viral (influenza A virus) infections, which reflected a failure of activated cells to persist or survive in inflammatory environments without the energy regulation induced by AMPK (93). Therefore, AMPK plays an important role in securing the fitness of activated T cells under conditions of metabolic stress. However, there is some controversy around these assertions, as others report that T cell-specific AMPKα1 deletion does not affect the proliferation of antigen specific cells in response to LmOVA *in vivo* (95).

Because T cells activated *in vitro* increase their glycolytic and anabolic metabolism and were observed to downregulate expression of *Cpt1a* and oxidation of external LCFAs (24, 25), it has been mostly assumed that $CD8^+$ T_{eff} cells do not require LC-FAO. This is supported by the fact that $CD8^+$ T cells lacking CPT1A are capable of differentiating into T_{eff} cells after TCR stimulation *in vitro* and after bacterial infection *in vivo* (25). However, *in vivo* studies suggest that OxPhos and FAO can play important roles in disease models that involve chronic stimulation with self-antigen or T cell exhaustion during chronic infection or tumour responses (19, 158, 159). T cells activated *in vivo* by MHC alloantigens as in the case of graft-versus-host disease (GVHD) seem to not upregulate Glut1 and display an increased OCR and mitochondrial membrane potential (160). Moreover, while having almost undetectable levels of pyruvate, alloreactive cells show elevated concentrations of medium and long-chain acylcarnitines (which are intermediate products of the oxidation of LCFAs) and increased uptake of external fatty acids and oxidation of palmitate (161, 162). Alloreactive cells also increase expression of *Cpt1a*, *Cpt2* and *Ppargc1a* (PGC1α), an activator of mitochondrial biogenesis and of the nuclear receptor peroxisome proliferative activated receptor gamma (PPARγ), which induces the uptake and cellular trafficking of lipids (162, 163). This oxidative metabolism has been associated to the increased expression in allogeneic T cells in GVHD of the inhibitory receptor Programmed cell Death 1 (PD-1) (164). PD-1 is a member of the CD28 family of receptors that is expressed in activated T cells. Upon ligation by PD-L1 or PD-L2, PD-1 prevents CD28-mediated activation of PI3K and thus of Akt (165) and therefore regulates T cell proliferation, cytokine secretion and survival (166). It has been reported that engagement of PD-1 in activated T cells, which causes overall reduction of T cell activation and IFN-γ production, also inhibits glycolysis and glutaminolysis, thus shifting the metabolic profile of activated cells (164, 165, 167). Moreover, PD-1

stimulation during *in vitro* activation of human $CD4^+$ T cells increased expression of *Cpt1a*, promoted lipolysis through upregulation of the adipose triacylglycerol lipase (ATGL) and supported oxidation of endogenous fatty acids (167). Interestingly, signalling from CTLA-4, another inhibitory receptor of the CD28 family which also reduces glycolysis and glutamine transport does not stimulate FAO and lipolysis, suggesting that the upregulation of FAO by PD-1 signalling is not solely a consequence of energy depletion (167).

The uptake of external fatty acids can also be important for T cell activation and proliferation. It has been recently reported that activation of naïve and Th2 $CD4^+$ T_{mem} cells is accompanied by an increase in PPARγ expression that is dependent on mTORC1, and supports the uptake of external fatty acids in addition to their endogenous synthesis (26). Inhibition of PPARγ with GW9662 or activation in fatty acid-free medium curtails proliferation of $CD4^+$ T cells and induces their apoptosis. It is unclear if this is also the case for $CD8^+$ T cell activation, as publications analysing the effect of mTORC1 inhibition in CTLs using proteomic and transcriptomic tools did not find meaningful regulation of *Pparg* by rapamycin (34).

It should also be considered that, in addition to energy storage, FAs can mediate other cellular functions in T cells (reviewed in (66)), such as building phospholipids and glycolipids in the cellular membranes, providing substrates for certain posttranslational modifications such as palmitoylation and myristoylation, or acting as ligands for nuclear receptors such as members of the PPAR family, that modulate lipid metabolism (168). Moreover, FAO can also provide precursors for synthesis of NADPH, a molecule with key roles in the conservation of cellular redox balance and in the synthesis of biomolecules. Through this pathway, which has been described in cancer cells, FAO can counteract the accumulation of reactive oxygen species (ROS), particularly in conditions of metabolic stress where synthesis of NADPH cannot proceed from glucose (123, 169-171). Although the relevance of this pathway in primary lymphocytes has, to our knowledge, not been evaluated, it could be also used by T cells to sustain redox balance under metabolic stress.

6. LOCALIZATION AND ENVIRONMENT MAY DICTATE T CELL METABOLIC PROGRAMS

Recent studies have now linked the maintenance of specific memory populations to their metabolic profile. As discussed before, most of the work analysing lipid metabolism in $CD8^+$ T_{mem} cells so far has focused on cells found in lymphoid organs like spleen or lymph nodes, which present a predominantly T_{CM} phenotype. Similarly, $CD8^+$ T_{mem}-like cells generated *in vitro* with IL-15 resemble T_{CM} and not T_{EM} or T_{RM} (129). To better understand the metabolic characteristics of $CD8^+$ T_{RM} cells, Pan and colleagues analysed OT-I T_{RM} cells produced after skin infection with vaccinia virus expressing OVA ($rVACV_{OVA}$)(69). In this study, they found that T_{RM} can actively take up and oxidize external palmitate to a higher degree than T_n, T_{EM} or T_{CM} cells and have a higher expression of PPARγ and the fatty acid binding proteins 4 and 5 (FABP4 and FABP5). FABP4/5 have been described to play an important role in the uptake and intracellular trafficking of FAs in adipocytes (172), where their expression is promoted by PPARγ. This was found to also be the case in $CD8^+$ T_{RM}, as deletion of FAPB4 and FAPB5 reduced the ability of OT-I T_{mem} cells in the skin to take up external palmitate and survive in the epithelium after viral infection, without affecting the survival of T_{mem} in spleen. The authors suggested that this PPARγ-coordinated metabolism was related to the location of $CD8^+$ T_{RM} cells in the epithelial tissue, where they have easier access to free fatty acids

(FFAs) than to other nutrients, and thus they can use external FAs as a source of energy. These results are an important example of the heterogeneity of the T cell memory population and point towards a paradigm where distinct metabolic profiles are required for T cell function and survival in specific tissue microenvironments.

Specific metabolic characteristics relating to their location and the surrounding metabolic environment have also been assigned to other lymphocyte populations. For instance, $Foxp3^+$ T_{reg} cells make up more than 50% of the $CD4^+$ lymphocytes found in the visceral adipose tissue (VAT) of lean mice (173). Despite sharing most of their transcriptional profile with T_{reg} cells from the spleen, these VAT T_{regs} have unique metabolic characteristics that are also coordinated by PPARγ, which is highly expressed in these cells. Activation of PPARγ in VAT T_{reg} upregulates the expression of *Cpt1a* and enzymes related to FA transport, triglyceride synthesis and lipid-droplet organization (174). As is the case with tissue resident memory $CD8^+$ T cells, PPARγ is crucial for the development of $CD4^+$ T_{reg} cells in the adipose tissue, but not for those in the spleen or other lymphoid tissues (174).

Similarly, cells in the tumour microenvironment (TME) can find themselves in a metabolically challenging situation (175-177). The heavily glycolytic metabolism of most tumours results in glucose depletion in the TME, complemented by reduced oxygen concentration, which impairs the function of the tumour-infiltrating lymphocytes (TILs) (178, 179). In contrast, increased abundance of free FAs produced by the tumour is often observed in the TME (180), which can potentially provide TILs an alternative source of energy. Indeed, it has been recently shown that, compared to T cells outside the tumour, the expression of the nuclear receptor PPARα is increased in TILs. PPARα induces the expression of genes related to lipid oxidation, such as *Cpt1a*, which could help TILs to adapt to oxidize FAs from the TME, and thus sustain some degree of effector activity (181). Supplementing vaccination with the PPARα agonist fenofibrate generated cells with enhanced antitumor activity *in vivo*, supporting the notion that favouring FA catabolism might be best suited to fight tumours in a hypoglycemic environment and generating exciting perspectives for the design of antitumor therapies (181).

7. MITOCHONDRIAL AND FATTY ACID METABOLISM IN HUMAN IMMUNE RESPONSES

The study of individuals with inborn metabolic disorders can provide insights to the metabolic pathways dominating the human immune response (182). Specific immune deficiencies can be found in individuals with genetic diseases that affect mitochondrial function. Leukopenia, hypogammaglobulinemia, opportunistic fungal infections and recurring infections have been reported in patients with mitochondrial disease (MD) (40, 182). In particular, upper respiratory tract infections are observed in 30-50% of patients with diagnosed MD (40, 183) and it has been reported that vaccination against *S. pneumoniae* fails to elicit protective immunity in these individuals (184).

Although rare, CPT1 deficiency is observed exclusively for the "liver" CPT1A isoform and it is characterized primarily by hypoglycemic attacks usually associated with fasting (117, 124, 185). Although most symptoms described for these patients are related to the metabolic defect of LC-FAO in liver and its systemic consequences, an association between carrying a hypomorphic variant of the enzyme and the risk of suffering infections of the lower respiratory tract or acute otitis media was found in children from an Alaskan population with a relatively high incidence of CPT1A-deficiency, although confounding factors preclude reliable

conclusions (186). To improve available information, a clinical study evaluating the immune phenotype in subjects with defects in mitochondrial metabolism is ongoing (clinicaltrials.gov, NCT01780168) and preliminary results indicate that absence of enzymes involved in the mitochondrial β-oxidation of fatty acids does not impair the generation of $CD8^+$ memory responses (25).

In healthy human individuals, there is a progressive accumulation of $CD8^+CD28^-$ T_{EM} cells that has been associated with loss of activity of the deacetylase sirtuin 1 (Sirt1) with age. Like other members of the sirtuin family, Sirt1 is a NAD^+-dependent deacetylase, that has many roles in modulating metabolism and the immune response (187). Similar to the differences observed between murine T_{EM} and T_{CM} cells, $CD8^+CD28^-$ cells exhibit increased glycolytic metabolism when compared to $CD8^+CD28^+$ T_{mem} cells or T_n, but no differences in their basal or maximal oxygen consumption (188). Interestingly, treatment of $CD8^+CD28^-$ memory cells with the Sirt1 activator resveratrol lowers glycolytic capacity through activation of the FOXO1 transcription factor that, in addition to regulating the expression of CD62L and CD127, was described to modulate the glycolytic phenotype. Treatment of naïve or $CD28^+$ memory cells with the FOXO1 inhibitor AS1842856 reduced expression of CD62L and increased granzyme B production in $CD28^+$ T_{mem} cells, presumably through the downregulation of the transcription factor T-bet (81), while also increasing glycolytic metabolism. Although the exact mechanism through which FOXO1 modulates glycolysis was not defined, the connection between Sirt1 activity, memory phenotype and metabolism provides an interesting model to comprehend the cues that guide T_{mem} differentiation.

Understanding the metabolic preferences and requirements of T cells can have an important impact in the development of adoptive therapies. CAR-T cells are transduced to express a synthetic polypeptide that was initially developed to contain an extracellular target binding module, which usually signals through an intracellular TCR domain, and thus can stimulate the natural TCR activation pathway. Second- and third-generation CAR-T cells include the expression of costimulatory molecules, such as CD28 or 4-1BB (CD137), which together with the receptor can dramatically improve antitumor activity. The effect of the co-stimulation is not limited to the immune signalling mediated by these co-receptors, but they can also differentially influence cellular metabolism favouring cellular fitness and improve both effector function and persistence of the CAR-T cell (189-192).

In particular, it has been found that co-stimulation through 4-1BB is associated to a longer survival and enhanced memory development when compared to CAR-T cells expressing CD28 (189, 192). Interestingly, 4-1BB-expressing cells were also characterized by enhanced mitochondrial biogenesis and metabolism, higher SRC and rates of FAO, as well as a differential expression of metabolic genes favouring FA metabolism over glycolysis, including *Cpt1a* and *Fabp5* (191). Signalling through CD28 intracellular domain was also described to increase *Cpt1a* expression when compared to T cells harbouring only the transgenic antigen-specific receptor (131).

8. CONCLUDING REMARKS

During the last few years, a major interest in understanding the metabolic requirements of immune cells has triggered the establishment of immunometabolism as a new, exciting area of research. Several studies have attempted to describe unique metabolic conditions that favour the development of long-lasting adaptive memory, as this information could be crucial to improve the effectiveness of vaccination or cancer treatment (176). Taken together, some

of these studies had ultimately proposed that $CD8^+$ T_{mem} cells present an exceptional requirement for mitochondrial oxidation of fatty acids to sustain their survival and secondary activation, and identified an important role for CPT1A, an enzyme that can control the rate of LC-FAO, in modulating this adaptation (70, 71, 106, 131). Due to the absence of sufficient *in vivo* data and specific genetic models to evaluate this hypothesis, the current paradigm was based on the use of a chemical inhibitor of CPT1, etomoxir, and the analysis of *in vitro* cultures with IL-15 of antigen-stimulated $CD8^+$ T cells. However, an often-ignored fact is that CPT1A can only modulate the oxidation of FAs with chains longer than 12 carbons, while not affecting the oxidation of shorter FAs that can also provide a source of energy (67, 153). It is therefore only correct to affirm that CPT1A regulates the oxidation of *long-chain* fatty acids, and not fatty acids in general, which leaves the potential of SCFAs and MCFAs to support – or even maybe favour – the development of $CD8^+$ T cell memory largely unexplored. Furthermore, it is important to consider the limitations of analysing memory development with *in vitro* cultures. It is likely that T cells differentiated *in vitro* with IL-15 will not share all features of long-lived $CD8^+T_{mem}$ cells obtained after an infection, as IL-15 is not the only factor that influences their development *in vivo*. In addition, as it has been pointed out (130), the concentration of nutrients such as glucose, lipids, glutamine or O_2 that lymphocytes are exposed to in cell culture medium is radically different to that encountered *in vivo*, and thus limit the application of the information obtained from cell cultures into a real infection setting (159).

New studies targeting *Cpt1a* in T cells challenge the current model of T_{mem} reliance on LC-FAO for establishing protective memory *in vivo* (25). Moreover, careful examination of the effect of etomoxir on T cell metabolism has suggested that, at high concentrations, this compound has multiple off-target effects on mitochondrial metabolism and other cellular processes, which may vary depending on the cellular context and requirements for activation, influencing the differentiation of not only T cells, but also other immune subsets such as macrophages (143, 146).

The arguments presented in this review do not preclude an important function for OxPhos in the development and survival of memory cells, or deny the possibility that T cells can profit from the oxidation of lipids to fuel their metabolism under certain circumstances. Instead, we seek to encourage the reader to adopt a more global view of the metabolic requirements of $CD8^+$ T_{mem} cells, considering both cellular state and tissue location. Future research should include use of *in vivo* and *in vitro* models that consider cellular location and nutrient that affect T cell metabolism. In this regard, an interesting topic of research involves the study of the PPAR family of nuclear receptors that have been reported to regulate lipid metabolism in T cells, remarkably in tissue resident cells that might have easier access to fatty acids than glucose or other metabolic substrates (26, 69, 181, 193, 194). As PPARs have the unique characteristic of being activated by lipid ligands (168, 194, 195), they could provide a link between environmental availability and the adoption of a lipid-based metabolism. Some of the work presented in this review has started to address this relationship, and we predict that future research will provide exciting new information. Moreover, we have discussed that mitochondrial respiration could favour the survival of memory cells after the resolution of the primary response, but it is also clear that protective immune memory can occur in the event of constitutive glycolysis, at least under certain circumstances (149).

Taken together, although T_{mem} cells can, in many cases, *upregulate* a certain metabolism to promote their survival, their *reliance* on this particular metabolic program might not be

universal for all T_{mem} subsets, or under all conditions. In the end, the choice of substrate might come down to one thing: enough ATP to get through the day.

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Conflict of interest statement

The authors declare no conflict of interest.

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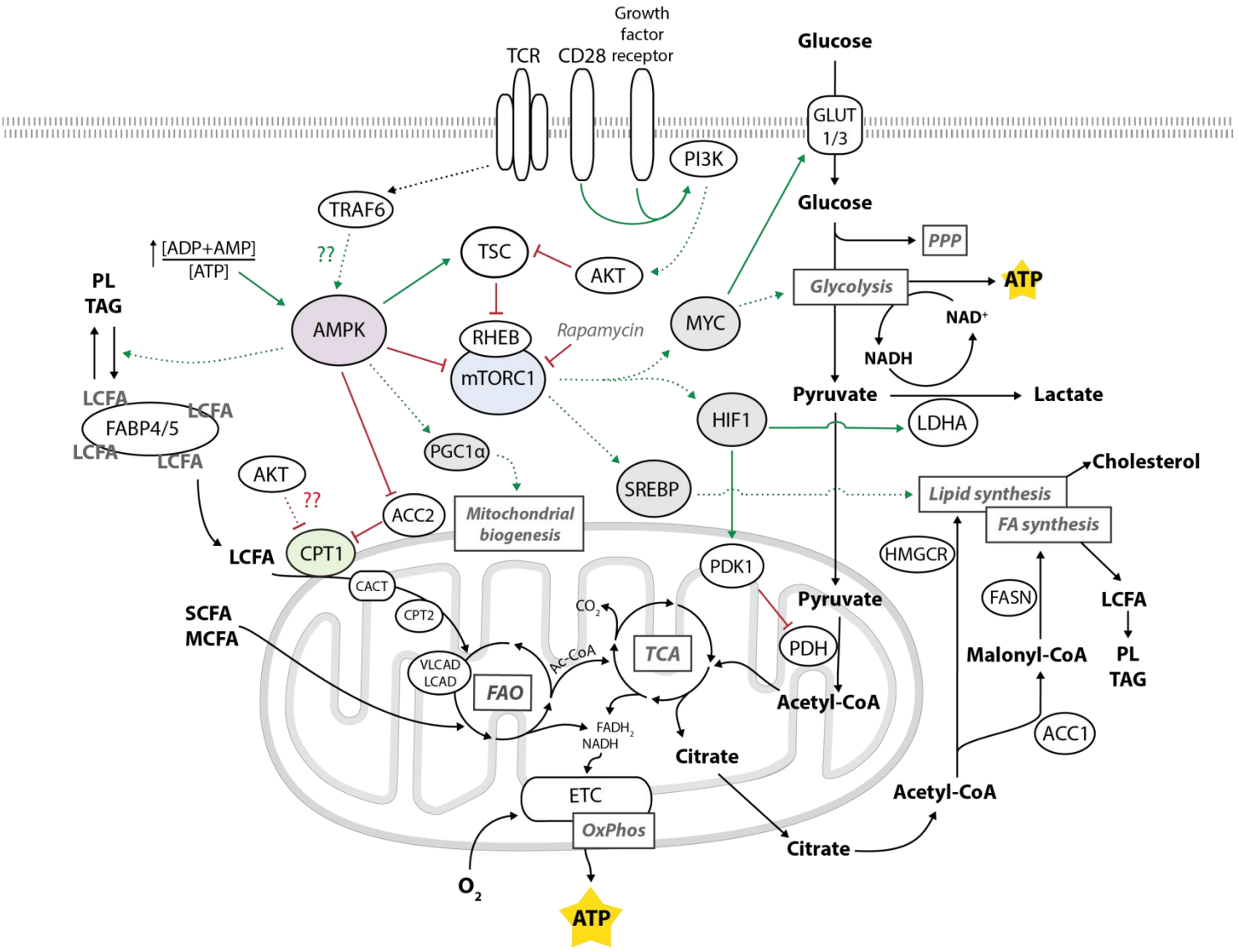

Figure 1. Overview of metabolic pathways regulated during T cell differentiation.

Immune signals from the TCR, co-stimulatory molecules and cytokine receptors activate PI3K and thus the tyrosine kinase AKT. Active AKT phosphorylates and inhibits the tuberous sclerosis complex (TSC), stimulating the activity of mTORC1. mTORC1 stimulates aerobic glycolysis and lipid synthesis indirectly through supporting the activity of transcription factors like MYC, HIF1 and SREBP that promote the expression of key enzymes in these processes. The AMP-activated-protein kinase (AMPK) is activated by high AMP and ADP (signalling metabolic stress) and antagonizes mTORC1 activity by stabilization of the repressor TSC and inhibition of the mTORC1 subunit Raptor. AMPK also indirectly promotes mitochondrial biogenesis through the activity of the transcriptional coactivator PGC1α. Moreover, AMPK can inhibit the synthesis of fatty acids and stimulate the oxidation of long-chain fatty acids (LCFA) by blocking the production of malonyl-CoA by the acetyl-CoA carboxylase (ACC1/2), and thus also relieving the inhibition on carnitine palmitoyltransferase 1 (CPT1) by malonyl-CoA. CPT1 is the rate-controlling enzyme for the transport of LCFAs into the mitochondrial matrix, where they can be metabolized into acetyl-CoA and reduced molecules through fatty acid oxidation (FAO). Short- and medium-chain fatty acids (SCFAs and MCFAs) enter FAO independently of CPT activity. In $CD8^+$ T cells, glucose enters the cell through the Glut1 and Glut3 glucose transporters and is converted into pyruvate through glycolysis. In resting cells, pyruvate dehydrogenase (PDH) in the mitochondria converts this pyruvate to acetyl-CoA, which is further oxidized in the tricarboxylic acid (TCA) cycle, together with acetyl-CoA obtained from FAO. The TCA cycle generates reduced NADH and $FADH_2$, whose oxidation by members of the electron transport chain (ETC) consumes molecular oxygen (O_2) and supports the generation of ATP by oxidative phosphorylation (OxPhos). In activated cells, a mayor part of the pyruvate produced after glycolysis is reduced to lactate by the enzyme lactate dehydrogenase (LDHA), and much of the acetyl-CoA obtained in the mitochondria is shuttled to the cytoplasm to sustain lipid synthesis. PDK1, pyruvate dehydrogenase kinase 1; PPP, pentose phosphate pathway; HMGCR hydroxy-methyl-glutaryl-CoA reductase; FASN, fatty acid synthase; CACT, carnitine-acylcarnitine translocase; (V)LCAD, (very) long-chain acyl-CoA dehydrogenase; RHEB, RAS homolog enriched in brain; FABP, fatty acid binding protein; PL, phospholipid; TAG, triacylglycerol; TRAF6, TNF-associated factor 6.

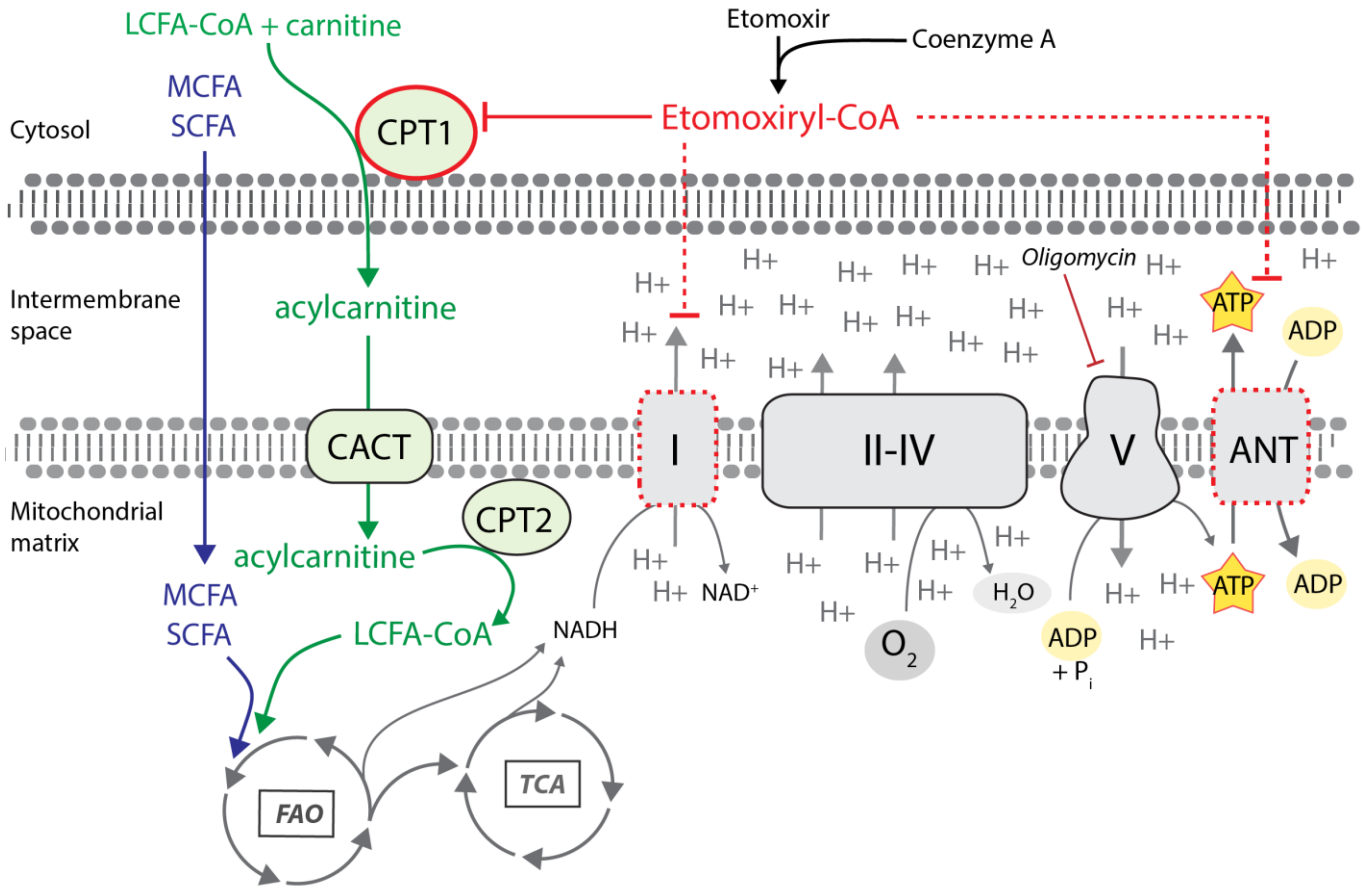

Figure 2. LC-FAO and off-target effects of etomoxir.

Medium- and short-chain fatty acids (MCFA and SCFA) enter the mitochondria freely, and undergo fatty acid oxidation (FAO). Long-chain fatty acids (LCFA) in the cytoplasm are found conjugated to coenzyme A (LCFA-CoA), which is replaced with carnitine by carnitine palmitoyltransferase 1 (CPT1), located at the outer mitochondrial membrane. Acylcarnitines are transported across the inner mitochondrial membrane by the carnitine-acylcarnitine translocase (CACT), and then the LCFA-CoA is restored by CPT2 in the matrix. The products of FAO are reduced molecules like NADH and acetyl-CoA that enters the tricarboxylic acid cycle (TCA). In the cell, etomoxir is conjugated to coenzyme A to form etomoxiryl-CoA. Etomoxiryl-CoA effectively inhibits the function of CPT1. At high concentrations, etomoxiryl-CoA can also inhibit the adenine nucleotide transporter (ANT) and mitochondrial complex I. ANT mediates the exchange of ATP obtained by oxidative phosphorylation in the matrix with cytosolic ADP, thus its inhibition results in ATP accumulation in the matrix, which impairs the ATP-synthetase activity of the mitochondrial complex V, similarly to the compound oligomycin. Inhibition of complex I interferes with oxidation of NADH. Both events result in a reduction of electron flow through the mitochondrial electron transport chain and the consumption of oxygen.