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Mitochondrial Dynamics Controls T Cell Fate Through Metabolic Programming

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Mitochondrial Dynamics Controls T Cell Fate Through Metabolic Programming

by

Michael D. Buck

A dissertation presented to
The Graduate School
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

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Michael Buck

Washington University in St. Louis

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ABSTRACT OF THE DISSERTATION

Mitochondrial Dynamics Controls T Cell Fate Through Metabolic Programming

by

Michael D. Buck

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Professor Erika L. Pearce, Chair

Professor Eugene M. Oltz, Co-chair

Activated effector T (T_E) cells augment anabolic pathways of metabolism, such as aerobic glycolysis, while memory T (T_M) cells engage catabolic pathways, like fatty acid oxidation (FAO). However, signals that drive these differences remain unclear. Mitochondria are metabolic organelles that actively transform their ultrastructure. Therefore, we questioned whether mitochondrial dynamics controls T cell metabolism. We show that T_E cells have punctate mitochondria, while T_M cells maintain fused networks. The fusion protein Opa1 is required for T_M , but not T_E cells after infection, and enforcing fusion in T_E cells imposes T_M cell characteristics and enhances antitumor function. Our data suggest that, by altering cristae morphology, fusion in T_M cells configures electron transport chain (ETC) complex associations favoring oxidative phosphorylation (OXPHOS) and FAO, while fission in T_E cells leads to cristae expansion, reducing ETC efficiency and promoting aerobic glycolysis. Thus mitochondrial remodeling is a signaling mechanism that instructs T cell metabolic programming.

Chapter 1: T Cell Metabolism Drives Immunity

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Abstract

Lymphocytes must adapt to a wide array of environmental stressors as part of their normal development, during which they undergo a dramatic metabolic remodeling process. Research in this area has yielded surprising findings on the roles of diverse metabolic pathways and metabolites, which have been found to regulate lymphocyte signaling and influence differentiation, function and fate. In this review, we integrate the latest findings in the field to provide an up-to-date resource on lymphocyte metabolism.

Introduction

“Part of the secret of success in life is to eat what you like and let the food fight it out inside.” –
Mark Twain

Simply stated, we are what we eat. Our genetics coupled with environmental influences dictate how we metabolize the nutrients we consume and shape our growth, function, and overall health. The same principles hold true at the cellular level. Just as a track runner quickly engages their muscles to propel themselves from rest to sprint in response to a starting gun, pathogen-derived or inflammatory signals drive T cells out of quiescence, resulting in rapid modulation of gene expression and the acquisition of new functions. These changes range from increased production of cytokines and cytolytic molecules to the ability to undergo cell division and migration. Intimately integrated into this program of activation is the regulation of cellular metabolism.

The engagement of specific metabolic pathways profoundly affects cell differentiation and function. Metabolic reprogramming is controlled by key receptor signaling events and growth factor cytokines, as well as availability of nutrients. In addition, metabolic products provide substrates that can alter the functional fate of a cell through post-translational modifications (PTMs) or epigenetic remodeling. Several recent articles have covered these and other emerging topics in T cell metabolism (Bird, 2015; Chapman and Chi, 2014; Lochner et al., 2015; O'Sullivan and Pearce, 2015; Palmer et al., 2015; Ramsay and Cantrell, 2015; Ron-Harel et al., 2015). In this review, we provide a general but comprehensive overview of lymphocyte metabolism integrated with current research. Our focus will be on data and concepts derived primarily from T cell studies with tie-ins from other fields when relevant.

T cell development and quiescence

Although the bulk of T-cell metabolism research centers on mature T cells, even at their inception and throughout their development, T cells cycle through states of metabolic quiescence and activation. Hematopoietic stem cell progenitors that are double negative (DN) for CD4 and CD8 co-receptors migrate from the bone marrow and seed the thymus where they rearrange their antigen receptor gene loci to produce a functional T cell receptor (TCR). Signals from the receptor Notch1 maintain cell survival and are required to promote T-cell lineage commitment (Maillard et al., 2006; Pui et al., 1999; Radtke et al., 1999). Induced deletion of Notch1 during neonatal development results in arrest at the most immature DN1 (CD44⁺CD25⁻) stage (Radtke et al., 1999), while enforced expression of constitutively active Notch1 in bone marrow cells blocks B cell differentiation and instead causes the ectopic development of CD4⁺CD8⁺ double positive (DP) T cells (Pui et al., 1999).

Successful expression of TCR β with pT α and CD3 forms the pre-TCR and signals with Notch1 to drive cells out of quiescence as they enter β -selection (Ciofani et al., 2004; Ciofani and Zuniga-Pflucker, 2005; Saint-Ruf et al., 1994). RAG recombinase expression declines and expression of the transferrin receptor CD71 and other nutrient transporters are induced as the cells proliferate (Ciofani and Zuniga-Pflucker, 2005; Kelly et al., 2007). Signaling from the pre-TCR, Notch1, and the chemokine receptor CXCR4 converge to activate phosphatidylinositol 3-kinase (PI3K); this stimulates the switch to anabolic metabolism (processes that supports the biosynthetic demands of proliferation) (Ciofani and Zuniga-Pflucker, 2005; Janas et al., 2010). Increased expression of the glucose transporter Glut1 is required during this stage and its

expression is dependent on activation of the kinase Akt by PI3K (Juntilla et al., 2007; Swainson et al., 2005; Wieman et al., 2007). PI3K-Akt signaling also activates the mechanistic target of rapamycin (mTOR), and signals from this kinase augment the glycolytic metabolism used to support cell growth and proliferation (MacIver et al., 2013).

Disruptions in PI3K signaling also affect the transition of DP thymocytes to single positive CD4 and CD8 T cells and the development of natural killer T (NKT) cells, which require sustained signaling in order to join *tcr α* Va to distal Ja gene segments that define their invariant TCR (D'Cruz et al., 2010; Rodriguez-Borlado et al., 2003). PTEN (phosphatase and tensin homolog) is the principal negative regulator of the PI3K pathway. Thymocytes from mice that lack the microRNA cluster miR-181a1b1 have altered cellular metabolism due to a significant increase in PTEN expression (Hena-Mejia et al., 2013). Glucose uptake, measured by acquisition of the fluorescent glucose analog 2-NBDG, and glycolytic rate are reduced in these cells, and nutrient transporter expression is diminished. As a result of dysregulated PI3K signals, these mice have deficiencies in DP cells and completely lack NKT cells (Hena-Mejia et al., 2013).

The cytokine interleukin (IL)-7 has a pivotal role in ensuring the survival of developing and quiescent naïve T cells by increasing expression of the anti-apoptotic factor Bcl-2 (B-cell lymphoma 2) (Akashi et al., 1997; Maraskovsky et al., 1997; Tan et al., 2001; Yu et al., 2003). Mice deficient in IL-7 or the IL-7R α chain have defects in T cell development (Peschon et al., 1994; von Freeden-Jeffry et al., 1995). IL-7 signals through the JAK3-STAT5 pathway, but can also activate PI3K (Pallard et al., 1999; Wofford et al., 2008). A recent study suggests that in addition to maintaining the survival of developing lymphocytes, IL-7 signaling promotes the

growth and proliferation of DN4 cells by increasing levels of trophic receptors, such as CD71 and the amino acid transporter CD98 (Boudil et al., 2015; Pearson et al., 2012), activities that previously were attributed mainly to Notch1 signaling. However, Notch1 can induce IL-7R α expression and therefore its effects could be downstream of IL-7 signals (Gonzalez-Garcia et al., 2009; Magri et al., 2009).

Mature naïve T cells exit from the thymus into the periphery. As quiescent cells they primarily oxidize glucose-derived pyruvate in their mitochondria via oxidative phosphorylation (OXPHOS), or use fatty acid oxidation (FAO) to generate ATP (**Figure 1**) (Fox et al., 2005; Pearce and Pearce, 2013; Pearce et al., 2013; van der Windt and Pearce, 2012; Wang et al., 2011). A balance between tonic TCR signals and IL-7 are needed to sustain naïve T cells. Homeostatic proliferation of naïve T cells is supported by TCR ligation with self-peptides presented on major histocompatibility complex (MHC) molecules in the periphery (Ernst et al., 1999; Goldrath and Bevan, 1999; Muranski et al., 2000). However, unrestrained Akt activation, or deletion of negative regulators of TCR stimulation, leads to loss of quiescence (Yang et al., 2011). T cells defective in tuberous sclerosis complex 1 (TSC1), a negative regulator of mTOR signaling, prematurely exit from quiescence and have increased rates of apoptosis and hyperactive responses to TCR stimulation (Yang et al., 2011). In addition, TCR mediated PI3K-Akt activation downregulates IL-7R α (Cekic et al., 2013), but as discussed above, IL-7 signaling is critically essential to prevent apoptosis and ensure survival of the naïve T cell pool (Rathmell et al., 2001; Surh and Sprent, 2008). A recent study showed that the metabolite adenosine, which is a byproduct of metabolic activity, suppresses TCR signaling in a dose dependent manner (Cekic et al., 2013). The G-protein-coupled adenosine receptor subtype A_{2A}R is predominantly

expressed in T cells. Binding with adenosine activates cAMP-dependent protein kinase A (PKA), which suppresses TCR mediated activation of the PI3K pathway and prevents IL-7R α downregulation (Cekic et al., 2013).

Activation and effector T cell differentiation

Metabolic reprogramming during T cell activation

Once in the periphery, a mature naïve T cell is like a bomb, lying dormant in the lymphoid organs and circulation until it is triggered to activate and ‘explode’ in a proliferative chain reaction. T-cell activation stimulated by TCR ligation and binding with costimulatory molecules induces metabolic remodeling of the naïve T cell to a program of anabolic growth and biomass accumulation; this is marked by the engagement of aerobic glycolysis, a process in which glucose is converted into lactate even though sufficient oxygen is present to support glucose catabolism via the tricarboxylic acid (TCA) cycle and OXPHOS (**Figure 1**) (MacIver et al., 2013; Vander Heiden et al., 2009). Although aerobic glycolysis is less efficient than OXPHOS at yielding an abundance of ATP per molecule of glucose, aerobic glycolysis can generate metabolic intermediates important for cell growth and proliferation, and provides a way to maintain redox balance (NAD⁺/NADH) in the cell (**Figure 2**) (Anastasiou et al., 2011; Macintyre and Rathmell, 2013; Vander Heiden et al., 2009). For example, glucose-6-phosphate and 3-phosphoglycerate (3PG) produced during glycolysis can be metabolized in the pentose phosphate and serine biosynthesis pathways, respectively, donating important precursors for nucleotide and amino acid synthesis (Pearce et al., 2013; Wang et al., 2011). Glucose can also enter the mitochondria as pyruvate, where it is converted to acetyl-CoA and joins the TCA cycle

by condensing with oxaloacetate to form citrate. Breakdown of substrates in the TCA cycle not only provides reducing equivalents for OXPHOS, but also precursors for biosynthesis. Glucose-derived citrate can be exported into the cytosol to generate acetyl-CoA by ATP citrate lyase (ACL) for use in lipid synthesis (Bauer et al., 2005; DeBerardinis et al., 2008; Hatzivassiliou et al., 2005). Similarly, oxaloacetate can be used to produce aspartate, an additional precursor for generating nucleotides (**Figure 2**) (DeBerardinis et al., 2007).

Several transcription factors and signaling pathways coordinately support and regulate this change in T-cell metabolic programs following activation. Growth factor cytokines such as IL-2 and ligation of costimulatory molecules promote the switch to glycolysis through the enhancement of nutrient transporter expression and activation of the key metabolic regulator mTOR (**Figure 1**) (Frauwirth et al., 2002; Jones and Thompson, 2007; Kolev et al., 2015; Wieman et al., 2007). Existing as two complexes, mTORC1 and mTORC2, this kinase integrates extrinsic and intrinsic signals related to nutrient levels, energy status, and stress to induce changes in cellular metabolism, growth, and proliferation (Laplanche and Sabatini, 2012). CD28 ligation enhances PI3K activity, which recruits 3-phosphoinositide-dependent protein kinase-1 (PDK1) and Akt. PDK1, together with mTORC2, phosphorylates Akt, which in turn activates mTORC1. Both Akt and mTOR promote aerobic glycolysis and support effector T-cell differentiation, growth, and function (Delgoffe et al., 2011; Pollizzi et al., 2015). Akt regulates nutrient transporter expression and can phosphorylate the glycolytic enzyme hexokinase II, promoting its localization to the mitochondria and augmenting its enzymatic activity (John et al., 2011; Miyamoto et al., 2008). mTORC1 activation increases protein translation via phosphorylation of 4E-BP1 and p70S6 kinase (Laplanche and Sabatini, 2012) and promotes lipid

synthesis by activating SREBP2 (sterol regulatory element-binding protein 2) (Porstmann et al., 2008).

The upregulation of transcription factors c-Myc, estrogen-related receptor α (ERR α), and hypoxia inducible factor-1 α (HIF-1 α) coordinately drive the expression of genes involved in intermediary metabolism that fuel the rapid proliferation of effector T cells during clonal expansion (Doedens et al., 2013; Michalek et al., 2011; Wang et al., 2011). First discovered as an oncogene important for cell growth and proliferation (Cole, 1986; Sheiness et al., 1978), c-Myc has been shown to be a critical regulator of metabolic reprogramming following T-cell activation (Wang et al., 2011). c-Myc drives the expression of enzymes that promote aerobic glycolysis and glutaminolysis and coordinates these metabolic pathways with lipid, amino acid, and nucleic acid synthesis. However, c-Myc expression is not continually sustained after T-cell activation (Best et al., 2013; Nie et al., 2012). A recent study suggests that c-Myc induces the transcription factor AP4, which maintains the glycolytic transcriptional program initiated by c-Myc to support T-cell population expansion (Chou et al., 2014; Karmaus and Chi, 2014). HIF-1 α , a transcription factor that responds to oxygen levels, also increases glucose uptake and catabolism through glycolysis (Finlay et al., 2012; Kim et al., 2006). Deletion of its negative regulator, von Hippel-Lindau (VHL), enhances HIF-1 α mediated CD8 T-cell glycolysis and effector responses to persistent viral infection (Doedens et al., 2013).

ROS signaling

Although much of the attention on metabolic reprogramming in activated T cells has focused on the engagement of aerobic glycolysis, recent research has revealed the importance of

mitochondrial-driven activities in this process. In addition to energy production, the electron transport chain (ETC) is a major source of reactive oxygen species (ROS) (Turrens, 2003), which are important for T-cell responses (**Figure 2**) (Chaudhri et al., 1988; Devadas et al., 2002; Jones et al., 2007). T cells deficient for Rieske iron sulfur protein (RISP), a subunit of mitochondrial complex III, have impaired activation and antigen-specific T-cell expansion *in vitro* and *in vivo* due to defects in mitochondrial-derived ROS signaling (Sena et al., 2013). More recently, using a forward genetic screen, another group identified mice with enhanced CD8 T-cell responses to viral and tumor challenge (Okoye et al., 2015). The source of the heightened immunity gained after germline mutagenesis was found to be due to the increased expression of an orphan protein, identified as Lymphocyte Expansion Molecule (LEM). Interestingly, augmented OXPHOS and mitochondrial ROS levels were detected in CD8 T cells isolated after infection from these mice, whereas heterozygous LEM-deficient CD8 T cells had reduced OXPHOS and mitochondrial ROS levels. LEM helps stabilize a protein involved in inserting ETC complex proteins in the mitochondrial membrane, which may account for the increased ROS and enhanced proliferation evident in CD8 T cells from these mice (Okoye et al., 2015).

While ROS is produced as a general by-product of mitochondrial metabolism, new studies have specifically linked the metabolite succinate to both the generation of ROS and activation of HIF-1 α in settings of inflammation or injury (Chouchani et al., 2014; Tannahill et al., 2013). Innate immune receptor activation increases intracellular succinate from glutamine via glutamine-dependent anaplerosis and the γ -aminobutyric acid shunt pathway and this leads to HIF-1 α stabilization and activation (Tannahill et al., 2013). During ischaemia reperfusion injury, which happens when blood supply to a tissue is disrupted and then restored, succinate accumulates

from reverse activity of the enzyme succinate dehydrogenase (SDH) and is rapidly oxidized upon reperfusion. This leads to over-reduction of the electron carrier coenzyme Q, causing reverse electron transport through mitochondrial complex I and subsequently, excessive ROS production (Chouchani et al., 2014; O'Neill, 2014). Given that mitochondrial ROS and HIF-1 α activity are important for the metabolic reprogramming of naïve T cells after activation, it is interesting to speculate that the metabolite succinate may also support the transition from a naïve to an activated effector T cell.

Metabolic programming of T helper cell differentiation

Activation of T cells is intimately tied to the engagement of specific metabolic pathways, so it is no surprise that distinct metabolic programs also support the differentiation of CD4 T helper (Th) cells into their separate lineages. Initial studies found that suppression of mTOR with rapamycin promoted the generation of FoxP3⁺ T regulatory (Treg) cells even in the presence of Th17 polarizing cytokines *in vitro* (Kopf et al., 2007), and genetic deletion of mTOR in T cells augmented production of Treg cells upon activation, but not Th1, Th2, or Th17 cells (Delgoffe et al., 2009). These results are consistent with the metabolic profiles of these cells: Th1, Th2, and Th17 cells strongly engage glycolysis via mTOR signaling, while Treg cells depend more on the oxidation of lipids (**Figure 1**) (Michalek et al., 2011). Th17 cells in particular have been found to heavily rely on glycolysis for their development and maintenance, stimulated by HIF-1 α activity downstream of mTOR. Mice deficient in HIF-1 α have increased generation of Treg cells, and blocking glycolysis with 2-Deoxy-D-glucose (2-DG) inhibits Th17-cell differentiation (Dang et al., 2011; Shi et al., 2011). Treg-cell homeostasis and survival depends on the delicate balance between mTORC1 activation from PI3K-Akt and regulation from PTEN (Huynh et al., 2015;

Shrestha et al., 2015; Zeng et al., 2013). Signaling through mTORC1 versus mTORC2 also selectively differentiates CD4 T cells into the Th1 and Th2 lineages, respectively, (Delgoffe et al., 2011; Lee et al., 2010), although activation of mTORC1 and its component Raptor is still required for T-cell exit from quiescence to begin the transition into Th2 cells (Yang et al., 2013). Less is known about T follicular helper (Tfh) cell metabolism compared to other T cell subsets, but their lineage-defining transcription factor Bcl6, has been shown to suppress glycolysis potentiated by c-Myc and HIF-1 α (Johnston et al., 2009; Nurieva et al., 2009; Oestreich et al., 2014).

Substrate utilization in activated T cells

Glucose is a key metabolic substrate for T cells. Upon T-cell activation, Glut1 traffics to the cell surface from intracellular vesicles (Frauwirth et al., 2002; Rathmell et al., 2000; Wieman et al., 2007). Overexpression of Glut1 in mice results in larger naïve T cells and an increased number of CD44^{hi} T cells, suggesting that glucose acquisition mediates early steps in T-cell activation, such as promoting activation marker expression and increasing cell size (Jacobs et al., 2008). Consistent with these observations, T cell-specific deletion of Glut1 impairs CD4 T-cell activation, clonal expansion, and survival (Macintyre et al., 2014). When deprived of glucose, CD8 T cells display defects in functional capacity with reduced IFN- γ , granzyme, and perforin production (Cham et al., 2008; Cham and Gajewski, 2005; Jacobs et al., 2008). More recently, it was shown that T cells can become activated and proliferate when glucose catabolism through aerobic glycolysis is limited as they can rely on OXPHOS (Chang et al., 2013; Sena et al., 2013). However, in this case, effector function is compromised such that cytokine production is

impaired due to posttranscriptional regulation by the glycolytic enzyme GAPDH. When disengaged from glycolysis, GAPDH can function as a RNA-binding protein (RBP) and prevent the translation of cytokine messenger RNAs containing AU rich elements in their 3'-UTRs (Chang et al., 2013). Therefore, in addition to providing precursors for biomass, augmenting aerobic glycolysis in activated T cells allows for the acquisition of full effector function.

Amino acids

While glucose is a critical substrate for T cells, glutamine is also essential during T-cell activation (Carr et al., 2010; Frauwirth et al., 2002; Wang et al., 2011). T cells increase the expression of glutamine transporters and their deletion impairs the transition to an effector T cell (Carr et al., 2010; Sinclair et al., 2013). Clear differences in concentrations of other amino acids also exist in quiescent compared to activated T cells, corresponding to their distinct metabolic requirements (Ananieva et al., 2014; Pearson et al., 2012). New research has begun to uncover the vast array of additional amino acid transporters and catabolizing enzymes that regulate amino acid levels, revealing previously unappreciated roles for amino acids in T-cell metabolism and function.

Deficiency in the neutral amino acid transporter Slc7a5 (LAT1), which transports leucine, was found to prevent the ability of both CD4 and CD8 T cells to metabolically reprogram and undergo clonal expansion or effector differentiation (Hayashi et al., 2013; Sinclair et al., 2013). These cells had impaired mTORC1 activation and the inability to induce key metabolic processes, such as glutamine and glucose uptake (Sinclair et al., 2013). This deficiency, however, did not impair the ability of CD4 T cells to differentiate into Treg cells. Leucine can

activate mTOR via leucyl-tRNA synthetase, and thus it is not surprising that reduced leucine uptake impairs mTOR activation (Han et al., 2012). However the effects of Slc7a5 deficiency was more severe than those induced by mTOR inhibition using rapamycin (Sinclair et al., 2013), suggesting either that rapamycin may not have completely blocked mTOR activation, or that leucine deficiency has effects over and above limiting mTOR activation (Powell, 2013; Thoreen et al., 2009). Additionally, it was observed that although no overt decrease in global protein expression occurred in Slc7a5-deficient cells, protein expression of the key metabolic transcription factor c-Myc was diminished, despite its increased mRNA expression upon activation (Sinclair et al., 2013). This raises the intriguing question of whether leucine deficiency results in posttranslational regulation of c-Myc expression. Alternatively this effect could simply be due to a limitation in the supply of amino acids in Slc7a5-deficient cells, which is not sufficient to keep up with the demands of synthesizing proteins such as c-Myc, which have a short half-life (Sinclair et al., 2013).

Results from another study suggest that modulation of intracellular leucine concentrations can be used to regulate metabolic reprogramming. It was found that the expression of the cytosolic branched chain aminotransferase (BCATc), which can reduce intracellular leucine concentrations through a transamination reaction, limited mTORC1 activation (Ananieva et al., 2014). BCATc expression was upregulated upon CD4 T-cell activation, and T cells that lacked BCATc had increased intracellular leucine, which correlated with enhanced activation of mTORC1 and glycolytic phenotype. Increased BCATc expression has been observed in anergic T cells, which have impaired metabolic function (Ananieva et al., 2014; Zheng et al., 2009). These data could

suggest that leucine depletion by BCATc contributes to T-cell anergy through suppression of mTOR activity.

The alanine serine and cysteine transporter system (ASCT2/Slc1a5), which also transports glutamine, is another solute carrier whose expression increases following T-cell activation (Levring et al., 2012). It was recently found that loss of ASCT2 decreased glutamine import and impaired OXPHOS and glucose metabolism in activated CD4 T cells (Nakaya et al., 2014). Surprisingly, the loss of ASCT2 did not inhibit proliferation or IL-2 production. However, ASCT2-deficient cells cultured *in vitro* had a decreased ability to differentiate into Th1 and Th17 cells, but not Th2 or Treg cells. Interestingly, glutamine transport into cells can substantially enhance leucine transport via Slc7a5, as increased intracellular glutamine levels result in glutamine export and concomitant import of leucine by this transporter (Nicklin et al., 2009). Supporting this additional role for glutamine in T-cell activation, addition of leucine to T cells lacking ASCT2 helps rescue their polarization defects (Nakaya et al., 2014).

Depletion of extracellular arginine has been found to impair T-cell proliferation and aerobic glycolysis, but not mitochondrial OXPHOS (Fletcher et al., 2015). However, provided that extracellular concentrations of citrulline are sufficient, T cells can partially compensate by synthesizing arginine *de novo* in an argininosuccinate 1 (ASS1)-dependent process (Fletcher et al., 2015; Qualls et al., 2012; Tarasenko et al., 2015). One study suggested that ASS1 activity may contribute to T-cell function in ways beyond simple synthesis of arginine, as deletion of ASS1 can negatively impact *in vitro* Th1 and Th17 cell polarization, even in the presence of extracellular arginine (Tarasenko et al., 2015). Arginine metabolism also has a role in

macrophage polarization and dictating metabolic phenotype (Galvan-Pena and O'Neill, 2014; Rath et al., 2014), but whether arginine metabolism also has such roles in T cells remains to be determined.

A recent study suggests that intracellular recycling of amino acids also contributes to T-cell amino acid homeostasis. Deficiency in cytosolic protease tripeptidyl peptidase II (TPPII), which digests proteins for the recycling of amino acids, lead to increased sensitivity to perturbations in intracellular amino acids concentrations, impaired IFN- γ production, and a susceptibility to viral infections (Lu et al., 2014). Lack of TPPII activity in both human and murine T cells resulted in impaired glycolysis due to enhanced degradation of the key glycolytic enzyme hexokinase II, an effect that likely contributed to their impaired cytokine production (Lu et al., 2014). Another study also found that TPPII deficiency caused susceptibility to viral infections, although under these particular experimental conditions, defects in cytokine production were not observed and the T-cell dysfunction was attributed to premature immunosenescence (Stepensky et al., 2015). Although it remains to be tested, it may be possible that this premature cell senescence could be linked to reduced glycolytic flux. In a model of oncogenic stress-induced senescence, inhibition of hexokinase II or glucose uptake induces senescence in human epithelial cells (Gitenay et al., 2014).

Many products of amino acid catabolism also have important non-anaplerotic roles that can alter cell signaling and function. The metabolic byproduct of tryptophan catabolism, kynurenine, can ligate the aryl hydrocarbon receptor and enhance polarization of CD4 T cells to a Treg phenotype (Mezrich et al., 2010; Opitz et al., 2011). Another example of this is catabolism of

phenylalanine by interleukin-4-induced gene 1 protein (IL4I1). When highly expressed by tumors or APCs, IL4I1 can inhibit T cell proliferation (Boulland et al., 2007; Lasoudris et al., 2011). This effect appears to be due to the production of H₂O₂, a product of phenylalanine catabolism. IL4I1 is also expressed in Th17 and Treg cells (Santarlaschi et al., 2012; Scarlata et al., 2015). The specific purpose for IL4I1 expression in these T-cell subsets remains ambiguous, although its expression in Th17 cells was speculated to have a self-regulatory role, where its induction led to diminished proliferation (Santarlaschi et al., 2012). However given that low concentrations of H₂O₂ can act as a signaling molecule (Veal et al., 2007), IL4I1 might also play a role in cell signaling pathways independent of mechanisms that inhibit proliferation.

Fluctuations in environmental amino acid concentrations, as well as metabolic products from amino acid catabolism, can dramatically alter T-cell activity and polarization. A well documented example of this is indoleamine-2,3-dioxygenase (IDO)-mediated tryptophan catabolism. IDO, which is often expressed at high levels by antigen presenting cells (APCs) or tumor cells, can deplete tryptophan within a tissue microenvironment, and this in turn can lead to inhibition of effector T-cell proliferation and induction of anergy (Munn et al., 2002; Uyttenhove et al., 2003). Depletion of tryptophan causes activation of the integrated stress response inducer general control nonderepressible 2 (GCN2) kinase, which results in the inhibition of translation initiation and metabolic remodeling (Castilho et al., 2014; Guo and Cavener, 2007; Munn et al., 2005).

Studies into the interactions of APCs with T cells have highlighted multiple pathways through which APCs modulate extracellular concentrations of amino acids, or their catabolic products, to

regulate T-cell responses. In a tumor, TGF- β -producing dendritic cells (DCs) can enhance expression of transporters for histidine, leucine, valine, and tryptophan, depleting these amino acids from the extracellular microenvironment and directly impairing T-cell proliferation (Angelini et al., 2002). Treg cells can also enhance expression of particular amino acid catabolizing enzymes, including arginase 1, histidine decarboxylase, threonine dehydrogenase, and IL4I1, in skin grafts and bone marrow derived DCs (Cobbold et al., 2009). Limitations in these amino acids, singularly or in combination, enhanced Treg-cell polarization when T cells were activated *in vitro* (Cobbold et al., 2009). Although depletion of amino acids from the microenvironment appears to be a way in which APCs can negatively regulate T-cell activity, the converse also occurs, whereby APCs can support T-cell activation through supplementing a microenvironment. For example, DCs and monocytes can release cysteine, which is thought to support T-cell activation and function (Angelini et al., 2002; Sido et al., 2000). Cysteine supply is a limiting factor in T-cell proliferation and is used extensively for protein and glutathione synthesis, as well as providing beneficial catabolic products, such as taurine, which may support T-cell function through regulating osmolality (Kaesler et al., 2012; Sikalidis, 2015).

Lipid metabolism

Lipids or fatty acids encompass another critical substrate group for T cells. They are a vital component of cell membranes, provide a high yielding energy source, and can also supply substrates for cell signaling and PTMs (Lochner et al., 2015; Thurnher and Gruenbacher, 2015). Following T-cell activation, the demand for lipids rapidly increases. Within 24 hours, *in vitro* activated T cells augment fatty acid synthesis (FAS) while concomitantly decreasing FAO, thus enhancing the accumulation of fatty acid metabolites needed for membrane synthesis (Wang et

al., 2011). c-Myc and mTOR have important roles in coordinating these metabolic changes (Wang et al., 2011; Yang et al., 2013), and SREBP transcription factors are critical for reprogramming lipid metabolism (Kidani et al., 2013). SREBPs induce expression of genes involved in FAS and mevalonate pathways, which supply *de novo* synthesized fatty acids and cholesterol, respectively (Thurnher and Gruenbacher, 2015). CD4 T cells deficient in Raptor, and thus mTORC1 signaling, have impaired *de novo* FAS, most likely due to reduced expression of SREBP1 and SREBP2 protein (Yang et al., 2013).

Loss of SREBP function in CD8 T cells results in a failure to induce metabolic pathways needed for clonal expansion during a viral infection (Kidani et al., 2013). Exogenous cholesterol rescues the defects in SREBP-deficient T cells, suggesting that at least within this context, a lack of cholesterol is the main limiting factor. This requirement for cholesterol synthesis is consistent with results which show that perturbing sterol homeostasis in activated T cells – by activating the liver X receptor (LXR), which targets genes that are involved in cholesterol cellular export – impairs T-cell proliferation. The inhibitory effect of LXR activation can be overcome through the addition of mevalonate, a cholesterol precursor (Bensinger et al., 2008). Inhibition of 3-hydroxy-3-methylglutaryl-coenzymeA (HMG-CoA) reductase, an enzyme in the mevalonate pathway, results in a Th2-cell bias in the experimental autoimmune encephalomyelitis (EAE) disease model, due to impaired biosynthesis of isoprenoids and a subsequent reduction in prenylation of Ras and RhoA GTPases (Youssef et al., 2002). These data suggest that in addition to cholesterol homeostasis, other products of the mevalonate pathway can also influence T-cell differentiation. The impact of commonly used drugs that lower cholesterol by inhibiting HMG-

CoA reductase can affect both prenylation and cholesterol synthesis, and thus it is plausible these drugs have multiple effects on activated T cells.

The synthesis of fatty acids is also important for effector T-cell function. Although activated T cells readily acquire and use extracellular fatty acids, it appears that there may also be cell-intrinsic requirements for *de novo* synthesized fatty acids (Berod et al., 2014; Lee et al., 2014; O'Sullivan and Pearce, 2014; O'Sullivan et al., 2014). Inhibition of acetyl-CoA carboxylase 1 (ACC1), an enzyme in FAS, was shown to limit Th17-cell differentiation and promote the development of Treg cells. This effect translated into improved disease outcomes in EAE (Berod et al., 2014). Inhibition of ACC1 impaired phospholipid synthesis in Th17 cells while also impairing glycolytic flux, both through aerobic glycolysis and the TCA cycle. In contrast, Treg cells were able to sustain their requirements for fatty acids through acquisition from extracellular sources (Berod et al., 2014). ACC1 deficiency also impairs Th1 and Th2 development, suggesting that CD4 effector T cells have a common requirement for FAS (Berod et al., 2014). In contrast, T cell-specific deletion of ACC1 does not impair CD8 effector T-cell development after infection, although effector T-cell expansion is diminished due to increased cell death, indicating that FAS is required for the persistence of CD8 effector T cells (Lee et al., 2014). Collectively, these findings suggest that there are varying requirements for *de novo* synthesized fatty acids between different T-cell subsets. Interestingly, defects following ACC1 inhibition in either Th17 cells or CD8 effector T cells can be rescued through the addition of excess free fatty acids to the media (Berod et al., 2014; Lee et al., 2014), indicating that these cells can compensate for the lack of FAS if the extracellular fatty acid supply is plentiful. It has also been observed that addition of extracellular fatty acids can enhance T-cell proliferation (Gorjao et al.,

2007). It is plausible that the demand for fatty acids is so substantial in these highly proliferative populations that *de novo* FAS can be supplemented through extracellular uptake. This concept would be consistent with a recent study suggesting that lipid released from adipose tissue may enhance T-cell proliferation *in vivo* (Kim et al., 2015b). Responses to TNF-mediated signaling in the hypothalamus induced B- and T-cell proliferation in the spleen, an effect mediated by an induction of lipolysis through sympathetic nervous system signaling to adipose tissue and a resultant increase in circulating leptin and free fatty acids (Kim et al., 2015a).

Although in general the balance of FAS to FAO within effector T-cell populations is weighted heavily towards FAS, effector T cells can utilize FAO (Byersdorfer et al., 2013; O'Sullivan et al., 2014). Given that the demand for energy is high in these cells, it is likely that they need some metabolic flexibility in their fuel sources, an idea that is consistent with recent work highlighting the importance of adenosine monophosphate-activated protein kinase (AMPK) in effector T-cell function (Blagih et al., 2015). The extent to which FAO occurs in effector T cells is likely to be highly context dependent, in part due to the heterogeneity of this population of cells during an immune response. Studies using animal models of graft versus host disease (GvHD) have found that alloreactive T cells increase fatty acid uptake and enhance FAO compared to other effector T cells (Byersdorfer et al., 2013; Gatza et al., 2011; Glick et al., 2014). Suppressing Akt during activation can induce a metabolic profile suggestive of FAO utilization (Crompton et al., 2015), and culturing CD8 effector T cells in low glucose enhances FAO (O'Sullivan et al., 2014). A recent report also found that inhibition of T cell signaling through ligation of PD-1 induces changes in the metabolic profile of activated T cells, including decreased aerobic glycolysis and enhanced FAO (Patsoukis et al., 2015). Collectively, these data suggest that the utilization of

FAO in effector T cells may be influenced by a number of factors such as activation state, exposure to antigen, inflammatory signals, and micro-environmental nutrient availability.

Memory T cell metabolism

Effector T cell populations contract following pathogen clearance and undergo apoptosis, leaving behind a small population of long-lived memory T cells that can respond vigorously upon antigen rechallenge (Williams and Bevan, 2007). Although both naïve and memory T cells acquire effector functions upon activation, memory T cells have an accelerated response to antigen, proliferate faster, and produce more cytokines than their naïve counterparts. Work from our laboratory, and others, have shown that changes in metabolism also drive memory T-cell development (**Figure 1**) (Araki et al., 2009; Pearce et al., 2009; Rao et al., 2010).

AMPK and mTOR

Increases in intracellular AMP to ATP concentrations activate the energy stress sensor AMPK, a signal that also promotes FAO (Jones and Thompson, 2007). AMPK is important for the development of memory T cells and administration of the metabolic stressor and AMPK activator metformin enhances the generation of memory T cells following infection (Pearce et al., 2009; Rolf et al., 2013). In addition, AMPK allows for effector T cells to metabolically adapt during nutrient stress and modulates T-cell effector function through suppression of mTOR (Blagih et al., 2015; MacIver et al., 2011; Tamas et al., 2006). Inhibiting mTOR with rapamycin boosts memory T-cell development *in vivo* (Araki et al., 2009; Pearce et al., 2009; Rao et al., 2010). Loss of the mTORC1 negative regulator TSC1 compromises formation of memory T-cell

precursors that are present during the primary effector response (Kaech et al., 2003; Shrestha et al., 2014). Similarly, suppressing mTORC2 fosters memory T-cell generation (Pollizzi et al., 2015). Inhibition of mTOR and activation of AMPK also strongly stimulate the catabolic process of autophagy. Autophagy has been shown to support T-cell viability and bioenergetics after activation (Hubbard et al., 2010; Pua et al., 2007). Consistent with the idea that activation of catabolic pathways promotes the development of memory T cells, a recent study found that deletion of the autophagy molecules Atg5 or Atg7 compromised the formation of CD8 memory T cells after viral infection (Xu et al., 2014).

FAO and mitochondria

Work from our lab has shown that CD8 memory T cells are dependent on FAO for their development, long-term persistence, and ability to respond to antigen stimulation (**Figure 1**) (Pearce et al., 2009; van der Windt et al., 2012; van der Windt et al., 2013). Enhancing FAO in memory T cells through increased expression of carnitine palmitoyltransferase 1a (CPT1a) – a critical mitochondrial transporter of long chain fatty acids and rate limiting step to β -oxidation – increases CD8 memory T-cell numbers after infection (van der Windt et al., 2012). During an immune response, common γ chain cytokines like IL-15 and IL-7 have an essential role in supporting catabolic metabolism by promoting mitochondrial biogenesis, CPT1a expression, and FAO (van der Windt et al., 2012). As a result, memory T cells have increased mitochondrial mass and greater spare respiratory capacity (SRC) compared to naïve and effector T cells, which endows them with a bioenergetic advantage for survival and recall after antigen rechallenge (Gubser et al., 2013; van der Windt et al., 2012; van der Windt et al., 2013). Interestingly, a study investigating heterologous prime-boost vaccination found that memory T-cell

differentiation is hastened and enhanced after secondary or tertiary immunization, including acquisition of substantially greater mitochondrial mass and SRC (Fraser et al., 2013). In a sense, memory T cells are metabolically primed and ready to respond to secondary infections. This idea has recently been extended to innate immune cells under the novel concept of “trained immunity” whereby metabolic priming confers superior immunity to a secondary pathogenic insult (Cheng et al., 2014). Previous engagement of aerobic glycolysis in monocytes driven by mTOR and HIF-1 α was found to induce epigenetic modifications that endowed them with enhanced function against other infections (Cheng et al., 2014).

The specific role of FAO in promoting memory T-cell development and survival remains to be elucidated, but it appears that metabolic reprogramming associated with FAO enhances mitochondria-associated processes. Induction of FAO in memory T cells enhances SRC, which is the reserve capacity of mitochondria to produce energy over and above normal energy outputs (van der Windt et al., 2012). This parameter is probably important for the longevity of memory cells, especially in times of stress or nutrient restriction, conditions that may present themselves when infection is resolved and growth factor signals are scarce. Surprisingly, endothelial cells, unlike most other cell types, use carbon derived from FAO for nucleotide synthesis and proliferation (Schoors et al., 2015), providing another way in which FAO supports cell function.

Another unexpected discovery was that memory T cells preferentially use *de novo* FAS to fuel FAO (O'Sullivan et al., 2014). Specifically, CD8 memory T cells utilize glucose to produce triacylglycerides (TAGs) that are subsequently hydrolyzed by lysosomal acid lipase (LAL) to support mitochondrial FAO (O'Sullivan and Pearce, 2014). It was also recently shown that

glucose metabolism is critical for CD4 memory T-cell survival, and this is controlled by Notch signaling (Maekawa et al., 2015). The requirement for FAS in CD8 memory T cells is supported by a recent study showing that glycerol import into the cell via IL-7-induced aquaporin-mediated transport is required for memory T-cell longevity (Cui et al., 2015). Glycerol is the molecular backbone for TAGs. Aquaporin 9 (AQP9)-deficient T cells had reduced glycerol import and TAG synthesis and impaired memory T-cell survival following viral infection (Cui et al., 2015).

The reasons why CD8 memory T cells synthesize and then catabolize fatty acids in an apparently futile cycle rather than simply acquire extracellular fatty acids are not understood. However, this synthesis/catabolism cycle has also been shown to occur in muscle and adipose tissues (Dulloo et al., 2004; Yu et al., 2002). If viewed on a purely energetic level, this process appears counter-productive, as there would be no net gain in ATP. It is possible that building and burning fatty acids allows memory T cells to sustain their glycolytic and lipogenic machinery while maintaining mitochondrial health during times of quiescence, allowing for the rapid recall ability that is characteristic of memory T cells following antigen recognition and activation (Gubser et al., 2013; van der Windt et al., 2013). It could also potentially provide a mechanism for balancing redox state or metabolic intermediates. An energetically futile cycle has been described in yeast grown in glucose rich media, whereby trehalose cycling provides a buffer system to maintain intracellular phosphate levels and balance glycolytic intermediates (van Heerden et al., 2014).

Emerging topics and concluding remarks

It is an exciting time for the field of immunometabolism. While the body of literature on this topic is increasing at an exponential rate, much remains to be explored. We are just beginning to understand the many connections between metabolism and gene regulation in T cells (Kaelin and McKnight, 2013; Lu and Thompson, 2012; Ost and Pospisilik, 2015; Wang and Green, 2012). Acetyl-CoA and NAD^+ generated from oxidative metabolism are used for histone acetyltransferase (HAT) and histone deacetylase (HDAC) activity (Canto and Auwerx, 2011; Imai and Guarente, 2010; Wellen and Thompson, 2012). Protein acetylation is a reversible PTM that influences epigenetic changes mediated by HATs and HDACs and also controls the actions of transcription factors and molecular chaperones (Glozak et al., 2005). T-cell metabolic reprogramming during activation increases cytosolic NAD^+ and citrate, the precursor of acetyl-CoA, which may direct cell-fate decisions through protein acetylation (Berger et al., 1987). In agreement with this idea, lineage-specific cytokine-encoding genes that affect T-cell differentiation undergo dynamic changes in histone acetylation after antigen-driven stimulation (Avni et al., 2002; Fields et al., 2002). The actions of the transcription factor FoxP3 in directing Treg-cell development are controlled through opposing activities of the HATs p300 and TIP60, and the HDAC Sirtuin1 (Sirt1) (Beier et al., 2011; Li et al., 2007; Tao et al., 2007; van Loosdregt et al., 2010). Acetylation also affects the activity of circadian clock proteins, and reciprocally, the circadian acetylome has been found to regulate the epigenome and mitochondrial metabolic pathways (Koike et al., 2012; Lu and Thompson, 2012; Masri et al., 2013). Organisms rely on the cell autonomous transcription–translation oscillator loop managed by solar time to accommodate physiological changes brought about by the daily pattern of rest, activity, and

feeding (Curtis et al., 2014). The circadian clock also in part regulates Th17 cell development (Yu et al., 2013). Circadian rhythm controls nutrient acquisition and metabolic flux and it will be interesting to see how the body's internal clock may connect to lymphocyte metabolism, regulation and function (Rey and Reddy, 2013).

Intermediates from glucose catabolism can be converted into substrates that are needed to support cell growth and proliferation. Recent studies of one-carbon metabolism in cancer research involving the serine and glycine biosynthetic pathways may have implications for T-cell metabolism, given the many common features shared between activated T cells and proliferating cancer cells. Although the role of one-carbon metabolism in generating units for nucleic acid synthesis from folate has long been appreciated, more recently it was recognized that this pathway is an important source of NADPH to maintain redox balance and methyl groups for methylation (Fan et al., 2014; Locasale, 2013). For example, depletion of methylene tetrahydrofolate dehydrogenase (MTHFD) in cancer cells results in a decreased cellular NADPH/NADP⁺ ratio and increased oxidized glutathione, enhancing sensitivity to oxidative stress (Fan et al., 2014). Serine and glycine metabolism also have a vital role in cell survival under harsh environmental conditions of nutrient scarcity and hypoxia (Kim et al., 2015a). Tumors foster these kinds of conditions, but they are able to survive and function under these stressors. A recent study found that increased expression of mitochondrial serine hydroxymethyltransferase (SHMT2) and glycine decarboxylase (GLDC) confer a survival advantage for glioma cells by allowing them to lower their oxygen consumption and metabolize toxic molecules in the tumor microenvironment (Kim et al., 2015a). T cells also migrate and travel to sites of infection or tumors and must adapt to these hypoxic or nutrient-depleted

environments (Pearce et al., 2013). It remains to be explored whether these metabolic pathways support lymphocyte survival by similar mechanisms or have evolved to serve other purposes.

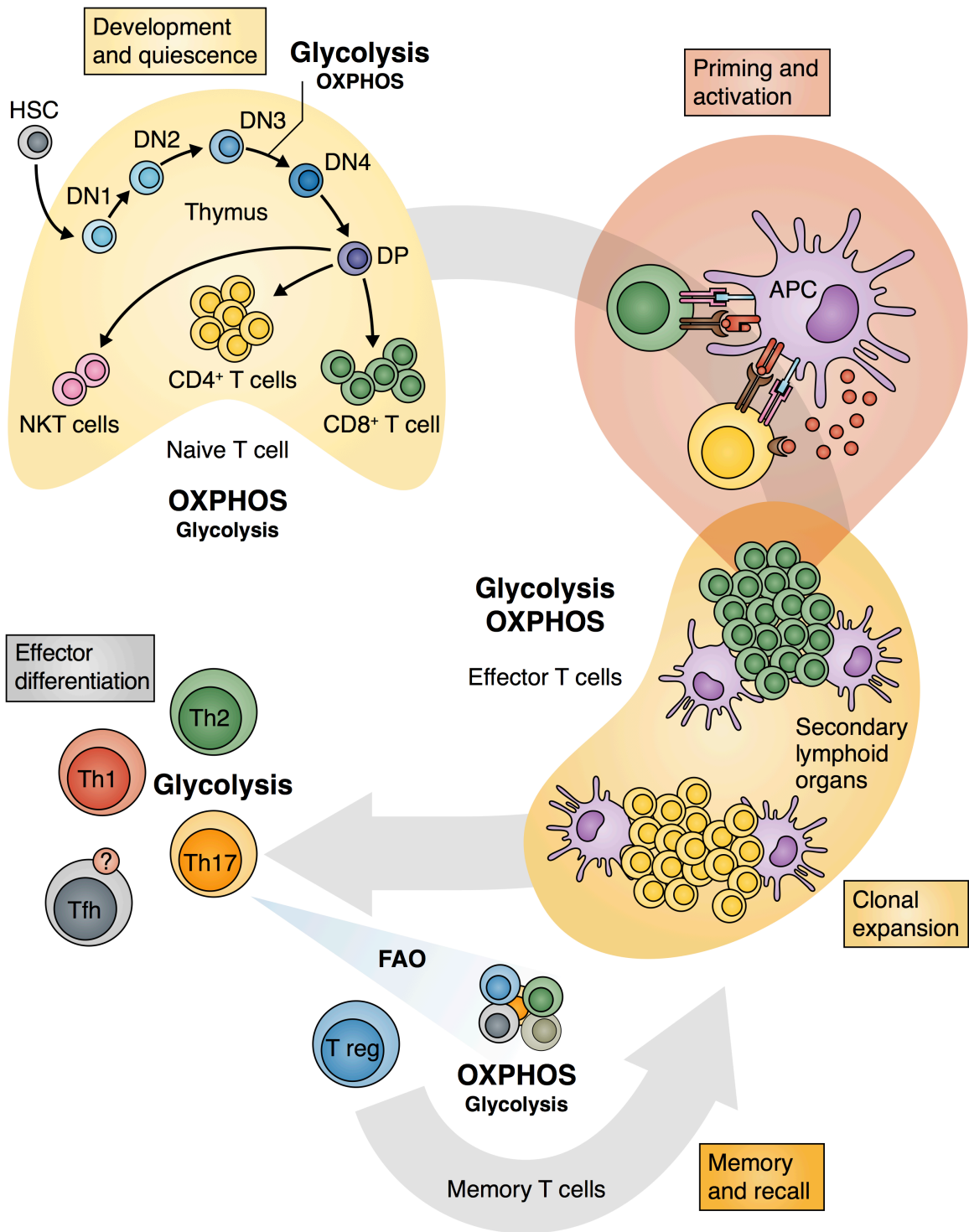
Another area of interest is how substrate availability affects T-cell differentiation and their functional fate. Studies investigating the relationship between the gut microbiome and lymphocytes have found that metabolites produced by commensal bacteria have important implications for maintaining immune cell gastrointestinal homeostasis and defense against pathogens. Short chain fatty acids (SCFA) such as butyrate, acetate, and propionate produced by bacteria induce differentiation of colonic Treg cells (Furusawa et al., 2013; Smith et al., 2013) and also Th17 cells under certain conditions (Park et al., 2015). The vitamin A metabolite retinoic acid can synergize with TGF- β to stimulate Treg conversion (Coombes et al., 2007; Denning et al., 2007; Elias et al., 2008; Mucida et al., 2007; Sun et al., 2007). However, vitamin A metabolite deficiency also abrogates Th1 and Th17 cell immunity (Hall et al., 2011) and, more recently, was found to diminish type 3 innate lymphoid cells (ILC3s), but expand ILC2 cells (Spencer et al., 2014). Lymphocytes can also regulate whole body metabolism by affecting the tissues in which they reside. ILC2s can promote 'beiging' of white adipose tissue and therefore control caloric expenditure through secretion of methionine-enkephaline peptides (Brestoff et al., 2015). Loss of insulin sensitivity due to inflammation of adipose tissue in obesity and type 2 diabetes results in part from deficiencies in adipose tissue-specific Treg cell populations controlled by the transcriptional regulator PPAR- γ (Cipolletta et al., 2012; Feuerer et al., 2009). These are just a few examples of what remains to be explored in the interplay between lymphocytes, their environment, and metabolism.

In the development of novel therapeutics for the treatment of human disease, targeting T-cell metabolism provides a unique opportunity to manipulate T-cell function (O'Sullivan and Pearce, 2015). For example, it has been demonstrated that compared to Th1 and Treg cells, Th17 cells have elevated pyruvate dehydrogenase kinase 1 (PDK1) expression, which promotes aerobic glycolysis through inhibition of pyruvate dehydrogenase (PDH). Inhibition of PDK1 using dichloroacetate (DCA) selectively impairs Th17 proliferation and survival and reduces T-cell mediated inflammation in models of inflammatory bowel disease and EAE (Gerriets et al., 2015). Targeting trophic transporters on T cells may also provide a way in which to manipulate T-cell function through altering their nutrient uptake; for example JPH203, which inhibits amino acid transporter Slc7a5, could be used to inhibit inflammatory T cells without impairing Treg-cell function (Hayashi et al., 2013; Sinclair et al., 2013). In the context of cancer, the development of adoptive cellular immunotherapies using *in vitro* expanded tumor infiltrating lymphocytes (TILs) could benefit from tailoring culture conditions to optimize TIL metabolism prior to transfer into the patient (Restifo et al., 2012). Exaggerated glycolysis and cell size resulting from *in vitro* expansion conditions, such as high glucose, can be detrimental to TIL survival and persistence *in vivo*. Strategies to limit glycolysis directly or suppress Akt activation in TILs have already shown promising results in this context (Crompton et al., 2015; Sukumar et al., 2013). Inhibiting glycolysis and oxidative metabolism with 2-DG and metformin may also hold therapeutic potential in other disease settings as highlighted by a recent study using models of system lupus erythematosus (Yin et al., 2015).

It is apparent that many diverse processes integrate with lymphocyte signaling, gene regulation, and function to shape T-cell metabolism. Understanding the metabolic regulation that dictates T-

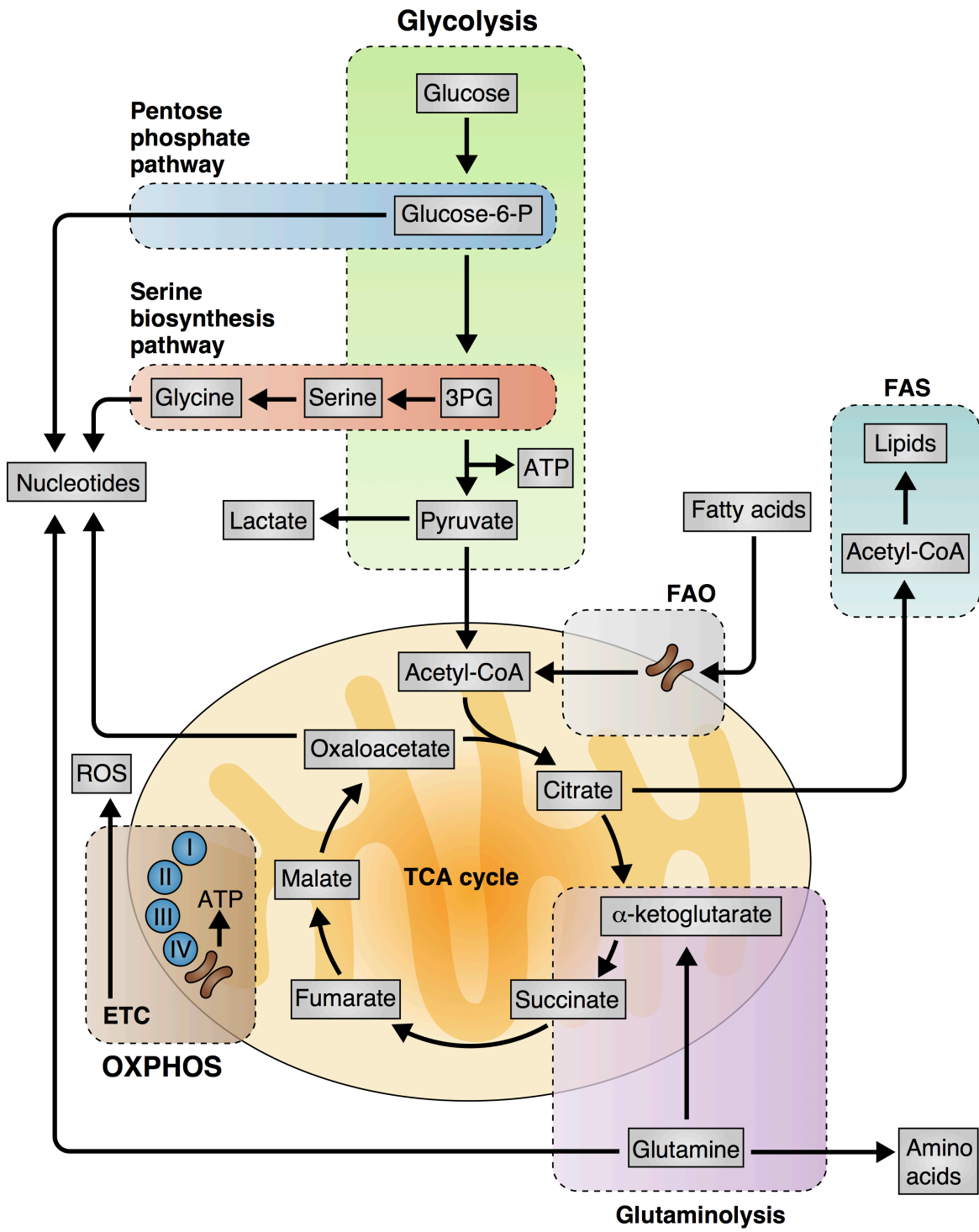
cell fate and how nutrient availability and micro-environmental factors influence T-cell function will provide further insight into immune cell biology and could lead to new approaches to treating human diseases.

Figure 1. Metabolism drives the life cycle of T cells.



T cells engage specific metabolic pathways during the course of their development that underpin their differentiation and function. Naïve T cells mature and exit from the thymus primarily relying on OXPHOS for their metabolic needs, although they augment glycolytic metabolism during times of proliferation that follow TCR gene rearrangements. In secondary lymphoid organs, TCR ligation, costimulation, and growth factor cytokine signals induce clonal expansion and metabolic reprogramming of an antigen-specific T cell. This conversion to an activated effector T cell is marked by the engagement of aerobic glycolysis and increased OXPHOS activity. Glycolytic metabolism differentiates CD4 Th1, Th2, and Th17 effector cells (and possibly Tfh cells) from Treg cells. Promoting FAO and catabolic metabolism enhances Treg and memory T cell development (blue arrow). Memory T cells are a quiescent population of cells that primarily use OXPHOS, but both OXPHOS and glycolysis increase rapidly after antigen rechallenge and facilitate their recall responses.

Figure 2. Metabolic pathways that support T cells.



ATP is the molecular currency of energy in the cell. It can be derived from glucose through two integrated pathways. The first of these, glycolysis (green), involves the enzymatic breakdown of glucose to pyruvate in the cytoplasm. The TCA cycle (orange) encompasses the second pathway where pyruvate is converted to acetyl-CoA in the mitochondria and shuttled through several enzymatic reactions to generate reducing equivalents to fuel OXPHOS (brown). Other substrates can also be metabolized in the TCA cycle, such as glutamine via glutaminolysis (purple) or fatty acids via β -oxidation (FAO) (gray). These connected biochemical pathways can also provide metabolic precursors for biosynthesis. Intermediates from glucose catabolism during glycolysis can shuttle through the pentose phosphate (dark blue) and serine biosynthesis pathways (red) to fuel nucleotide and amino acid production. Oxaloacetate from the TCA cycle can similarly be used to generate aspartate for use in nucleotide synthesis. Precursors for amino acid and nucleotide biosynthesis can be obtained from glutamine. Citrate from the TCA cycle can be exported from the mitochondria and converted to acetyl-CoA for FAS (light blue). ROS generated from the ETC during OXPHOS can also act as secondary signaling molecules.

Chapter 2: Metabolic Instruction of Immunity

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Abstract

Choices. Choices have consequences. Immune cells survey and migrate throughout the body and sometimes take residence in niche environments with distinct communities of cells, extracellular matrix, and nutrients that may differ from those in which they matured. Imbedded in immune cell physiology are metabolic pathways and metabolites that not only provide energy and substrates for growth and survival, but also instruct effector functions, differentiation, and gene expression. This review of immunometabolism will reference the most recent literature to cover the choices that environments impose on the metabolism and function of immune cells and highlight their consequences during homeostasis and disease.

Introduction

Cells of the immune system possess particular sets of skills – skills that are vital for host defense and tissue homeostasis, but also cause disease if not properly controlled – skills that make them altogether fairly peculiar. Unlike other cells in the body, immune cells possess the ability to respond to environmental signals and assume a wide variety of distinct functional fates. Immune cells can morph from dormant sentinels into pathogen killing machines, migrate from one tissue to another, modulate surface receptor expression, clonally expand, secrete copious amounts of effector molecules, or exert controlling effects over neighboring cells. After the burst of activity following an immune response, these specialized cells can die, creating space and limiting tissue damage in a particular environment, or return to resting states that allow them to persist for extended periods of time in readiness for a secondary response.

The activation, expansion, engagement of effector functions, and return to homeostasis of immune cells are intimately linked and dependent on dynamic changes in cellular metabolism. The utilization of particular metabolic pathways is controlled on one level by growth factors and nutrient availability dictated by competition between other interacting cells, and on another level by the exquisite balance of internal metabolites, reactive oxygen species (ROS), and reductive and oxidizing substrates. Studying immune cells, particularly lymphocytes and myeloid cells, has lent deep insight into how cells differentiate and coordinate their behaviors with metabolism under a wide array of settings.

Leukocytes are also nomads and settlers. They migrate from the place where they develop to survey the entire body, and sometimes take up residence in tissues in which they did not originate. In doing so, they must adapt to an ecosystem comprised of unique cells, extracellular matrix, growth factors, oxygen, nutrients, and metabolites. How do they do this and what are the genetic, metabolic, and immunological consequences of these adaptations? In this review, we will explore the interactions between immune cells and the tissue environments they inhabit, how these impinge on their metabolism, how their metabolism instructs their function and fate, and how these relationships contribute to tissue homeostasis and disease pathology. The central concepts of immune cell metabolism have been covered extensively in several reviews (Buck et al., 2015; MacIver et al., 2013; O'Neill and Pearce, 2016; O'Neill et al., 2016; Pearce et al., 2013), and thus will not be discussed at length here.

The tumor microenvironment

Recent breakthroughs in immunotherapy have shown that eliciting immune responses against multiple types of cancer can lead to considerably longer-lasting remissions, or in some cases complete regression of metastatic disease (Ribas, 2015). Although it is well known that cancer cells can evade immune recognition through “immunoediting”, the process by which antitumor immune responses, especially those from tumor infiltrating T lymphocytes (TILs), select for cancer cell clones that no longer express detectable tumor antigens (Vesely and Schreiber, 2013), the advent of effective cancer immunotherapies has shown that additional mechanisms of immunosuppression exist that limit or impair antitumor immunity. Thus, considerable efforts are underway to elucidate other mechanisms that restrain antitumor responses to develop new and more efficacious forms of immunotherapy.

At the forefront of these mechanisms to consider is how immune cell metabolism, and thus immune cell function, is altered by the tumor microenvironment. Tumors are a major disturbance to tissue homeostasis, creating metabolically demanding environments that encroach on the metabolism and function of infiltrating immune and stromal cells. The unrestrained cell growth seen in cancer is often supported by aerobic glycolysis, the same metabolic pathway needed to fuel optimal effector functions in many immune cells (Pearce et al., 2013; Warburg, 1956; Warburg et al., 1958). At minimum, this similarity creates a competition for substrates between tumors and immune cells. The demand for nutrients, essential metabolites, and oxygen imposed by proliferative cancer cells, in combination with their immunosuppressive by-products, creates harsh environmental conditions in which immune cells must navigate and adapt (**Figure 3**). How

tumor and immune cells share or compete for resources in this environment, and how such relationships regulate antitumor immunity are important questions to address.

Hypoxia

When tumor growth exceeds the vasculature's ability to fully perfuse the tumor microenvironment with oxygen, regions of hypoxia are established and induction of the hypoxia-responsive transcription factor HIF-1 α intensifies cancer cell glucose utilization and lactate release (Eales et al., 2016). Using ¹³C-labeled glucose, Hensley and colleagues traced the fate of glucose within healthy lung tissue and tumors of patients with non-small lung cell carcinoma and found that even within a single tumor, heterogeneity in glucose utilization exists (Hensley et al., 2016). Lesser-perfused regions of the lung tumors were associated with higher glucose metabolism whereas higher-perfused regions could utilize circulating lactate, transported through monocarboxylate transporter 1 (MCT1), as an alternative TCA cycle substrate (Sonveaux et al., 2008). Lactate metabolism in oxygenated cancer cells also increases glutaminolysis (Pérez-Escuredo et al., 2015). How this metabolic heterogeneity in tumor cells relates to intratumoral immune cell function has not been well elucidated, but exposure of NK and T cells to high concentrations of lactate impairs their activation of the transcription factor NFAT and production of the cytokine IFN- γ (Brand et al., 2016). Lactic acid also disrupts T cell motility and causes loss of cytolytic function in CD8 T cells (Haas et al., 2015). Moreover, decreasing conversion of pyruvate to lactate by genetic targeting of lactate dehydrogenase A (LDHA) in tumors helps to restore T cell infiltration and function (Brand et al., 2016), linking lactate production to immunosuppression observed in the tumor microenvironment (**Figure 3**).

Lactate uptake by tumor-associated macrophages (TAMs) also stimulates tumor progression by inducing vascular endothelial growth factor (VEGF) and arginase I (Arg1) expression through HIF-1 α (Colegio et al., 2014). Moreover, chronic VEGF signaling in hypoxic areas leads to elevated glycolysis in endothelial cells, resulting in excessive endothelial sprouting and abnormal leaky vasculature (Goveia et al., 2014). Interestingly, inhibition of REDD1, a hypoxia-induced inhibitor of mTOR, in TAMs increases their rates of glycolysis to a level that competes with neighboring endothelial cells for glucose and suppresses their angiogenic activity. This metabolic tug-of-war over glucose helps restore vascular integrity, improve oxygenation within the tumor, and prevent metastases (Wenes et al., 2016), providing an example of an intimate metabolic relationship that exists between cells in tumors.

Hypoxia also has considerable effects on TIL function, proliferation, and migration (Vuillefroy de Silly et al., 2016). Increases in HIF-1 α activity by culturing T cells in physiologic normoxia (~3-5% O₂), genetic deletion of von Hippel-Lindau (VHL) factor, or inhibiting activity of the oxygen-sensing prolyl-hydroxylase (PHD) family of proteins, enhances CD8 T cell glycolysis and effector functions and promotes antitumor immunity (Clever et al., 2016; Doedens et al., 2013; Finlay et al., 2012; Wang et al., 2011). HIF-1 α is also needed for the production of the metabolite S-2-hydroxyglutarate (S-2HG), which can drive epigenetic remodeling in activated CD8 T cells and enhance IL-2 production and antitumor defenses (Tyraakis et al., 2016).

Thus, one may expect that hypoxia would potentiate HIF-1 α activity and TIL effector functions in tumors, however this is not what is observed. In addition to signals received from TIL IFN- γ (Noman et al., 2014), HIF-1 α also induces the expression of the suppressive ligand PD-L1 in

tumor cells, TAMs, and myeloid-derived suppressor cells (MDSCs), and this can lead to TIL suppression via PD-1 (**Figure 3**). Moreover, recent work in both mouse and human tumors showed that CD8 TILs lose mitochondrial mass, membrane potential, and oxidative capacity, particularly within the most dysfunctional PD-1⁺ CD8 T cells (Scharping et al., 2016). The loss of mitochondrial function in TILs correlated with diminished expression of PPAR-gamma coactivator 1 α (PGC1 α) over time and a block in their proliferation and IFN- γ production. Perhaps severe hypoxia ultimately diminishes TIL effector functions. Indeed, respiratory supplementation of oxygen or treatment with metformin decreased intratumoral hypoxia and relieved several immunosuppressive features in the tumor microenvironment; the latter also served as an adjunct therapy that enhanced the antitumor effects of PD-1 blockade (Hatfield et al., 2015; Scharping et al., 2017). These findings suggest that remodeling the hypoxic tone in tumors may be an essential component to developing more efficacious forms of immunotherapy for patients.

Nutrient alterations and competition within the tumor microenvironment

Apart from hypoxia, the competition for nutrients and metabolites between tumor cells and infiltrating immune and stromal cells can be fierce, consequently influencing signal transduction, gene expression, and the metabolic activities of these neighboring cells. For example, tumor cells manipulate surrounding adipocytes to increase lipolysis to whet their appetite for fatty acids (Nieman et al., 2011). Cancer associated fibroblasts degrade tryptophan that not only starves immune cells in the local environment of an essential amino acid, but also leads to the production of the immunosuppressive metabolite kynurenine (Hsu et al., 2016). Moreover, glucose is a critical substrate for the antitumor functions of effector T cells and M1 macrophages,

which both require engagement of aerobic glycolysis for their activation and full effector functions (Buck et al., 2015; O'Neill and Pearce, 2016). Augmented aerobic glycolysis in cancer cells and endothelial cells places immune cells and their neighbors at odds (**Figure 3**). Glucose deprivation represses Ca^{2+} signaling, IFN- γ production, cytotoxicity, and motility in T cells and pro-inflammatory functions in macrophages (Cham et al., 2008; Chang et al., 2013; Chang et al., 2015; Macintyre et al., 2014). Several recent studies have demonstrated that the glycolytic activities of cancer cells may restrict glucose utilization by TILs, thereby impairing antitumor immunity (Chang et al., 2015; Ho et al., 2015; Zhao et al., 2015). Increasing glycolysis rates of tumor cells through overexpression of the glycolytic enzyme hexokinase 2 (HK2) suppressed glucose-uptake and IFN- γ production in TILs and created more immunoevasive tumors (Chang et al., 2015; Ho et al., 2015). Zhao and colleagues found that glucose restriction imposed by ovarian cancer leaves microRNA repression of the methyltransferase EZH2 intact in CD8 T cells, reducing their survival and functional capacity (Zhao et al., 2015). Thus, tumor cells can selfishly coerce or outcompete neighboring cells for glucose to supply their own metabolic demands in a manner that simultaneously suppresses immune defenses.

Amino acid deprivation in the tumor microenvironment serves as another metabolic checkpoint regulating antitumor immunity. Glutaminolysis in tumor cells is critical to replenish metabolites through anaplerotic reactions, which could result in competition for glutamine between tumor cells and TILs (Jin et al., 2015; Pérez-Escuredo et al., 2015). Glutamine controls mTOR activation in T cells and macrophages and is also a key substrate for protein O-GlcNAcylation and synthesis of S-2HG that regulate effector T cell function and differentiation (Sinclair et al., 2013; Swamy et al., 2016; Tyrakis et al., 2016). TAMs, MDSCs, and tolerizing dendritic cells

(DCs) can suppress TILs through expression of essential amino acid (EAA)-degrading enzymes such as Arg1 and indoleamine-2,3-dioxygenase (IDO) (**Figure 3**) (Lee et al., 2002; Munn et al., 2002; Rodriguez, 2004; Uyttenhove et al., 2003). Indeed, inhibitors of Arg1 and IDO are under investigation as therapeutic targets in clinical trials (Adams et al., 2015). Several recent studies have highlighted the critical roles of other amino acids such as arginine, serine, and glycine in driving T cell expansion and antitumor activity, but how the availability of these fluctuate within the tumor microenvironment is not clear (Geiger et al., 2016; Ma et al., 2017). Currently, a knowledge gap exists of how the availability of various nutrients and metabolites vary across tumor types, genotypes or even spatially within tumors to affect antitumor immune responses.

Bioactive lipids, modified lipoproteins, and cholesterol metabolism within the tumor are also important mediators of immune cell function. Like macrophages in atherosclerotic plaques, DCs in the tumor can accumulate oxidized lipoproteins through scavenger receptor mediated internalization and formation of lipid droplets, which can ultimately impair their ability to cross-present tumor antigens and activate T cells (Cubillos-Ruiz et al., 2015; Ramakrishnan et al., 2014). Expression of lectin-type oxidized LDL receptor 1 (LOX-1) selectively marks MDSCs and oxidized lipid uptake and lipoprotein metabolism contributes to their T cell suppressive functions (Condamine et al., 2016). In addition, blocking cholesterol esterification in TILs by targeting ACAT1 pharmacologically or genetically increases intracellular levels of cholesterol and confers superior T cell responses in a model of melanoma (Yang et al., 2016). It is possible that as immune cells adapt to different tumor microenvironments and the limited availability of “immune stimulatory” nutrients, they become more dependent on alternative fuel sources (such as fats or lactate) that are less conducive to supporting antitumor effector functions. In summary,

more elaborate knowledge of these forms of metabolic cross talk or competition between cells within tumors is needed before one can begin to think about how to manipulate these relationships in a manner that alters tumor progression.

Metabolic exhaustion in TILs and checkpoint blockade

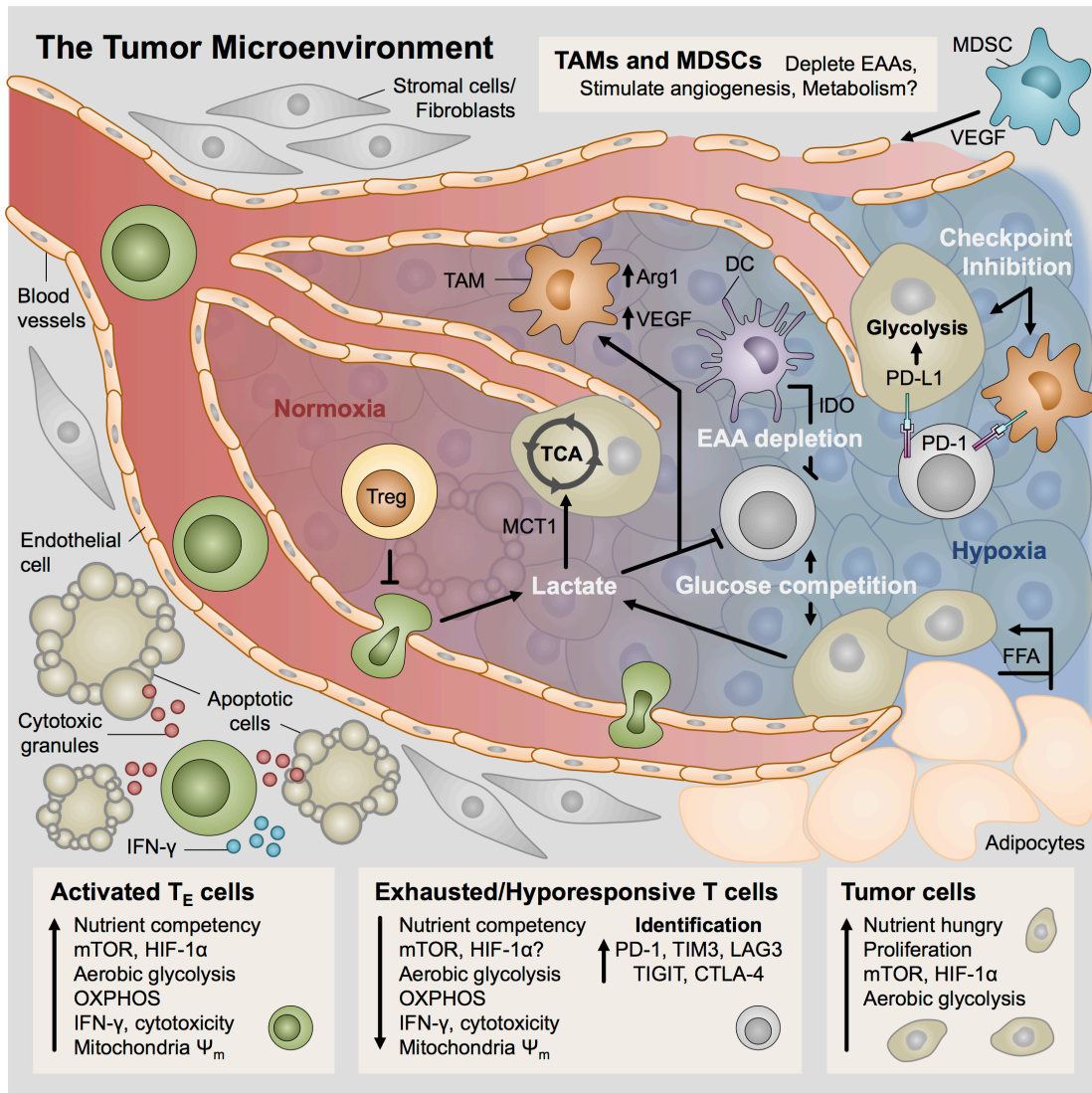
As TILs adapt to the tumor microenvironment, they progressively lose their ability to respond to T cell receptor (TCR) stimuli, produce effector cytokines, and proliferate – a process termed functional exhaustion or hyporesponsiveness. This is in part due to the upregulation of several inhibitory receptors like PD-1, LAG3, TIGIT, and CTLA-4 that desensitize T cells to tumor antigens (Wherry and Kurachi, 2015) (**Figure 3**). PD-1, its ligand PD-L1, and CTLA-4 are important checkpoints for T cells in tumors and the targets of a new and powerful class of cancer treatments that elicit effective and durable responses in patients across multiple cancer types (Ribas, 2015). Interestingly, both chronic exposure to antigen or environmental triggers such as glucose deprivation can upregulate PD-1 (Chang et al., 2013; Wherry and Kurachi, 2015). PD-1 not only suppresses TCR, PI3K and mTOR signaling in T cells, but also dampens glycolysis and promotes fatty acid oxidation (FAO) – features that may enhance the accumulation of suppressive regulatory CD4 T (Treg) cells in tumors (Bengsch et al., 2016; Parry et al., 2005; Patsoukis et al., 2015). Indeed, blockade of PD-1 re-energizes anabolic metabolism and glycolysis in exhausted T cells in an mTORC1 dependent manner (Chang et al., 2015; Staron et al., 2014). This breathes caution into the types of drug combinations one may consider with α -PD-1:PD-L1 blockade or other forms of immunotherapy. Metabolic interventions, such as the use of mTOR inhibitors, must be targeted specifically to avoid unintended compromises of immune cell function. The PD-1:PD-L1 axis may also directly affect the metabolic activity of

tumor cells (**Figure 3**). It was shown that PD-L1 expression correlated with the rates of glycolysis and the expression of glycolytic enzymes in those cells (Chang et al., 2015). Furthermore, checkpoint blockade antibodies including α -PD-L1 led to an increase in extracellular glucose in tumors *in vivo* that likely contributed to the improved TIL function and subsequent tumor regression observed. On this note, tumor cell-intrinsic PD-1 expression has also been observed that counter intuitively may increase intrinsic mTOR signaling and tumor growth (Kleffel et al., 2015). Collectively, these findings suggest there may be broader role of the PD-1:PD-L1 axis in cellular metabolism that extends beyond T cells.

Improving the proportion of patients that respond to immunotherapy is an intense area of study, ranging from the search for biomarkers of response, target discovery, to testing new combination therapies. Likely, the most effective therapies will coordinately target co-inhibitory receptor to ligand interactions and restore a T cells' ability to utilize metabolic substrates necessary to sustain their effector functions. Seemingly paradoxical is the observation that dampening effector T cell differentiation by impairing glycolysis and boosting mitochondrial FAO and OXPHOS potentiates their survival and functional capacity (Buck et al., 2016; Sukumar et al., 2013). Additionally, manipulating metabolic enzyme expression to help T cells adapt to metabolic perturbations in the tumor microenvironment may be other viable strategies (Clever et al., 2016; Doedens et al., 2013; Ho et al., 2015; Scharping et al., 2016), especially for adoptive cell therapy, a personalized form of cancer treatment that allows for the manipulation and expansion of a patient's antitumor T cells prior to re-infusion (O'Sullivan and Pearce, 2015). As our capability to selectively reprogram T cell metabolism and reinvigorate tumor-specific T cells

improves, there is much promise to provide greater therapeutic benefits to more patients, especially to those with previously incurable cancers.

Figure 3. Metabolic tug-of-war within the tumor microenvironment.



The balance of nutrients and oxygen within the tumor microenvironment controls immune cell function. Glucose and amino acid consumption by tumor cells can outpace that of infiltrating immune cells, specifically depriving them of nutrients to fuel their effector function. Poorly perfused tumor regions drive hypoxia response programs in tumor cells, macrophages, and T cells. Increased HIF-1 α activity in response to hypoxia or other mechanisms promotes glycolysis and increases concentrations of suppressive metabolites and acidification of the local environment. As a by-product of glycolysis, lactate concentration increases, which is coordinately utilized by tumor cells to fuel their metabolism, promotes macrophage polarization, and directly suppresses T cell function. The ability of T cells to target tumors is further limited by their upregulation of co-inhibitory receptors and engagement with their ligands on neighboring tumor cells and macrophages. As T cells progressively enter a dysfunctional state, their mitochondrial mass and oxidative capacity declines ultimately leading to their failure to meet bioenergetic demands to sustain effector functions and control tumor cell growth.

The gut environment

While the tumor microenvironment is often depicted as nutrient restrictive, the mammalian gastrointestinal tract represents a metabolically rich and diverse tissue system. Its primary function is to digest and absorb nutrients with the aid of microbial species contained within the lumen. A single layer of epithelial cells is all that separates these commensal microbes from the rest of the body. The majority of intestinal epithelial cells (IECs) are absorptive enterocytes that digest and transfer nutrients, however additional specialized epithelial lineages exist with a diverse array of functions. For example, goblet and Paneth cells secrete mucins and antimicrobial peptides that fortify the barrier against potentially pathogenic microbes, microfold (M) and goblet cells assist in the transferring of luminal antigens across the epithelial barrier for sampling by mucosal DCs, and Tuft cells are important for sensing and responding to protozoa and helminths. Together with intestinal resident immune cells including innate lymphoid cells (ILCs), intraepithelial lymphocytes (IELs), helper T cells and B cells, a balancing act between barrier protection and microbial tolerance with surveillance and inflammation is maintained (**Figure 4**). While the relationship between gut commensal microbes and immune cell development and function and also how IECs interface with immune system regulation has recently been reviewed (Kurashima and Kiyono, 2017), we examine the unique constraints that this environment presents on cellular immunometabolism.

While IECs control the intake of nutrients from the luminal environment of the gut, a recent study provides evidence that the way they are structured and uniquely placed controls their metabolic activity and function (Kaiko et al., 2016). The layer of epithelia in the small intestine

are organized into crypts and villi, which form invaginations that serve to optimize surface area whereby nutrients can be absorbed. At the base of the colonic crypt lie epithelial stem/progenitor cells that differentiate into specialized IECs as they migrate up the crypt-villus axis until they are eventually lost from the epithelial layer. This process of self-renewal from the crypt is continuous and therefore is a site of active proliferation (Kurashima and Kiyono, 2017). Kaiko, Ryu and colleagues screened microbiota derived products for their impact on intestinal epithelial progenitors and identified the short chain fatty acid (SCFA) butyrate as a potent inhibitor of intestinal stem cell proliferation at physiologic concentrations present within the lumen (Kaiko et al., 2016). They further found that differentiated colonocytes located at the forefront of the villi metabolized butyrate to fuel OXPHOS, thereby limiting its access to underlying progenitor cells, which do not readily utilize this substrate. Either removal of the ability to metabolize butyrate via deletion of acyl-CoA dehydrogenase or increased abundance of butyrate prevented the rapid regeneration of epithelial tissue after gut injury. Thus a combination of physical separation in the crypt and fermentation of butyrate by mature colonocytes protects the proliferating progenitor pool of IECs (**Figure 4**).

B and Tfh cell metabolism

Another unique structural feature of the gut are the Peyer's patches, aggregates of gut associated lymphoid tissue (Reboldi and Cyster, 2016). Found in the small intestine, they represent a specialized lymphoid compartment continuously exposed to food- and microbiome-derived antigens. Due to this exposure, Peyer's patches are rich in germinal centers (GCs), which are comprised of B, T, stromal, and follicular DCs. B cells segregate into zones where they undergo

cycles of proliferation and differentiation and compete for signals directing class switch recombination (CSR) and survival from T follicular helper (Tfh) cells, allowing further maturation of the antibody repertoire.

Although the literature on B and Tfh cell metabolism is still developing, it has been shown that B cell activation induced by either α -IgM ligation or LPS increases Glut1 expression and glucose uptake downstream of PI3K and mTOR signaling (Caro-Maldonado et al., 2014; Doughty et al., 2006; Jellusova and Rickert, 2016; Lee et al., 2013; Woodland et al., 2008). Glycolysis and OXPHOS are augmented as well as mitochondrial mass (Caro-Maldonado et al., 2014; Doughty et al., 2006; Dufort et al., 2007). Increased glucose acquisition also fuels *de novo* lipogenesis necessary for B cell proliferation and growth of their intracellular membranes. Inhibition of the fatty acid synthesis (FAS) enzyme ATP-citrate lyase in splenic B cells results in reduced expansion and expression of plasma cell differentiation markers (Dufort et al., 2014). Although apoptosis inducing factor (AIF) is required for T cell survival via electron transport chain (ETC) complex I function and respiration, AIF deficiency in B cells has no impact on their development or survival because of their reliance on glucose metabolism (Milasta et al., 2016). B cells cultured in galactose fail to expand unlike T cells, which can activate and proliferate in the presence of either galactose or glucose (Chang et al., 2013; Milasta et al., 2016). On the other hand, the transition to durable humoral immunity by long-lived plasma cells (LLPCs) was shown to be dependent on mitochondrial pyruvate import (**Figure 4**). Glucose supports antibody glycosylation, but LLPCs acquire more glucose than their short-lived counterparts and their long-term survival is dependent on their ability to siphon glucose-derived pyruvate into the mitochondria during times of metabolic stress (Lam et al., 2016).

It is interesting to speculate that with the constant proliferation of GC B cells in the gut and the importance of glucose and glycolysis in activated plasma cells, access to glucose would become limiting for other cells that occupy this microniche. A few studies suggest that Tfh cells have evolved to be uniquely suited to survive under these constraints. It has been shown that Tfh cells have less mTORC1 activation and reduced glycolysis compared to Th1 cells (Ray et al., 2015). In part, this may be due to expression of their lineage defining transcription factor Bcl6, which can suppress glycolysis potentiated by c-Myc and HIF-1 α (Johnston et al., 2009; Nurieva et al., 2009; Oestreich et al., 2014). Consistent with this, overexpression of Bcl6 reduces glycolysis in T cells, and ablation of mTOR using shRNA favors Tfh cell development over Th1 cells *in vivo* after viral infection (Ray et al., 2015) (**Figure 4**). However, a more recent study using mice with conditional deletions of mTORC1 and mTORC2 via OX40 and CD4 cre recombinase observed a requirement of mTOR signaling in Tfh cell development and GC formation within Peyer's patches (Zeng et al., 2016). The former applied retroviral mTOR shRNA, which requires T cells be fully activated prior to knockdown, while this more recent report used mice where mTOR was excised during T cell development or at the moment of T cell activation, which may explain the disparity between the studies.

In addition to possibly limiting quantities of glucose substrate within GCs, these microniches contain areas of hypoxia, resulting in HIF-1 α activation (Abbott et al., 2016; Cho et al., 2016). B cells placed under hypoxic conditions had reduced activation induced deaminase expression and subsequently underwent less CSR to the pro-inflammatory IgG2c isotype when cultured in conditions that promote IgG production (Cho et al., 2016). In contrast, B cells cultured in IgA-

promoting conditions during hypoxia were unaffected, yielding comparable levels of IgA to cells kept at normoxia and highlighting how lymphocyte function may be fine-tuned to varying oxygen tension in tissues (**Figure 4**). B cells isolated from mice with constitutive activation of HIF-1 α by deletion of its suppressor VHL had defects in IgG2c production, which was attributed to diminished mTORC1 activation. B cells from Raptor deficient heterozygotes also yielded fewer IgG antibodies (Cho et al., 2016). A separate study found that the mTOR inhibitor rapamycin dampens CSR, yielding the formation of lower affinity, more cross reactive B cell antibodies, which offered broad protection against heterosubtypic flu infection (Keating et al., 2013). Both mTORC1 and HIF-1 α promote aerobic glycolysis (O'Neill and Pearce, 2016). However, the metabolic activities of the cells cultured under different isotype conditions while under hypoxia were not explored. Cytokines initiate CSR to distinct isotypes and signals derived from these growth factors might be responsible for the differences in metabolic signaling and suggest varying requirements to initiate metabolic programs and CSR in B cells. Secretion of IgA predominates the gut and is critical to maintaining barrier protection and bacterial homeostasis (Kurashima and Kiyono, 2017). The apparent stability of CSR to the IgA isotype under hypoxia and impaired pro-inflammatory IgG2c subtype might have evolved to ensure tolerance with the microbiome, while concurrently providing a stringent method of selection of antibodies produced during inflammatory responses to pathogen-derived antigens.

Nutrients and immune signals in the gut

The metabolic relationship between commensals and immune cells in the gut is further illustrated by the finding that SCFAs derived from the fermentation of dietary fiber by gut microbiota promote B cell metabolism and boost antibody responses in both mouse and human B cells (Kim

et al., 2016). Supplementation with dietary fiber or the SCFAs acetate, propionate, and butyrate increases intestinal IgA production, as well as systemic IgG during infection (**Figure 4**). Culturing B cells with SCFAs was shown to raise acetyl-CoA levels and increase mitochondrial mass, lipid content, and FAS leading to increased plasma cell differentiation and metabolic activity (Kim et al., 2016). Part of this phenotype could be attributed to histone deacetylase (HDAC) inhibition, an established effect of SCFA supplementation.

In addition to their effects on B Cells, SCFAs have been found to promote the development and function of colonic Treg cells via induction of Foxp3 in a HDAC dependent manner (Arpaia et al., 2013; Furusawa et al., 2013; Smith et al., 2013) (**Figure 4**). Treg cells are critical to maintaining commensal tolerance by the immune system through suppression of aberrant T cell responses. Unlike other activated T helper subsets, Treg cells have been described to primarily rely on OXPHOS driven by FAO (Newton et al., 2016). However, signals downstream of TLR ligation can augment glycolysis and proliferation of Treg cells and reduce their ability to suppress T cell responses (Gerriets et al., 2016). Retroviral enforced expression of Foxp3 promotes OXPHOS and dampens glucose uptake and glycolysis, whereas Treg cells transduced with Glut1 decreased Foxp3 expression after adoptive transfer *in vivo* and fail to suppress T cell-mediated colitis in a model of inflammatory bowel disease. These findings suggest that during inflammation and microbial infection, Treg cells may temporarily lose their regulatory function to give way to robust T cell responses and participate as more conventional effector helper T cells. Increases in NaCl either from supplementation *in vitro* or diet *in vivo* inhibit the suppressive function of human Treg cells via serum/glucocorticoid-regulated kinase 1 (SGK1), which integrates signals from PI3K and mTORC2 to regulate sodium controlled signal

transduction (Hernandez et al., 2015). However, a study of human Treg cells found that the glycolytic enzyme enolase-1 was required for their suppressive activity through its control of Foxp3-E2 splice variants (De Rosa et al., 2015). Depending on environmental cues and metabolites, it appears that Treg cell metabolism can be modulated, affecting their function.

As discussed, increases in SCFAs either from diet, infection, or exogenous treatment impinge on metabolic process including HDAC activation (Rooks and Garrett, 2016). A recent study suggests that activation of the HDAC sirtuin 1 (SIRT1) negatively impacts Th9 cell differentiation (Wang et al., 2016). The exposure of CD4 T cells to distinct cytokine cocktails differentiates them into separate helper lineages. However, perturbing metabolism also modulates CD4 T cell fate. A yin and yang relationship between Th17 and Treg cell differentiation has been established. Th17 cells are particularly glycolytic and depend on engagement of this pathway downstream of mTOR and HIF-1 α activation. Dampening glycolysis through deletion of HIF-1 α or with the inhibitor 2-DG in T cells impairs Th17 development and instead promotes Treg cells, even under Th17-inducing culture conditions (Dang et al., 2011; Shi et al., 2011). Suppression of mTOR with rapamycin or genetic ablation also augments production of Treg cells (Delgoffe et al., 2009; Kopf et al., 2007), and pharmacological inhibition of *de novo* fatty acid synthesis prevents Th17 differentiation and instead enforces a Treg cell phenotype (Berod et al., 2014).

Although the metabolic characteristics of other CD4 T cell subsets have been compared (Michalek et al., 2011), little was known about Th9 cell metabolism. Th9 cells are characterized by their ability to produce IL-9 and can be generated from naïve cells in culture using the

cytokines TGF- β and IL-4. They have implicated roles in autoimmunity, melanoma, and worm infections (Kaplan et al., 2015). Wang and colleagues found that Th9 cells are highly glycolytic, in part from their active suppression of SIRT1 expression via the kinase TAK1 (Wang et al., 2016). SIRT1 was previously shown to negatively control HIF-1 α as well as mTOR (Lim et al., 2010; Liu et al., 2013). In line with this, Th9 cell development was augmented in SIRT1-deficient T cells whereas retroviral enforced expression of SIRT1 or dampening of aerobic glycolysis by chemical or genetic means impaired Th9 cell differentiation (Wang et al., 2016). Th9, Th17, and Treg cells all share the cytokine TGF- β for their development, but then depend on additional cytokine signals for their eventual fates. Given their divergent metabolic phenotypes, as well as HDAC requirements, it would be interesting to explore further whether variances in intracellular levels of SCFA metabolites for example, might couple with environment signals to influence their eventual metabolic and development pathway.

Apart from its affect on CD4 T cells, the SCFA acetate also has been shown to affect secondary recall responses from CD8 memory T cells (Balmer et al., 2016). Germ-free mice reconstituted with commensal microbes, or oral or systemic infection with bacterial species, elevated serum acetate concentrations. Memory T cells generated *in vitro* or *in vivo* cultured with acetate levels observed during these infections secreted more IFN- γ and augmented glycolysis after restimulation. Acetate can be quickly converted into acetyl-CoA, which can condense with oxaloacetate into citrate in the mitochondria to fuel the TCA cycle and OXPHOS, be used as a substrate for FAS, or participate in post translational modification (PTM) of proteins including histones (Pearce et al., 2013). Balmer and colleagues mechanistically tied their results to lysine acetylation of the glycolytic enzyme GAPDH. GAPDH activity has been shown to regulate T

cell production of IFN- γ (Chang et al., 2013; Gubser et al., 2013). Although the study demonstrated that the enzymatic activity of GAPDH was altered by acetylation of K217, whether this PTM was critical to acetate-dependent increases in IFN- γ protein was not explored. In a separate report, CD4 T cells deficient in LDHA expression had defects in IFN- γ production, which stemmed from widespread lack of acetylation of the *Ifng* locus (Peng et al., 2016). LDHA is the critical enzyme that defines aerobic glycolysis, converting pyruvate to lactate. Culturing cells in galactose impairs aerobic glycolysis, as galactose enters glycolysis through the Leloir pathway at a significantly lower rate than glucose (Bustamante and Pedersen, 1977), a result confirmed by tracing galactose metabolism in T cells (Chang et al., 2013). Reducing GAPDH engagement from glycolysis in this fashion permits moonlighting function by this abundantly expressed protein. It was shown that GAPDH binds to the 3'UTR of AU-rich containing cytokine mRNAs, preventing their efficient translation (Chang et al., 2013). Peng and colleagues argue against GAPDH posttranscriptional control of T cell function during aerobic glycolysis deficiency because modification of the 3'UTR of *Ifng* did not rescue defects in cytokine production in their system. However as the authors demonstrated, LDHA-deficient cells have defects in *Ifng* mRNA production, whereas cells forced to respire in galactose remain as transcriptionally competent for *Ifng* as those cultured in glucose. Supplementation with the SCFA acetate rescued their epigenetic defect and cytokine production. These studies show that aerobic glycolysis regulates both transcriptional and translational functions in T cells.

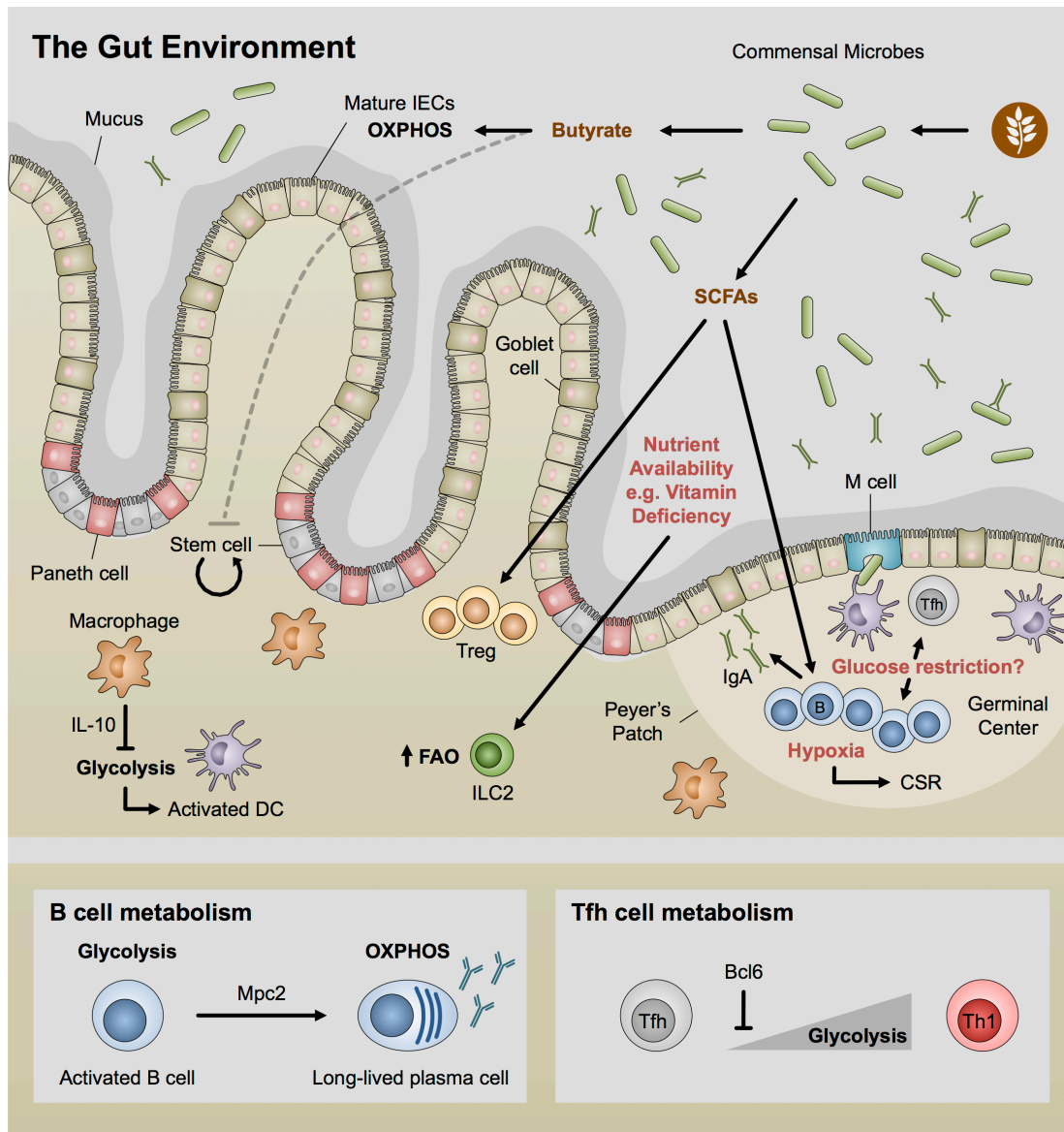
While products generated from the microbiome can modulate the metabolism of immune cells and shift the balance between tolerance and inflammation, there are hints that immune driven signals central to gut homeostasis may also mediate their effects through metabolic modulation.

One such example is the pleotropic anti-inflammatory cytokine IL-10. Most hematopoietic cells produce and sense IL-10 and its importance for maintaining tolerance with the intestinal microbiota is clearly evident from observations that IL-10 or IL-10R deficient mice develop spontaneous colitis (Kuhn et al., 1993; Spencer et al., 1998). IL-10R deficiency in macrophages is also sufficient to recapitulate onset of severe colitis in mice (Shouval et al., 2014; Zigmond et al., 2014). Further, mice with a myeloid cell-specific deficiency in STAT3, which is activated downstream of the IL-10R by JAK1, develop chronic enterocolitis as they age (Takeda et al., 1999). In experiments that shed light on the importance of aerobic glycolysis engagement in DC activation, it was found that treatment of DCs with recombinant IL-10 blocked increases in their glycolytic rate after LPS stimulation (Krawczyk et al., 2010). Cells subjected to IL-10R blockade further upregulated glycolysis after activation compared to controls. It is tempting to speculate that one of the ways IL-10 might be anti-inflammatory is through inhibition of metabolic reprogramming to aerobic glycolysis during innate immune cell activation (**Figure 4**). Coincidentally, STAT3 was found to localize to mitochondria and interact with ETC complexes, which helped maintain efficient OXPHOS in the heart (Wegrzyn et al., 2009). Whether traditional cell surface cytokine-receptor signaling could modulate levels of mitochondrial STAT3 was not explored. Of interest, CD8 T cells with a conditional deletion of the IL-10R fail to form memory T cells (Laidlaw et al., 2015), which depend on FAO driven OXPHOS for their generation after infection (Cui et al., 2015; Pearce et al., 2009; van der Windt et al., 2012).

The gut is one example of a tissue that presents distinct metabolic challenges for immune cells, which affect their steady state and protective versus inflammatory responses. Other examples, such as skin, provide the potential for commensal organisms to metabolically affect immune cell

function, a topic reviewed elsewhere (Hand et al., 2016). The intestinal tract is constantly subject to fluctuations in diet and sometimes the intake of invasive pathogens can also deprive metabolic substrates from immune cells. The bacterium *Salmonella typhimurium* produces a putative type II asparaginase that depletes available asparagine needed for metabolic reprogramming of activated T cells via c-Myc and mTOR (Torres et al., 2016). The use of asparaginase for acute lymphoblastic leukemia treatment highlights the potential for the depletion of extracellular arginine to significantly affect cellular function (DeBerardinis and Chandel, 2016). Lack of dietary vitamin B1 decreases the number of naïve B cells in Peyer's patches due to their dependence on this TCA cycle cofactor, while leaving IgA⁺ plasma cells intact in the lamina propria (Kunisawa et al., 2015). Although ILC metabolism has only recently been explored (Monticelli et al., 2016; Wilhelm et al., 2016), it was found that in settings of vitamin A deficiency, type 2 ILCs sustain their function via increased acquisition and utilization of fatty acids for FAO (Wilhelm et al., 2016) (**Figure 4**). The internal balance between polyunsaturated fats and saturated fatty acids can also determine the pathogenicity of Th17 cells; cells that help maintain mucosal barrier immunity and contribute to pathogen clearance (Wang et al., 2015a). Long chain fatty acids (LCFA) promote Th1 and Th17 cell polarization and mice fed with LCFA have exacerbated T cell-mediated autoimmune responses, whereas mice fed with SCFA are protected (Haghikia et al., 2015). If the internal lipidome of Th17 cells can alter their function from protective to inflammatory as well as their access to LCFA versus SCFA in the small intestine, it begs the question of how other tissue systems with a rich diversity of fat deposits and cells, such as the adipose tissue, modulate the metabolism and function of resident immune cells.

Figure 4. Models of metabolic relationships in the gastrointestinal tract



The gut serves as a direct interface with the outside world and the foods we consume. A single epithelial cell layer separates the contents of the intestinal lumen from the lamina propria where DCs, macrophages, ILCs, and T cells reside. Peyer's patches are interspersed along the epithelium, which in addition to supporting sampling of luminal antigens by DCs and M cells, house germinal centers that mature IgA-secreting B cells with Tfh cell help. B cells augment glycolysis upon activation and depend on pyruvate import via Mpc2 for longevity as long-lived plasma cells (LLPCs). Plasma cell hunger for glucose may restrict this nutrient from Tfh cells, however Tfh cells downregulate glycolysis in response to expression of their lineage defining transcription factor. In addition, GCs contain areas of hypoxia that impinge on B cell function like class switch recombination (CSR). Commensal bacteria produce metabolites such as short chain fatty acids (SCFAs) from the fermentation of dietary fiber, which influence B cell metabolism and promote IgA secretion. The presence of SCFAs and vitamins support maintenance of barrier function by promoting the development and survival of Tregs and ILCs, respectively. Homeostatic signals secreted by gut resident immune cells (e.g. IL-10) may also modulate metabolism and therefore control their activation state.

Restructuring of intracellular metabolism

Thus far, we have scrutinized some of the extracellular environmental factors, such as substrate availability, that influence immune cell metabolism and function. However, other more cell-intrinsic processes determine the balance in metabolic pathway usage and subsequent immune response. Termed “restructuring of intracellular metabolism” here, these internal influences range from cytoskeletal and organelle structural remodeling to metabolite and redox balance (**Figure 5**).

Cytoskeleton and asymmetric cell division

Perhaps one of the most fundamental ways that cellular metabolism is altered in response to external stimuli is through gross structural changes in intracellular architecture. In an elegant dissection of PI3K signaling in epithelial cells, Hu and colleagues found cytoskeletal dynamics and glycolysis to be uniquely intertwined (Hu et al., 2016). The authors showed that addition of growth factors or insulin activated Rac downstream of PI3K, causing a disruption of the actin cytoskeleton. Loss of this structural integrity released bound aldolase from filamentous F-actin, increasing its catalytic activity. Chemical and genetic inhibition of PI3K, Rac, or actin dynamics modulated glycolysis via mobilization of aldolase (**Figure 5**). It will be interesting in future studies to explore how other glycolytic enzymes or even the mitochondria in immune cells restructure their metabolic activity through changes in cytoskeletal morphology in response to growth factor or pathogen-derived signals, especially since directed cellular migration is such an integral feature of how the disseminated immune system can focus its attention on points of infection or damage. On a related note, two complementary studies have found that in response

to activation by DCs, lymphocytes asymmetrically partition the metabolic regulators c-Myc and mTORC1 during the first cell division (Pollizzi et al., 2016; Verbist et al., 2016). This distribution event determines their eventual destiny wherein the daughter cell that inherits high c-Myc expression and mTORC1 activity greatly augments glycolysis and undergoes higher rates of proliferation during a primary immune response and the other daughter cell maintains the metabolic profile of a memory T cell with increased persistence and secondary response capability. The patterning of Myc expression at the inception of the proliferative burst following activation also regulates a T cell's capacity to sustain cellular division (Heinzel et al., 2016).

Redox balance and mitochondrial dynamics

The ability to maintain internal metabolite balance is also crucial for supporting the equilibrium of reductive and oxidizing equivalents such as NAD^+/NADH and FAD/FADH_2 , which participate in redox reactions that release energy from the oxidation of substrates. Two recent back-to-back studies demonstrate a requirement for the synthesis of aspartate to balance redox that may have implications for highly proliferative lymphocytes, such as B and T cells (Birsoy et al., 2015; Sullivan et al., 2015). For about 20 years, it has been known that cells lacking a functional ETC possess a defect in proliferation that can be rescued by exogenous pyruvate (King and Attardi, 1989). However, how pyruvate accomplishes this feat remained untested. In the absence of a functional ETC, presumably NADH produced from cellular metabolism cannot be adequately oxidized. Sullivan and colleagues hypothesized that pyruvate may act as a surrogate electron acceptor to restore redox balance by generating NAD^+ during its conversion to lactate. Indeed, when they added alternative substrates that also result in the oxidation of NADH, such as α -ketobutyrate, proliferation of cells with impaired ETC function was restored. Birsoy

and colleagues identified GOT1 through a CRISPR-Cas9 genetic screen as a critical factor for Jurkat leukemic T cell viability during ETC inhibition (Birsoy et al., 2015). Ultimately both studies recognized that aspartate, produced via GOT1, to be a critical metabolite for cells to expand and grow. When the ETC is dysfunctional, electron acceptors become limiting and aspartate synthesis is impaired that results in a block in cellular proliferation.

As seen above, genetic selection or chemical impairment of the ETC perturbs redox balance, but a closer examination of mitochondrial dynamics in T cells suggests that physiologic restructuring of the mitochondria during an immune response also generates changes in redox flow that mediate metabolic pathway choice during activation and quiescence. Activation initiated by TCR ligation and binding with costimulatory molecules metabolically reprograms T cells for rapid anabolic growth and biomass accumulation. Concomitantly, mitochondria undergo biogenesis and take on a grossly punctate and dispersed morphology with expanded cristae junctions and augment aerobic glycolysis and OXPHOS (Buck et al., 2016; Ron-Harel et al., 2016). During this process, the mitochondrial proteome remodels itself to increase mitochondrial one-carbon metabolism. Knockdown of SHMT2, the first enzyme in this pathway, impairs CD4 T cell survival and proliferation *in vivo* (Ron-Harel et al., 2016). It has also been observed that effector and memory T cells have morphologically distinct mitochondria. Effector T cells have more “fissed” mitochondria whereas memory T cells have more “fused” mitochondrial networks with tight cristae suggesting a requirement for mitochondrial fusion in memory T cell metabolism and homeostasis. Consistent with this observation, antigen-specific T cells lacking the inner mitochondrial membrane fusion protein Opa1 fail to generate memory T cells after bacterial infection and have impaired survival *in vitro* (Buck et al., 2016). The activation, proliferation,

and function of Opa1 deficient effector T cells however, remain intact. Opa1 KO T cells have augmented rates of glycolysis and possess mitochondria with diminished OXPHOS efficiency and malformed cristae compared to controls. We found that in quiescent T cells, such as naïve and memory T cells, mitochondrial fusion ensured tight cristae associations that allowed for the ETC to function efficiently. On the other hand, mitochondrial fission directed cristae expansion during immune cell activation physically separated ETC complexes and decreased ETC efficiency, thereby disrupting cellular redox balance. In order to correct this redox imbalance, cells exported lactate from pyruvate conversion and in doing so, supported their commitment to aerobic glycolysis. These data suggest a model whereby the structural remodeling of mitochondria can signal the engagement of a particular metabolic pathway (**Figure 5**).

Tight associations of mitochondrial cristae result in dense packing of ETC complexes, which have been found to associate in specialized configurations termed respiratory supercomplexes or respirasomes. Supercomplexes facilitate efficient transfer of electrons and minimize proton leak during ATP production (Cogliati et al., 2013). Research into this area is still growing, but a couple of studies have highlighted their contributions to immune responses. CD8 T cells express high levels of methylation-controlled J protein (MCJ), a member of the DnaJ family of chaperones (Champagne et al., 2016). MCJ localizes to the inner membrane of mitochondria and associates with complex I of the ETC. By doing so, it negatively regulates the assembly of complex I into supercomplexes. MCJ deficiency was found to enhance naïve and activated CD8 T cell OXPHOS and a unique attribute was its role in the secretion, but not the translation, of effector cytokines (Champagne et al., 2016). Increased respiration efficiency improved the survival of the MCJ KO effector T cells, which also induced superior protective immunity

against viral challenge. Boosting oxidative capacity through enforcement of mitochondrial fusion also extends the survival and function of CD8 T cells *in vivo* (Buck et al., 2016). A separate study in myeloid cells found that TLR and inflammasome dependent bacterial recognition by macrophages induces a transient decrease of complex I respirasome assembly while enhancing complex II activity (Garaude et al., 2016). Inhibition of complex II (a.k.a. SDH – succinate dehydrogenase) *in vivo* disrupted production of IL-1 β after infectious challenge while enhancing levels of the anti-inflammatory cytokine IL-10.

Itaconate and ROS

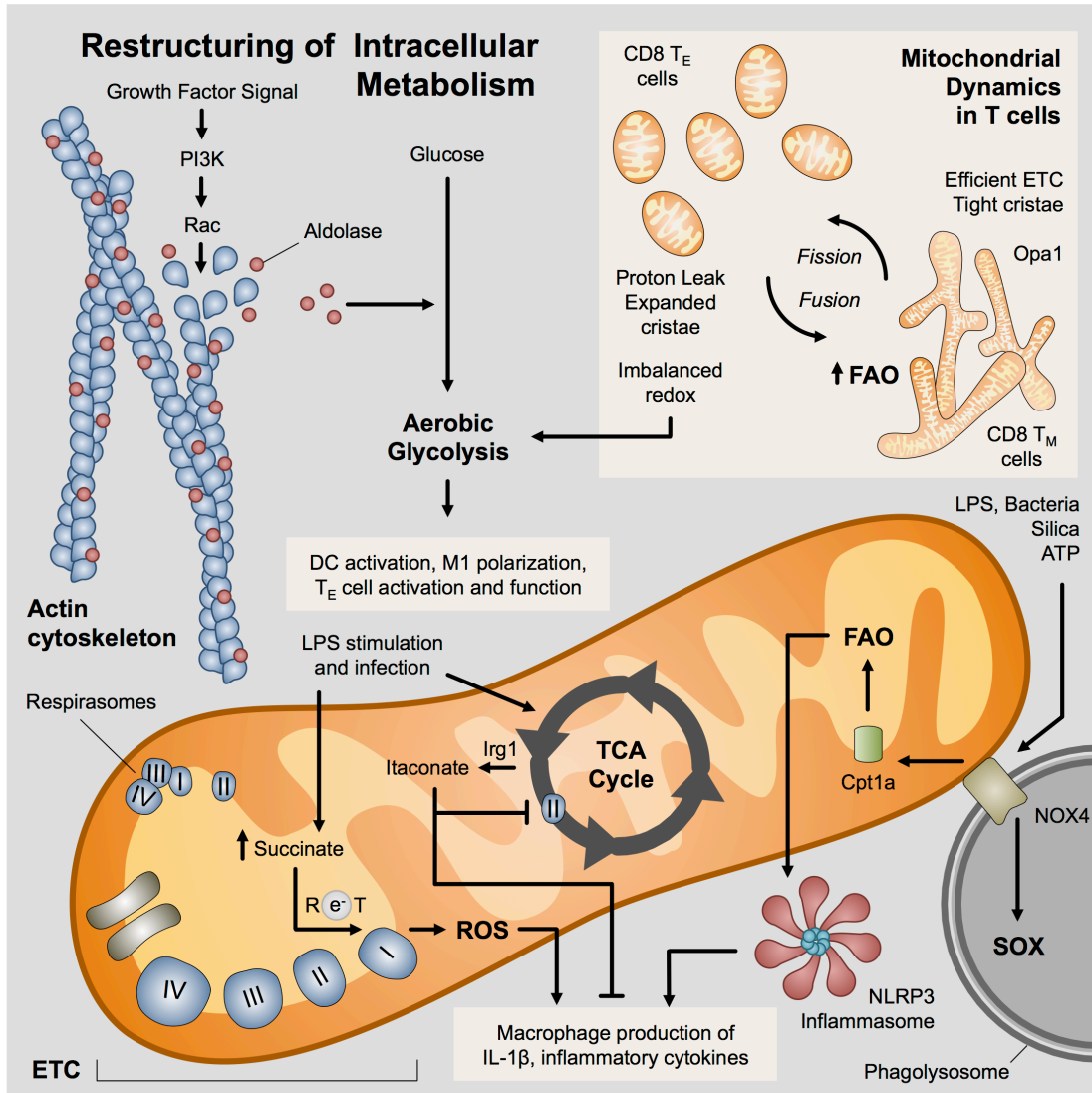
In relation to the role of SDH in macrophage cytokine production, a key example of an intracellular metabolite impinging on immune cell metabolism and response outcome is provided by itaconate. A comprehensive description of its discovery as an antimicrobial metabolite and modulator of immune cell bioenergetics has been reviewed recently (Luan and Medzhitov, 2016). Upon activation, downstream of inflammatory stimuli such as TLR signaling, macrophages increase their internal concentrations of the metabolite itaconate by conversion of TCA cycle derived aconitate via the mitochondria-associated enzyme immunoresponsive gene 1 (Irg1). Itaconate in turn competitively inhibits SDH function, resulting in succinate accumulation (Lampropoulou et al., 2016). Itaconate can also impair OXPHOS in a dose dependent manner (Cordes et al., 2016). Therefore, production of itaconate may necessitate the switch to aerobic glycolysis that occurs in myeloid cells after LPS stimulation by introducing a brake in the TCA cycle and ETC through SDH inhibition. However the situation is a bit more complicated (**Figure 5**). Inflammatory cytokine production from activated myeloid cells is also coupled to commitment to ATP generation via aerobic glycolysis (O'Neill and Pearce, 2016). Exogenous

treatment with itaconate inhibits production of IL-1 β , IL-6, IL-12, and ROS in response to inflammatory signals, while Irg1 deficient macrophages have increased inflammatory cytokine output (Lampropoulou et al., 2016). Irg1 KO cells have intact SDH activity and their hyper inflammatory phenotype agrees with a recent study demonstrating a requirement for SDH dependent IL-1 β generation in LPS activated macrophages (Mills et al., 2016). Mills, Kelly and colleagues propose a model whereby IL-1 β production is dependent on ROS generated from the oxidation of accumulated succinate that is driven by reverse electron transport (RET) from intact SDH. This builds from a previous report demonstrating that glutamine dependent succinate accumulation after LPS stimulation via glutamine-dependent anaplerosis and the γ -aminobutyric acid shunt pathway is important for augmenting glycolysis through HIF-1 α stabilization and IL-1 β production from macrophages (Tannahill et al., 2013). However, Lampropoulou and colleagues describe succinate accumulation driven by itaconate inhibition of SDH. One possible explanation that unifies these observations is that initial signals downstream of TLR activation drive succinate production from glutamine to initiate ROS and generate IL-1 β . Itaconate, later accumulates as Irg1 levels increase and feeds back on SDH to inhibit its activity and sets a threshold for inflammatory cytokine production (Luan and Medzhitov, 2016). In doing so, both processes may also initiate and sustain myeloid cell commitment to aerobic glycolysis.

The generation of mitochondrial-derived ROS has been shown to be important for macrophage activation and inflammatory cytokine production and also critical for the activation and expansion of antigen-specific T cells (Mills et al., 2016; Sena et al., 2013; Tannahill et al., 2013). However, immune cells can generate ROS from other intracellular compartments apart from the mitochondrial ETC. Cellular superoxide anions can be released from the NADPH oxidase

(NOX), which assembles on the phagolysosome to aid in the destruction of ingested microorganisms. ROS derived from NOX has been previously shown to be critical for the activation of the NLRP3 inflammasome (Abais et al., 2015). However, a study from Moon and colleagues extends a role for NOX4 in the activation of the NLRP3 inflammasome through its modulation of FAO (Moon et al., 2016). NOX4 deficient mouse and human macrophages stimulated *in vitro* with ATP, nigericin, or silica had impaired caspase-1 activation and subsequent IL-1 β and IL-18 maturation, although TNF- α production remained intact. Inflammasome activation was also diminished in NOX4 KO mice after *S. pneumoniae* challenge. This defect was identified as an inability of NOX4 deficient cells to augment Cpt1a dependent FAO during inflammasome activation. Consistent with this observation, Cpt1a deficient macrophages were unable to activate the NLRP3 inflammasome, but enforced expression of Cpt1a in NOX4 KO macrophages rescued inflammasome activation and cytokine release (**Figure 5**). However, exactly how NOX4 regulates Cpt1a protein levels and FAO is unresolved.

Figure 5. Restructuring of intracellular architecture and metabolism.



Immune cell function is a product of their metabolic state. Growth factor signaling, actin rearrangement, and glucose metabolism are closely intertwined. Actin-bound aldolase can be freed from the cytoskeleton downstream of growth factor signaling to mediate glycolysis. Engagement of this pathway is central to the activation and downstream effector functions of DCs, M1 macrophages, and T cells. T cells can dynamically restructure their mitochondria through processes like mitochondrial fission and fusion to signal changes in metabolism and to promote long-term survival as they transition to memory cells. Inflammatory signals repurpose TCA cycle enzymes and metabolic intermediates such as itaconate, succinate, and processes like FAO to serve as internal signals that modulate ROS production, inflammasome activation, and cytokine production.

Outlook

Immunology for some time became a field that sought reductionist approaches to simplify a complex network of cells. Out of necessity, immunologists had to speak a common language or jargon that often excluded scientists in other disciplines. After many decades of hard work, great strides have been made in our understanding of the immune system and now immunologists are better equipped to cross over into other disciplines. It is this ‘take a step back and look’ approach that has led a number of laboratories to focus on metabolism and how this affects the immune system as well as its greater impact in tissue physiology.

It is obvious to most biologists that metabolism is integrated into every cellular process and fate decision. After all, everything must eat to survive. However, what is perhaps less appreciated is that the immune system is like a liquid organ unto itself. At their inception, immune cells are poised to respond to unknown stimuli, nutrients, pathogens and are akin to special agents with contingency plans, ready to respond to one disaster scenario after another, or may be relegated to pushing paperwork at the office maintaining the status quo. We have only highlighted some examples of the complexity of the situations and environments that immune cells face that provide various metabolic instructional cues throughout this review. This ability to rapidly change and adapt at any given second means that immune cells must intimately integrate their cellular metabolism in a way that most other organ and cell systems in the body do not have to, which we hope this review has shown and inspires research into many questions that remain to be explored. Coupling the unique benefits of studying immunometabolism is the added bonus of

the enormous clinical relevance of these cells in human health and disease. First defining and then exploiting their unique metabolism may continue to yield new targets for therapy.

Chapter 3: Materials and Methods

Mice and immunizations

C57BL/6, C57BL/6 CD45.1, C57BL/6 CD90.1, photo-activatable mitochondria (PhAM), and major histocompatibility complex (MHC) class I-restricted OVA specific TCR OT-I transgenic mice were purchased from The Jackson Laboratory. *Mfn1* and *Mfn2* conditional floxed mice were obtained from Dr. David C. Chan (California Institute of Technology, Pasadena, CA). *Opa1* conditional floxed mice were obtained from Dr. Hiromi Sesaki (Johns Hopkins University School of Medicine, Baltimore, MD). All conditional floxed mice were crossed to OT-I CD4 Cre transgenic mice to generate OT-I *Mfn1*^{F/F} CD4 Cre, OT-I *Mfn2*^{F/F} CD4 Cre, and OT-I *Opa1*^{F/F} CD4 Cre mice. All mice were bred and maintained under specific pathogen free conditions under protocols approved by the AAALAC accredited Animal Studies Committee of Washington University School of Medicine, St. Louis, MO USA and the Animal Welfare Committee of the Max Planck Institute of Immunobiology and Epigenetics, Freiburg, Germany. Age matched mice were injected intraperitoneally (i.p.) or intravenously (i.v.) as indicated with a sublethal dose of 1×10^6 colony forming units (CFU) of recombinant *Listeria monocytogenes* expressing OVA deleted for *actA* (LmOVA) for primary immunizations and challenged with 5×10^7 CFU for secondary immunizations. For tumor experiments, 1×10^6 EL4 lymphoma cells expressing OVA (EL4-OVA) were injected subcutaneously (s.c.) into the right flank of mice.

Cell culture and drug treatments

OT-I splenocytes were activated with OVA-peptide (SIINFEKL, New England Peptide) and IL-2 (100 U/mL) for 3 days and subsequently cultured in the presence of either IL-2 or IL-15 (10 ng/mL) for an additional 3 days in TCM (RPMI 1640 media supplemented with 10% FCS, 2 mM L-glutamine, 100 U/mL penicillin/streptomycin, and 55 μ M β -mercaptoethanol). For drug

treatment experiments, vehicle control (DMSO) or 10 μ M Mdivi-1 + 20 μ M M1 (Sigma) were added to cultures daily starting on day 3. For *in vitro* survival assays, cells were activated for 3 days as described, then cultured in either IL-2 at 5×10^4 cells/mL or IL-15 at 1×10^5 cells/mL in 96 well round bottom plates. Survival was analyzed by 7AAD exclusion using flow cytometry. Bone marrow cells were differentiated for 7 days into BM-Macs by culturing in complete medium (RPMI 1640 supplemented with 10% FBS, 100 U/mL penicillin/streptomycin, 2 mM L-glutamine) with 20 ng/mL mouse macrophage colony-stimulating factor (M-CSF; PeproTech) or into BM-DCs using 20 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF; PeproTech). BM-Macs and BM-DCs were stimulated using 20 ng/mL LPS (Sigma), 50 ng/mL IFN- γ (R&D Systems), or 20 ng/mL IL-4 (PeproTech). BM-DCs were cultured in 5 ng/mL GM-CSF during stimulation experiments.

Flow cytometry and spinning disk confocal microscopy

Fluorochrome-conjugated monoclonal antibodies were purchased from eBioscience, BD Pharmingen, or Biolegend and staining performed as previously described (Chang et al., 2015). OVA-specific CD8⁺ T cells from spleen, lymph node, or blood were quantified by direct staining with H2-K^bOVA₂₅₇₋₂₆₄ (K^bOVA) MHC-peptide tetramers. MitoTracker, TMRM, CMxROS, MitoSOX, and Hoechst staining was performed according to the manufacturer's instructions (Life Technologies). Nos2 protein levels in BM-Macs were quantified after fixation and permeabilization using the transcription factor staining buffer set (eBioscience) and a directly conjugated antibody against Nos2 (clone CXNFT, eBioscience). Cells were collected on FACS Calibur, Canto II, LSR II, and Fortessa flow cytometers (BD Biosciences) and analyzed using FlowJo (TreeStar) software. Cells were sorted using a FACS Aria II. Cells were imaged live on

glass bottom dishes coated with fibronectin or poly-D-lysine (Sigma) in TCM containing IL-2 or IL-15 (MatTek) using a LSM 510 META confocal scanning microscope (Zeiss), an Olympus Confocal Microscope FV1000, or a Zeiss spinning disk confocal with an Evolve (EMCCD) camera. Cells were kept in a humidified incubation chamber at 37°C with 5% CO₂ during image collection. Images were deconvolved and analyzed using ImageJ (NIH). Brightness and contrast were adjusted in Adobe Photoshop CS.

Transmission electron microscopy

Cells were fixed in 2% paraformaldehyde, 2.5% glutaraldehyde in 100 mM sodium cacodylate containing 0.05% malachite green. Following fixation, samples were washed in cacodylate buffer and post fixed in 1% osmium tetroxide. After extensive washing in H₂O, samples were stained with 1% aqueous uranyl acetate for 1 hour and washed again. Samples were dehydrated in ethanol and embedded in Eponate 12 resin (Ted Pella). Cut sections were stained with uranyl acetate and lead citrate and then imaged using a JOEL 1200 EX transmission electron microscope equipped with an 8 MP ATMP digital camera (Advanced Microscopy Techniques).

Metabolism assays

Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were measured in XF media (non-buffered RPMI 1640 containing 25 mM glucose, 2mM L-glutamine, and 1 mM sodium pyruvate) under basal conditions and in response to 200 μM etomoxir (Tocris), 1 μM oligomycin, 1.5 μM fluoro-carbonyl cyanide phenylhydrazone (FCCP) and 100 nM rotenone + 1 μM antimycin A, or 50 ng/mL phorbol 12-myristate 13-acetate (PMA) + 500 ng/mL ionomycin (all Sigma) using a 96 well XF or XFe Extracellular Flux Analyzer (EFA) (Seahorse Bioscience)

(van der Windt et al., 2016). For mitochondrial fission inhibition experiments, cells were plated in XF media containing 10 μ M Mdivi-1 or vehicle control (DMSO), followed by injection into port A with XF media, α CD3/CD28- conjugated beads (1 bead/cell; Dynabeads), or 20 ng/mL LPS \pm 50 ng/mL IFN- γ .

Glucose tracing

Cells were activated with OVA peptide and cultured in glucose free TCM (prepared with dialyzed FBS) supplemented with 11 mM glucose. On day 3 of culture, cells were washed and cultured overnight in TCM replaced with 11 mM D-[1,2-¹³C] labeled glucose. For harvest, cells were rinsed with cold 150 mM ammonium acetate (NH₄AcO), and metabolites extracted using 1.2 mL of 80% MeOH kept on dry ice. 10 nM norvaline (internal standard) was added. Following mixing and centrifugation, the supernatant was collected, transferred into glass vials and dried via centrifugal evaporation. Metabolites were resuspended in 50 μ L 70% ACN and 5 μ L of this solution used for mass spectrometer-based analysis performed on a Q Exactive (Thermo Scientific) coupled to an UltiMate 3000RSLC (Thermo Scientific) UHPLC system. Mobile phase A was 5 mM NH₄AcO, pH 9.9, B was ACN, and the separation achieved on a Luna 3u NH₂ 100A (150 \times 2.0 mm) (Phenomenex) column. The flow was kept at 200 μ L/min, and the gradient was from 15% A to 95% A in 18 min, followed by an isocratic step for 9 min and re-equilibration for 7 min. Metabolites we detected and quantified as area under the curve (AUC) based on retention time and accurate mass (\leq 3 p.p.m.) using TraceFinder 3.3 (Thermo Scientific) software.

Adoptive transfers

For *in vivo* memory T cell experiments, $\leq 1 \times 10^4$ OT-I⁺ CD8⁺ cells/mouse from donor splenocytes were transferred intravenously (i.v.) into congenic recipient mice. Blood samples or spleens were collected at indicated time points and analyzed by flow cytometry. For *in vivo* survival experiments, $1-2 \times 10^6$ day 6 IL-2 T_E treated cells/mouse were injected i.v. into naïve C57BL/6 mice. Cells were recovered two days later from the spleen or lymph nodes and analyzed by flow cytometry or isolated from spleens >3 weeks 6 days after LmOVA infection. For adoptive cellular immunotherapy experiments, $1-5 \times 10^6$ day 6 IL-2 T_E treated cells/mouse were injected i.v. into previously EL4-OVA tumor inoculated mice and measured for tumor volume growth.

RT-PCR and western blotting

RNA isolations were done by using the RNeasy kit (Qiagen) and single-strand cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Genomic DNA was extracted using the QIAamp DNA micro kit (Qiagen) to determine mtDNA/nDNA ratios. All RT-PCR was performed with Taqman primers using an Applied Biosystems 7000 sequence detection system. The expression levels of mRNA were normalized to the expression of a housekeeping gene (β -actin). For western blot analyses, cells were washed with ice cold PBS and lysed in 1x lysis buffer (Cell Signaling Technologies) supplemented with 1 mM PMSF. Samples were freeze-thawed 3 times and centrifuged at 20,000xg for 10 min at 4°C. Cleared protein lysate was denatured with LDS loading buffer for 10 min at 70°C. For native lysis, cells were resuspended in native lysis buffer (Life Technologies), supplemented with increasing percentages of digitonin, MgCl₂, and micrococcal nuclease. After nuclease incubation at RT for 1h, lysates were cleared by centrifugation at 20,000xg for 30 min at 4°C.

For mitochondrial membrane solubilization analyses, both the cleared supernatant and pellet were denatured with LDS loading buffer for 10 min at 70°C. Samples were run on precast 4-12% bis-tris protein gels (Life Technologies). Proteins were transferred onto nitrocellulose membranes using the iBLOT 2 system (Life Technologies). Membranes were blocked with 5% w/v milk and 0.1% Tween-20 in TBS and incubated with the appropriate antibodies in 5% w/v BSA in TBS with 0.1% Tween-20 overnight at 4°C. The following antibodies were used: Opa1 (BD), rodent OXPHOS complex proteins cocktail (Abcam), Calnexin (Santa Cruz), and β -Actin, Mfn2, Drp1, Drp1^{PS616} (Cell Signaling Technologies). All primary antibody incubations were followed by incubation with secondary HRP-conjugated antibody (Pierce) in 5% milk and 0.1% Tween-20 in TBS and visualized using SuperSignal West Pico or femto Chemiluminescent Substrate (Pierce) on Biomax MR film (Kodak).

Retroviral transduction

Activated OT-I splenocytes were transduced with control (empty vector) or Mfn1, Mfn2, Opa1 expressing retrovirus by centrifugation for 90 minutes in media containing hexadimethrine bromide (8 μ g/mL; Sigma) and IL-2 (100 U/mL). GFP or human CD8 were markers for retroviral expression.

Cytotoxicity assay

EL4-OVA tumor cells were pre-treated with 100 U/mL murine IFN- γ for 24 hours before use. To generate target cells, 1×10^6 tumor cells were labeled with 0.5 μ M Cell Proliferation Dye e670 (eBioscience) in PBS for 8 minutes at room temperature, washed twice with PBS and 10,000 cells were seeded per well in 96-well round bottom plates. IL-2 T_E cells treated with DMSO or

M1+Mdivi-1 were co-cultured with target cells at the indicated effector/target cell ratios and incubated for 12 hours at 37°C in 5% CO₂. To generate reference cells, 1x10⁶ tumor cells were labeled with 5 μM Cell Proliferation Dye e670 in PBS and incubated on ice. 10,000 reference cells were added before cells were stained with Po-ProTM-1 dead cell staining dye (Life Technologies). IL-2 T_E cell killing efficiency was analyzed by flow cytometry and data defined as percentage of live cells normalized to reference cells.

Statistical analysis

Comparisons for two groups were calculated using unpaired two-tailed student's *t*-tests, comparisons for more than two groups were calculated using one-way ANOVA followed by Bonferroni's multiple comparison tests. Comparisons over time were calculated using two-way ANOVA followed by Bonferroni's multiple comparison tests.

**Chapter 4: Mitochondrial Dynamics Controls T Cell Fate Through Metabolic
Programming**

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Introduction

T cells are important mediators of protective immunity against pathogens and cancer and have several unique properties, not least of which is their ability to proliferate at a rate arguably unlike any other cell in an adult organism. In this regard, one naïve T cell can clonally expand into millions of ‘armed’ T_E cells in just a few days (Williams and Bevan, 2007). Concomitant with T cell activation is the engagement of Warburg metabolism, a metabolic phenotype shared by cancer cells and unicellular organisms (Fox et al., 2005; Vander Heiden et al., 2009). Once the source of antigen is cleared, most antigen-specific cells die, but a subset of long-lived, resting T_M cells persists (Kaech et al., 2003). T_M cells have a unique metabolism that renders them equipped to rapidly respond should infection or tumor growth recur (Pearce et al., 2013). These extensive changes in phenotype and function of T cells go hand in hand with a highly dynamic metabolic range (Buck et al., 2015; MacIver et al., 2013). As such, these cells represent a distinctive and amenable system in which we can study marked changes in cellular metabolism that occur as part of normal cellular development, and not as a result of transformation.

Both OXPHOS and aerobic glycolysis generate energy in the form of ATP, but importantly, are also critical for other essential processes such as the building of biosynthetic precursors for biomass, the production of reactive oxygen species (ROS), and the balance of reducing/oxidizing equivalents like NADH/NAD⁺ which take part in redox reactions that release energy from nutrients. Naïve T (T_N) cells use OXPHOS for their metabolic needs, but both OXPHOS and aerobic glycolysis are augmented upon activation (Chang et al., 2013; Sena et al., 2013). The latter is characterized by the preferential conversion of pyruvate to lactate in the cytoplasm rather

than its oxidation in the TCA cycle. T_M cells predominantly use OXPHOS like T_N cells, but have enhanced mitochondrial capacity that is marked by their reliance on FAO to fuel OXPHOS (van der Windt et al., 2012; van der Windt et al., 2013). Failure to engage specific metabolic programs impairs the function and differentiation of T cells (Pearce and Pearce, 2013). Establishing the precise reasons why, and how, these and other cells emphasize one particular metabolic pathway over another remains a challenging prospect.

Mitochondria are essential hubs of metabolic activity, antiviral responses, and cell death (Nunnari and Suomalainen, 2012). These dynamic organelles constantly remodel their structure through fission and fusion events mediated by highly conserved nuclear encoded GTPases (Ishihara et al., 2013; Youle and van der Bliek, 2012). Mitochondrial fission generates smaller, discrete and fragmented mitochondria that can increase ROS production (Yu et al., 2006), facilitate mitophagy (Frank et al., 2012), accelerate cell proliferation (Marsboom et al., 2012; Taguchi et al., 2007), and mediate apoptosis (Youle and Karbowski, 2005). Dynamin-related protein 1 (Drp1) is a cytosolic protein that translocates to the outer mitochondrial membrane (OMM) upon phosphorylation to scission mitochondria (Ingeman et al., 2005; Labrousse et al., 1999; Wakabayashi et al., 2009). Fusion of mitochondria into linear or tubular networks limits deleterious mutations in mitochondrial DNA (mtDNA) (Santel et al., 2003), induces supercomplexes of the ETC maximizing OXPHOS activity (Cogliati et al., 2013; Mishra et al., 2014; Zorzano et al., 2010), and enhances endoplasmic reticulum (ER) interactions important for calcium flux (de Brito and Scorrano, 2008). In addition, mitochondria elongate as a survival mechanism in response to nutrient starvation and cell stress, linking fusion to cellular longevity (Friedman and Nunnari, 2014; Gomes et al., 2011; Rambold et al., 2011a). OMM fusion is

mediated by mitofusin 1 and 2 (Mfn1, Mfn2) isoforms (Chen et al., 2003), while inner membrane fusion is controlled by optic atrophy 1 (Opa1) protein (Cipolat et al., 2004). Complete organismal deficiency in any of these proteins is embryonically lethal and mutations in the genes that encode them underlie the cause of several human diseases (Archer, 2014; Chan, 2012; Chen et al., 2007; Zhang et al., 2011).

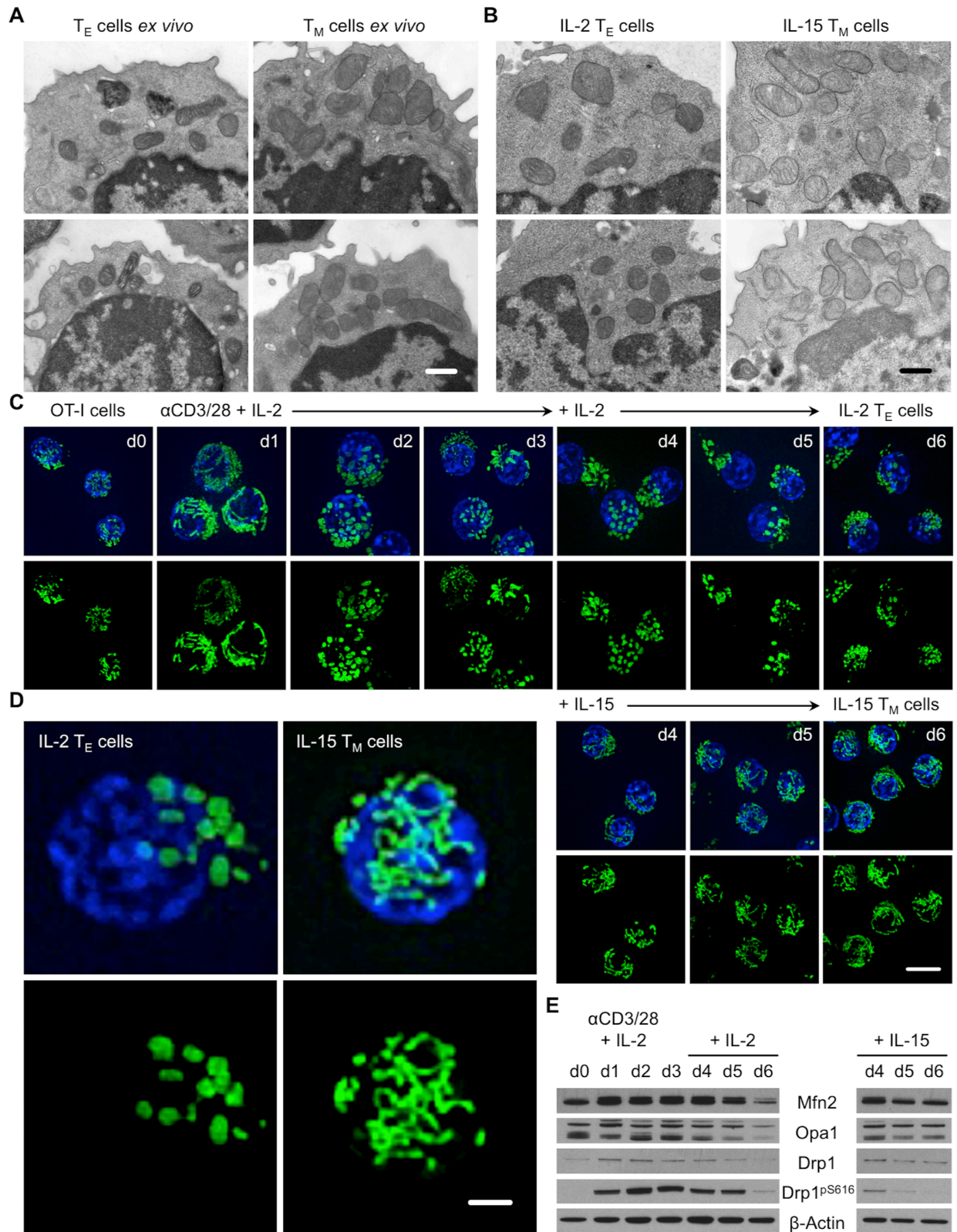
Mitochondrial membrane remodeling has been largely demonstrated to be acutely responsive to changes in cellular metabolism (Mishra and Chan, 2016; Wai and Langer, 2016), but whether it plays a dynamic role in shaping metabolic pathways has been inferred but not extensively studied. At the cellular level, deletion of any of the fission and fusion machinery perturbs OXPHOS and glycolytic rates at baseline (Liesa and Shirihai, 2013). Tissue-specific deletion of Mfn2 in muscles of mice disrupts glucose homeostasis (Sebastian et al., 2012) and Drp1 ablation in the liver results in reduced adiposity and elevated whole-body energy expenditure, protecting mice from diet-induced obesity (Wang et al., 2015b). A recent study has also suggested a link between Drp1 mediated fission and its affect on glycolysis during cell transformation (Serasinghe et al., 2015). The central question of whether fission/fusion and associated changes in cristae morphology actively control the adoption of distinct metabolic programs and therefore regulates T cell responses however, remains unanswered.

Unlike T_E cells, T_M cells maintain a fused mitochondrial network

We reported that T_M cells have more mitochondrial mass than T_E or T_N cells and suggested that mitochondria in these T cell subsets possess distinct morphologies (van der Windt et al., 2012; van der Windt et al., 2013). These observations prompted us to more closely assess mitochondrial structure in T cells. We infected mice with *Listeria monocytogenes* expressing ovalbumin (OVA) (LmOVA) and isolated T_E and T_M cells for ultrastructure analysis by electron microscopy (EM). We found that T_E cells had small, distinct mitochondria dispersed in the cytoplasm, while T_M cells had more densely packed, somewhat tubular, mitochondria (**Figure 6A**). In order to more thoroughly investigate these morphological differences, we differentially cultured activated OVA-specific T cell receptor (TCR) transgenic OT-I cells in interleukin-2 (IL-2) and IL-15 to generate IL-2 T_E and IL-15 T_M cells (**Figure 7**) (Carrio et al., 2004). These culture conditions approximate T cell responses *in vivo* and allow us to generate large numbers of cells amenable to further experimentation *in vitro* (O'Sullivan et al., 2014). We found that IL-2 T_E and IL-15 T_M cells possessed similar mitochondrial ultrastructure as their *ex vivo* isolated counterparts (**Figure 6B**). Next, we acquired live Z-stacked images of these T cells over time by confocal microscopy and found that while at day 1 after activation the mitochondria appeared fused, from days 2-6 after activation, IL-2 T_E cells exhibited predominantly punctate mitochondria (**Figure 6C**). In contrast, once cells were exposed to IL-15, a cytokine that supports T_M cell formation (Schluns et al., 2002), the mitochondria formed elongated tubules (**Figure 6C**). Magnified 3D rendered images from these experiments emphasized the marked differences in mitochondrial morphology between the IL-2 T_E and IL-15 T_M cells (**Figure 6D**). Together these data suggest that the mitochondria in T_E cells are actively undergoing fission,

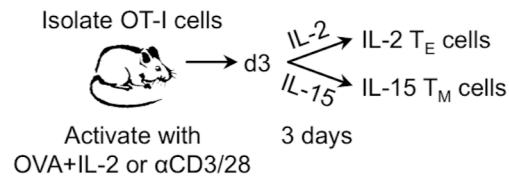
while in T_M cells, these organelles exist in a fused state. To further investigate these changes in mitochondrial morphology, we assessed the expression of several critical protein regulators of mitochondrial dynamics. We found that by day 6, fusion mediators Mfn2 and Opa1 were lower in T_E cells compared to T_M cells, while fission factor Drp1 was more highly phosphorylated at its activating site Ser616 in T_E cells (**Figure 6E**) (Marsboom et al., 2012). These data are consistent with our observations that mitochondria in T_M cells appear more fused than those in T_E cells.

Figure 6. Effector and memory T cells possess distinct mitochondrial morphologies.



(A) C57BL/6 mice were infected i.p. with 1×10^7 CFU LmOVA. Effector (T_E , $CD44^{hi} CD62L^{lo}$, 7 days post infection) and memory T (T_M , $CD44^{hi} CD62L^{hi}$, 21 days post infection) cells were sorted and analyzed by EM as well as (B) IL-2 T_E and IL-15 T_M cells generated from differential culture of OT-I cells activated with OVA peptide and IL-2 using IL-2 or IL-15, scale bar = 0.5 μm . (C-D) Mitochondrial morphology was analyzed in live OT-I PhAM cells over time before and after $\alpha CD3/CD28$ activation and differential cytokine culture by spinning disk confocal microscopy. Mitochondria are green (GFP) and nuclei are blue (Hoechst). (C) Scale bar = 5 μm , (D) Scale bar = 1 μm . (E) Immunoblot analysis of cell protein extracts from (C), probed for Mfn2, Opa1, Drp1, phosphorylated Drp1 at Ser616 ($Drp1^{pS616}$), and β -actin. (A-E) Results representative of 2 experiments.

Figure 7. *In vitro* differentiation of IL-2 T_E and IL-15 T_M cells approximate T cell response conditions that generate T_E and T_M cells *in vivo*.



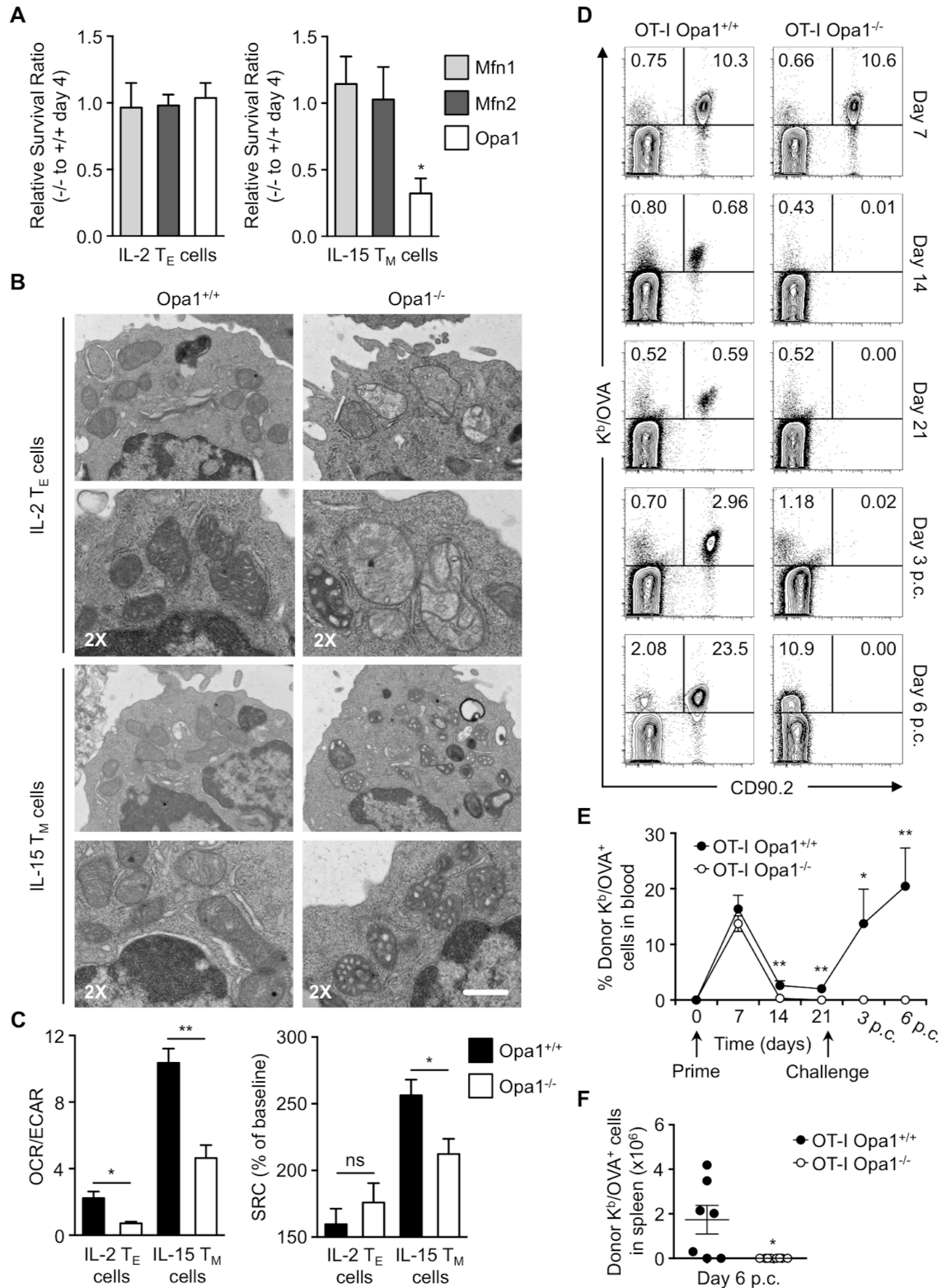
OT-I cells were activated with IL-2 and either OVA peptide or α CD3/CD28 for 3 days and then differentially cultured in IL-2 or IL-15 for an additional 3 days to generate IL-2 T_E and IL-15 T_M cells, respectively.

Mitochondrial inner membrane fusion protein Opa1 is necessary for T_M cell generation

We questioned next whether mitochondrial fusion was important for T_M cell generation and survival. We crossed Mfn1, Mfn2, and Opa1 floxed mice to OT-I CD4 Cre transgenic mice to conditionally delete these proteins in T cells. Peripheral T cell frequencies in these mice were grossly normal (**data not shown**). We differentially cultured these Mfn1^{-/-}, Mfn2^{-/-}, and Opa1^{-/-} OT-I T cells in IL-2 and IL-15 and found that only Opa1^{-/-} T cells displayed a selective defect in survival when cultured in IL-15 (**Figure 8A**). Opa1 deficiency did not effect IL-2 T_E cell survival. We measured the efficiency of gene deletion by mRNA and/or protein analyses (**Figure 9A-C**). While Mfn1 and 2 were efficiently deleted, we found some residual expression of Opa1 particularly in IL-15 T_M cells, suggesting that we were selecting for cells that retained expression of Opa1 in IL-15 culture conditions, albeit at a diminished level as most of these cells die (**Figure 8A**). We also assessed mitochondrial ultrastructure and, in agreement with published results for other cell types (Cogliati et al., 2013; Zhang et al., 2011), mitochondrial cristae were significantly altered and disorganized in the absence of Opa1 (**Figure 8B**). Consistent with their survival defect, Opa1^{-/-} IL-15 T_M cells exhibited decreased OXPHOS activity, as measured by O₂ consumption rate (OCR, an indicator of OXPHOS) to extracellular acidification rate (ECAR, an indicator of aerobic glycolysis) ratio, and spare respiratory capacity (SRC), compared to normal cells (**Figure 8C**). SRC is the extra mitochondrial capacity available in a cell to produce energy under conditions of increased work or stress and is thought to be important for long-term cellular survival and function (measured as OXPHOS activity above basal after uncoupling with FCCP) (Choi et al., 2009; Ferrick et al., 2008; Nicholls, 2009; Nicholls et al., 2010; van der Windt et al., 2012; Yadava and Nicholls, 2007). To determine whether Opa1 function is required for T_M cell

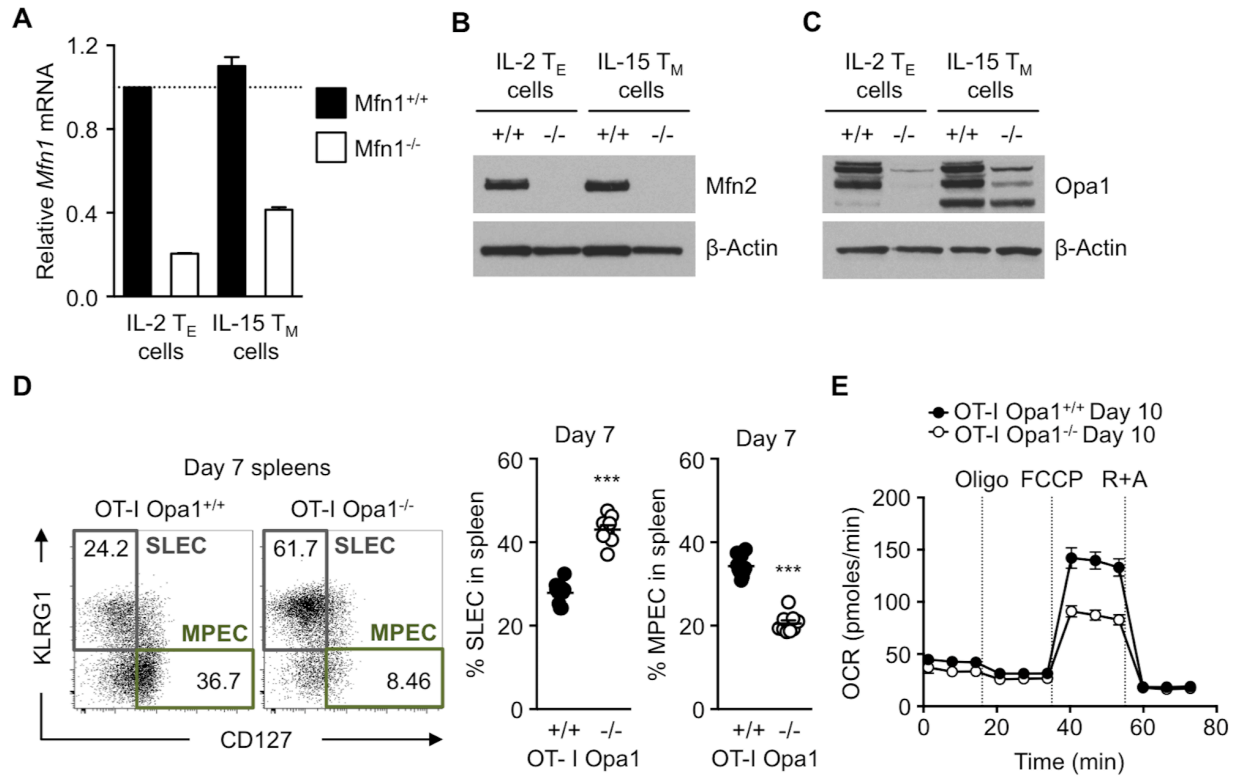
development *in vivo*, we adoptively transferred naïve Opa1^{-/-} OT-I T cells into congenic recipients, infected these mice with LmOVA, and subsequently assessed T_M cell formation in the weeks after infection. Control and Opa1^{-/-} OT-I T cells mounted normal T_E cell responses (day 7) to infection, while Opa1^{-/-} OT-I T_M cell formation (days 14-21) was drastically impaired (**Figure 8D**). Consistent with diminished T_M cell development, a significantly higher proportion of short-lived effector cells to memory precursor effector cells were present within the Opa1^{-/-} OT-I donor cell population 7 days after infection (**Figure 9D**) (Kaech et al., 2003). In addition, at day 10 post-infection, a time point at which T_E cells contract, while T_M cells emerge, Opa1^{-/-} T cells isolated *ex vivo* had decreased SRC compared to control cells (**Figure 9E**), correlating with their decreased survival. To assess whether Opa1^{-/-} T_M cells existed in too low an abundance to be discerned by flow cytometry, we challenged these mice with a second infection. We observed no recall response (day 3 and 6 p.c.) from Opa1^{-/-} T cells when assessing frequency (**Figure 8E**) or absolute numbers (**Figure 8F**), while there was considerable expansion of control donor cells. These data illustrate that Opa1 function is required for T_M cell, but not T_E cell generation.

Figure 8. Memory T cell development and survival, unlike effectors, requires mitochondrial fusion.



(A) Relative *in vitro* survival ratios of Mfn1, Mfn2, or Opa1 deficient (CD4 Cre⁺, -/-) to wild-type control (CD4 Cre⁻, +/+) OT-I IL-2 T_E and IL-15 T_M cells (*p=0.0465). Data normalized from 2-3 independent experiments shown as mean ± SEM. (B) Mitochondrial morphology of OT-I Opa1 wild-type and Opa1 knockout IL-2 T_E and IL-15 T_M cells analyzed by EM (scale bar = 0.5 μm, one experiment represented) and (C) Seahorse EFA. (Left) bar graph represents ratios of O₂ consumption rates (OCR, an indicator of OXPHOS) to extracellular acidification rates (ECAR, an indicator of aerobic glycolysis) at baseline and (right) spare respiratory capacity (SRC) (% max OCR after FCCP injection of baseline OCR) of indicated cells (*p<0.03, **p=0.0079). Data from 3 experiments shown as mean ± SEM. (D-F) 10⁴ OT-I Opa1^{+/+} or Opa1^{-/-} T cells were transferred i.v. into C57BL/6 CD90.1 mice infected i.v. with 1x10⁷ CFU LmOVA. Blood was analyzed by flow cytometry at indicated time points post infection. After 21 days, mice were challenged i.v. with 5x10⁷ CFU LmOVA and blood analyzed post challenge (p.c.). (D) % Donor K^b/OVA⁺ and CD90.2⁺ live cells shown in representative flow plots and (E) line graph with mean ± SEM. (*p=0.0238, **p<0.005). (F) Number of donor K^b/OVA⁺ cells isolated from spleens of infected mice shown as mean ± SEM (*p=0.0126). (D-F) Results representative of 2 experiments (n=9-11 per group).

Figure 9. Assessment of genetic deletion of mitochondrial fusion proteins in IL-2 T_E and IL-15 T_M cells and of donor T_E cells generated from infection.



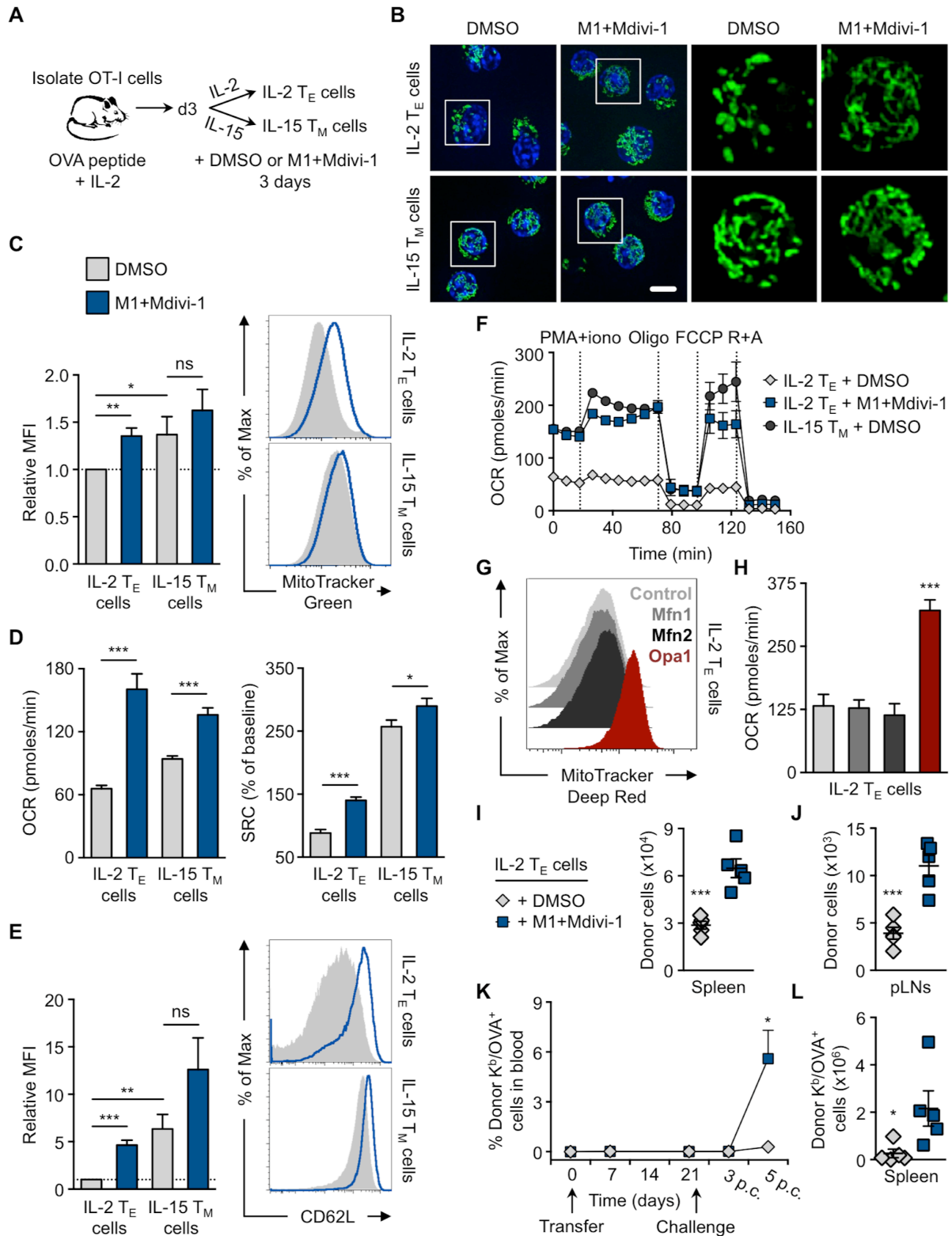
(A-C) IL-2 T_E and IL-15 T_M cells were cultured from (A) OT-I Mfn1 floxed, (B) OT-I Mfn2 floxed, (C) OT-I Opa1 floxed mice crossed to CD4 Cre transgenic mice to generate T cells conditionally deleted for proteins that mediate mitochondrial fusion (+/+ are CD4 Cre⁻ and -/- are CD4 Cre⁺). Efficiency of deletion by cre recombinase analyzed by (A) qPCR and (B-C) immunoblot. (D) Flow cytometry analysis of short-lived effector cells (SLEC, KLRG1^{hi} CD127^{lo}) and memory precursor effector cells (MPEC, KLRG1^{lo} CD127^{hi}) generated at day 7 post infection from OT-I Opa1^{+/+} and OT-I Opa1^{-/-} cells transferred into congenic recipients infected with LmOVA. Representative flow dot plots (left) and scatter dot plots (right) with mean ± SEM bars. Each dot represents individual mice (n=8-9 per genotype), ***p<0.0001. (E) OCR analysis of day 10 post-infection OT-I Opa1^{+/+} and Opa1^{-/-} donor cells at baseline and after oligomycin (Oligo), FCCP, and rotenone plus antimycin A (R+A) injections. Data is representative of 2 experiments shown as mean ± SEM.

Mitochondrial fusion imposes a T_M cell phenotype, even in the presence of activating signals

Genetic loss of function of Opa1 revealed that this protein is critical for T_M cell formation. Given the fused phenotype of mitochondria in these cells, we hypothesized that Opa1-mediated mitochondrial fusion supports the metabolism needed for T_M cell development. We used a gain of function approach to enhance mitochondrial fusion. Culturing T cells with the ‘fusion promoter’ M1 (Wang et al., 2012), and the ‘fission inhibitor’ Mdivi-1 (Cassidy-Stone et al., 2008) (**Figure 10A**), induced mitochondrial fusion in IL-2 T_E cells, rendering them morphologically similar to IL-15 T_M cells (**Figure 10B**). Treatment with these drugs enhanced other T_M cell properties in activated IL-2 T_E cells, including increased mitochondrial mass (**Figure 10C**), OXPHOS and SRC (**Figure 10D**), CD62L expression (**Figure 10E**) and robust metabolic activity, as indicated by bioenergetic profiling of the cells in response to secondary stimulation with PMA/ionomycin, followed by addition of oligomycin (ATP synthase inhibitor), FCCP, and rotenone plus antimycin A (ETC complex I and III inhibitors), all drugs that stress the mitochondria (**Figures 10F and 11A**) (Nicholls et al., 2010). However, we did not observe increased mtDNA in these cells (**Figure 11B**). We found that ECAR and the OCR/ECAR ratio increased after drug treatment (**Figure 11C**), indicating elevated metabolic activity overall, with a predominant increase in OXPHOS over glycolysis. While we observed these changes in mitochondrial activity, we did not measure any significant differences in mitochondrial membrane potential or ROS production after drug treatment (**Figure 11D**). The expression of other activation markers were also not substantially affected, although a small decrease in KLRG1 and increase in CD25 was measured (**Figure 11E**). Additionally, we performed a genetic gain of function experiment and transduced activated IL-2 T_E cells with retrovirus

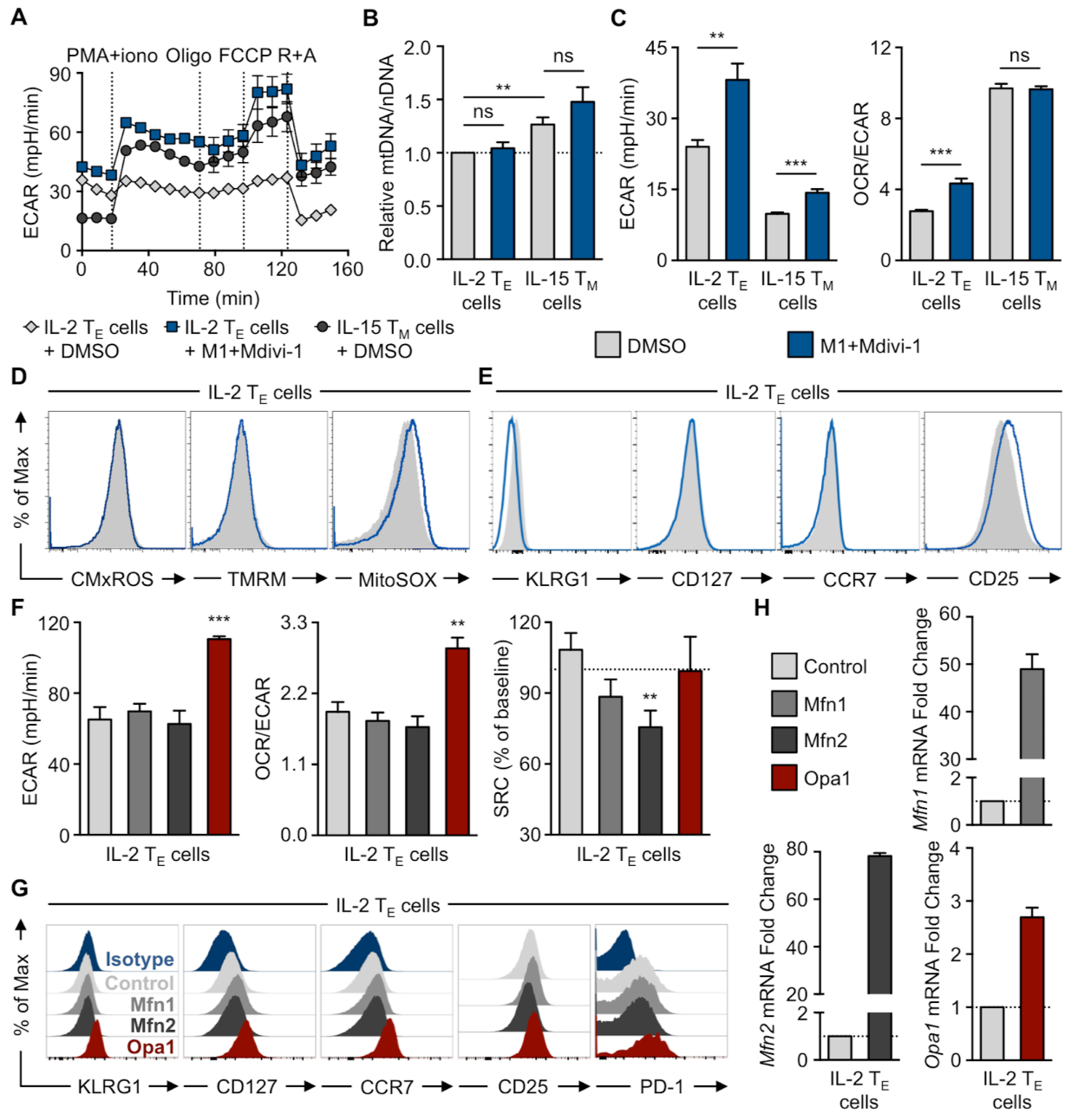
expressing Mfn1, Mfn2, or Opa1. Similar to enforcement of fusion pharmacologically, we found that cells transduced with Opa1 had more mitochondria (**Figure 10G**) and OXPHOS (**Figure 10H**), than empty vector control or Mfn-transduced T cells, as well as increased overall metabolic activity, with a predominant increase in OXPHOS over glycolysis (**Figure 11F**). T_M cell associated markers such as CCR7 and CD127 were increased on transduced cells, as well as T_E cell proteins, such as PD-1 (**Figure 11G**). We confirmed by mRNA expression that each target gene had increased expression after transduction over the control (**Figure 11G**). Together our results show that mitochondrial fusion confers a T_M cell phenotype on activated T_E cells even in culture conditions that program T_E cell differentiation.

Figure 10. Enhancing mitochondrial fusion promotes the generation of memory-like T cells.



(**A-F, I-L**) OVA peptide and IL-2 activated OT-I cells were differentiated into IL-2 T_E or IL-15 T_M cells for 3 days in the presence of DMSO control or fusion promoter M1 and fission inhibitor Mdivi-1 (M1+Mdivi-1) as shown in (**A**) pictorially. (**B**) Representative spinning disk confocal images from 3 experiments of live cells generated from OT-I PhAM mice. Mitochondria are green (GFP) and nuclei are blue (Hoechst), scale bar = 5 μ m. (**C**) Cells stained with MitoTracker Green and analyzed by flow cytometry. Relative MFI (left) from 6 experiments shown as mean \pm SEM (*p=0.0394, **p=0.0019) with representative histograms (right). (**D**) Baseline OCR and SRC of indicated cells from 3-4 experiments shown as mean \pm SEM (*p=0.0485, ***p<0.0001). (**E**) CD62L expression analyzed by flow cytometry of indicated cells. Relative MFI (left) from 7 experiments shown as mean \pm SEM (*p=0.0325, **p=0.0019, ***p<0.0001) with representative histograms (right). (**F**) OCR of indicated cells at baseline and in response to PMA and ionomycin stimulation (PMA+iono), oligomycin (Oligo), FCCP, and rotenone plus antimycin A (R+A). Data represents 2 experiments shown as mean \pm SEM. (**G-H**) OT-I cells were transduced with either empty (Control), Mfn1, Mfn2, or Opa1 expression vectors, sorted, and cultured to generate IL-2 T_E cells. (**G**) Histograms representative of 4 experiments of cells stained for MitoTracker Deep Red and (**H**) OCR data at baseline of transduced cells from 2 experiments. (**I-L**) 1-2x10⁶ IL-2 T_E cells cultured with DMSO (gray diamonds) or M1+Mdivi-1 (blue squares) were transferred into congenic C57BL/6 recipient mice. Cell counts of donor cells recovered 2 days later from the (**I**) spleen (***p=0.005) and (**J**) peripheral lymph nodes (pLNs, ***p=0.0006). (**K**) Blood from recipient mice analyzed for % donor K^b/OVA⁺ cells post transfer and challenge with 1x10⁷ CFU LmOVA by flow cytometry (*p=0.0150, n=5 per group). (**L**) Donor K^b/OVA⁺ cells recovered from recipient spleens 6 days post challenge (*p=0.0383). (**I-L**) Data represents 2 experiments shown as mean \pm SEM.

Figure 11. Assessment of T cell phenotype and metabolism following pharmacological or genetic enhancement of mitochondrial fusion.



(**A-E**) IL-2 T_E and IL-15 T_M cells generated from OT-I mice were treated with DMSO control or M1+Mdivi-1. (**A**) ECAR of indicated cells at baseline and after PMA and ionomycin (PMA+iono) stimulation, oligomycin (Oligo), FCCP, and rotenone plus antimycin A (R+A). (**B**) qPCR analysis of relative mitochondrial DNA (mtDNA) to nuclear DNA (nDNA) ratios of indicated cells. (**C**) ECAR (left) and OCR/ECAR ratios (right) of indicated cells under basal conditions. (**D**) Histograms of membrane potential (CMxROS, TMRM) and mitochondrial ROS (MitoSOX) using indicated fluorescent dyes and (**E**) KLRG1, CD127, CCR7, and CD25 surface marker expression of indicated cells analyzed by flow cytometry. (**F-H**) OT-I IL-2 T_E cells were activated and transduced with empty vector (Control), Mfn1, Mfn2, or Opa1 expressing retrovirus. (**F**) ECAR, OCR/ECAR, and SRC analyzed by Seahorse EFA, (**G**) KLRG1, CD127, CCR7, CD25 and PD-1 surface marker expression assessed by flow cytometry, and (**H**) gene expression analysis by qPCR. (**A-G**) Data are shown as mean \pm SEM and are representative or (**B-C, F**) combined from 2-3 experiments, not significant (ns), **p<0.001, ***p<0.0001.

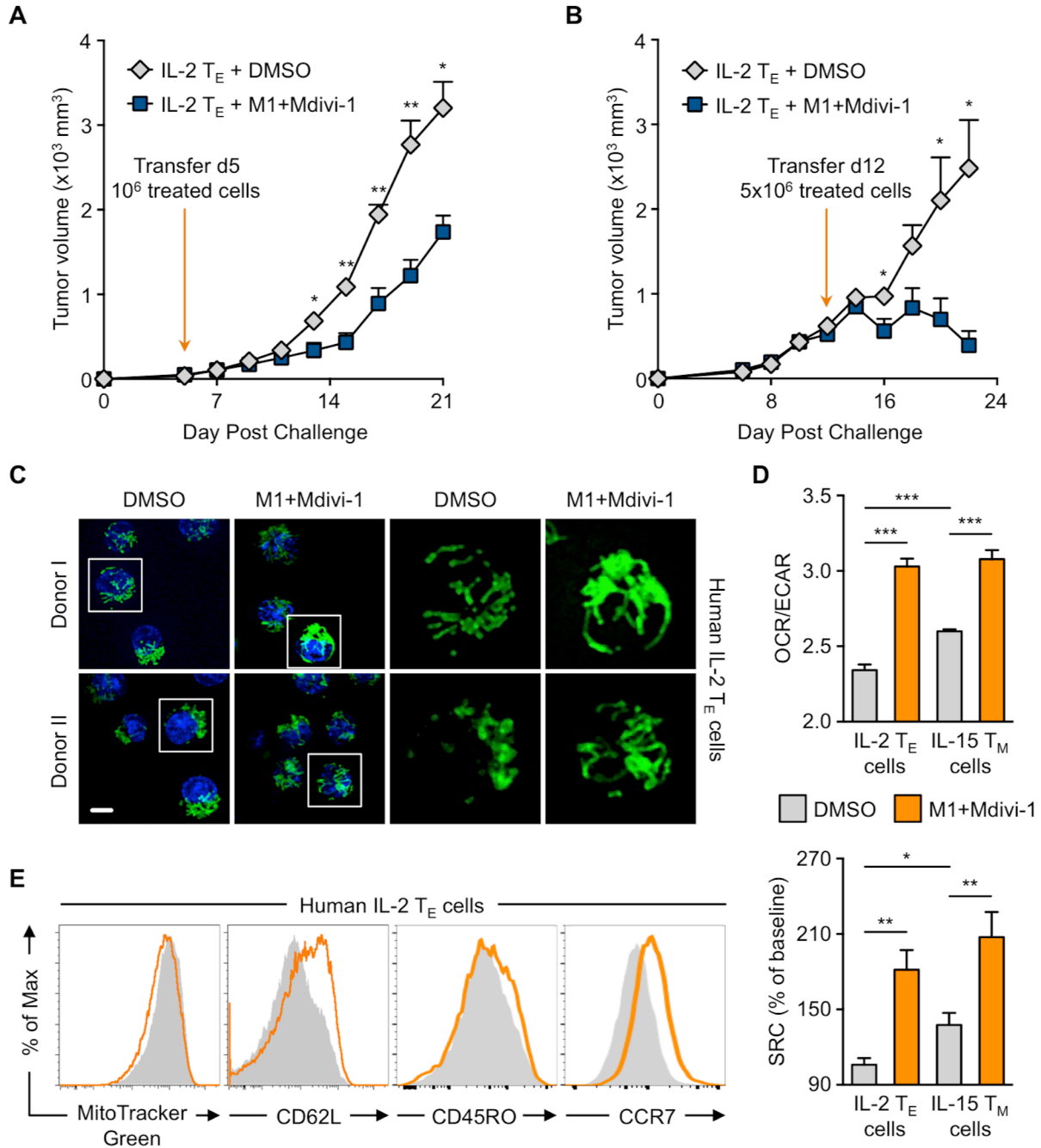
T cell mitochondrial fusion improves adoptive cellular immunotherapy against tumors

A major consideration when designing adoptive cellular immunotherapy is to improve T cell fitness during *ex vivo* culture, so that when T cells are re-introduced into a patient they are able to function efficiently and persist for long periods of time (Maus et al., 2014; O'Sullivan and Pearce, 2015; Restifo et al., 2012). Our data showed that fusion-promoting drugs created metabolically fit T cells. We hypothesized that enforced fusion would also enhance the longevity of IL-2 T_E cells *in vivo*. To test this, we adoptively transferred control and M1+Mdivi-1 treated OT-I T cells into congenic mice and tracked donor cell survival. We found significantly more drug treated T cells in the spleen (**Figure 10I**) and lymph nodes (**Figure 10J**) 2 days after transfer. To determine if the persistence of these cells would be maintained better long term than control cells, we infected mice with LmOVA more than 3 weeks later and measured T cell responses against the bacteria. We found that drug-treated cells selectively expanded in response to infection (**Figure 10K**) and could be recovered in significantly greater numbers in the spleen 6 days post-challenge (**Figure 10L**).

Next, we assessed whether these drugs could be used to promote T cell function in a model of adoptive cell immunotherapy. We injected EL4-OVA tumor cells into mice. Then either 5 or 12 days later we adoptively transferred IL-2 T_E cells that had been previously treated with DMSO or M1+Mdivi-1. In both settings, mice that had received 'fusion-promoted' T cells were able to control tumor growth significantly better than mice that had received control treated cells (**Figures 12A and B**). The cytolytic ability (**Figure 13A**) and proliferation (**Figure 13B**) of the modified IL-2 T_E cells were similar to control cells, however, fusion enforced IL-2 T_E cells

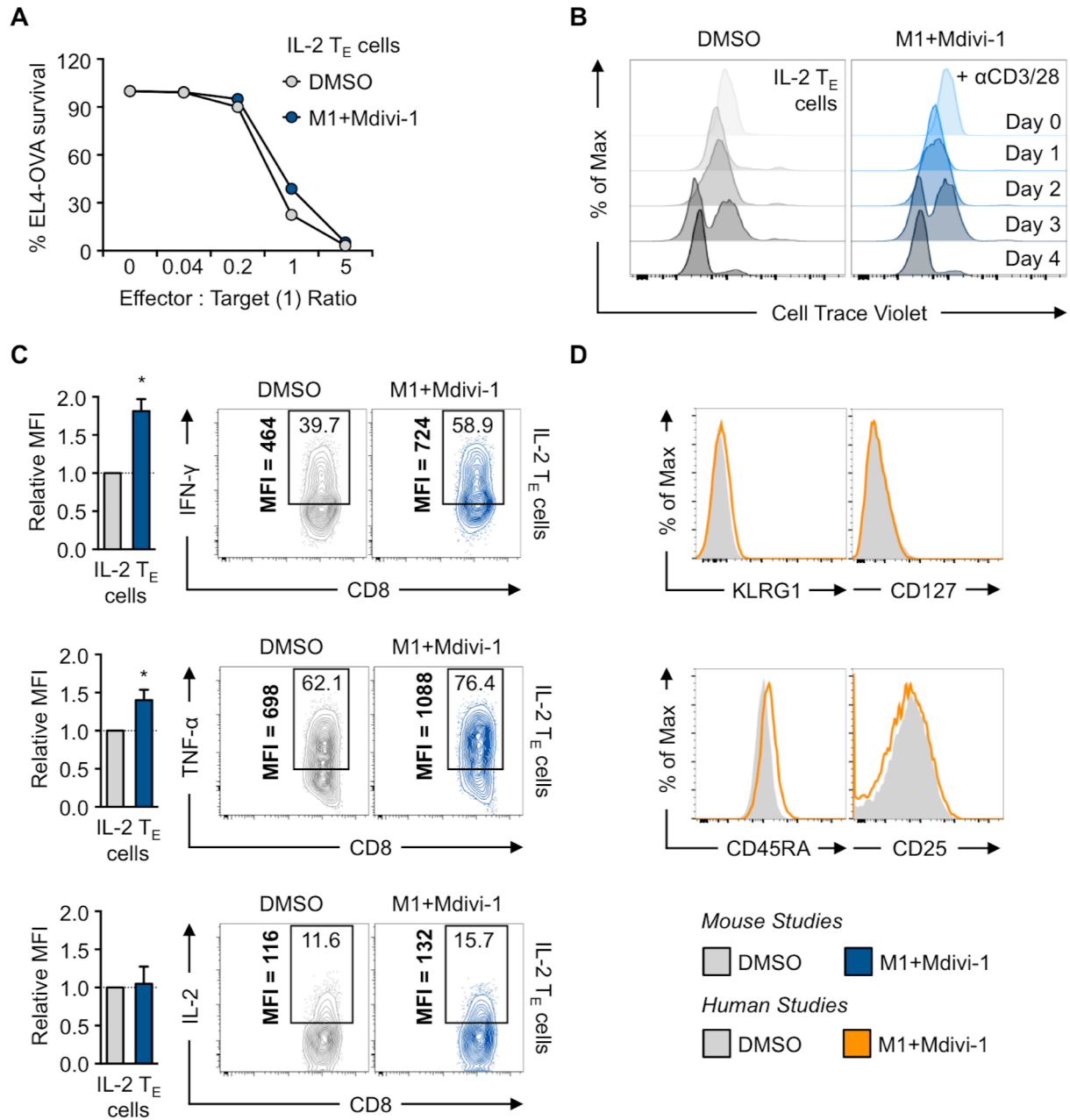
expressed significantly higher levels of IFN- γ and TNF- α when restimulated with PMA and ionomycin *in vitro* (**Figure 13C**). We also exposed activated human T cells to M1+Mdivi-1 treatment *in vitro* and found that activated human IL-2 T_E cells had visibly more fused mitochondria (**Figure 12C**), and exhibit the bioenergetic profile (**Figure 12D**), and surface marker expression (**Figure 12E**) characteristic of T_M cells, compared to control treated cells. Parameters such as mitochondrial mass (**Figure 12E**) and other surface markers (**Figure 13D**) were not significantly altered. These data suggest that promoting fusion in T cells may be a translatable treatment for enhancing human therapy.

Figure 12. Mitochondrial fusion improves adoptive cellular immunotherapy against tumors.



(**A-B**) C57BL/6 mice were inoculated s.c. with 1×10^6 EL4-OVA cells. (**A**) After 5 or (**B**) 12 days, 1×10^6 or 5×10^6 OT-I IL-2 T_E cells cultured with DMSO or M1+Mdivi-1 were transferred i.v. into recipient mice and tumor growth assessed. Data represents 2 experiments shown as mean \pm SEM (n=5 per group, *p<0.05, **p<0.005). (**C-E**) Human CD8⁺ PBMCs were activated with α CD3/CD28 + IL-2 to generate IL-2 T_E cells. (**C**) Confocal images of indicated treated cells where mitochondria are green (MitoTracker) and nuclei are blue (Hoechst). Representative images from 2 of 4 biological donors, scale bar = 5 μ m. (**D**) OCR/ECAR ratios and SRC of indicated cells from 2 separate donors shown as mean \pm SEM (*p=0.0303, **p<0.005, ***p<0.0001). (**E**) MitoTracker Green staining and CD62L, CD45RO, and CCR7 expression analyzed by flow cytometry shown with representative histograms from 4-6 biological replicates.

Figure 13. Examination of mouse and human IL-2 T_E cells after enforcing mitochondrial fusion with drugs.



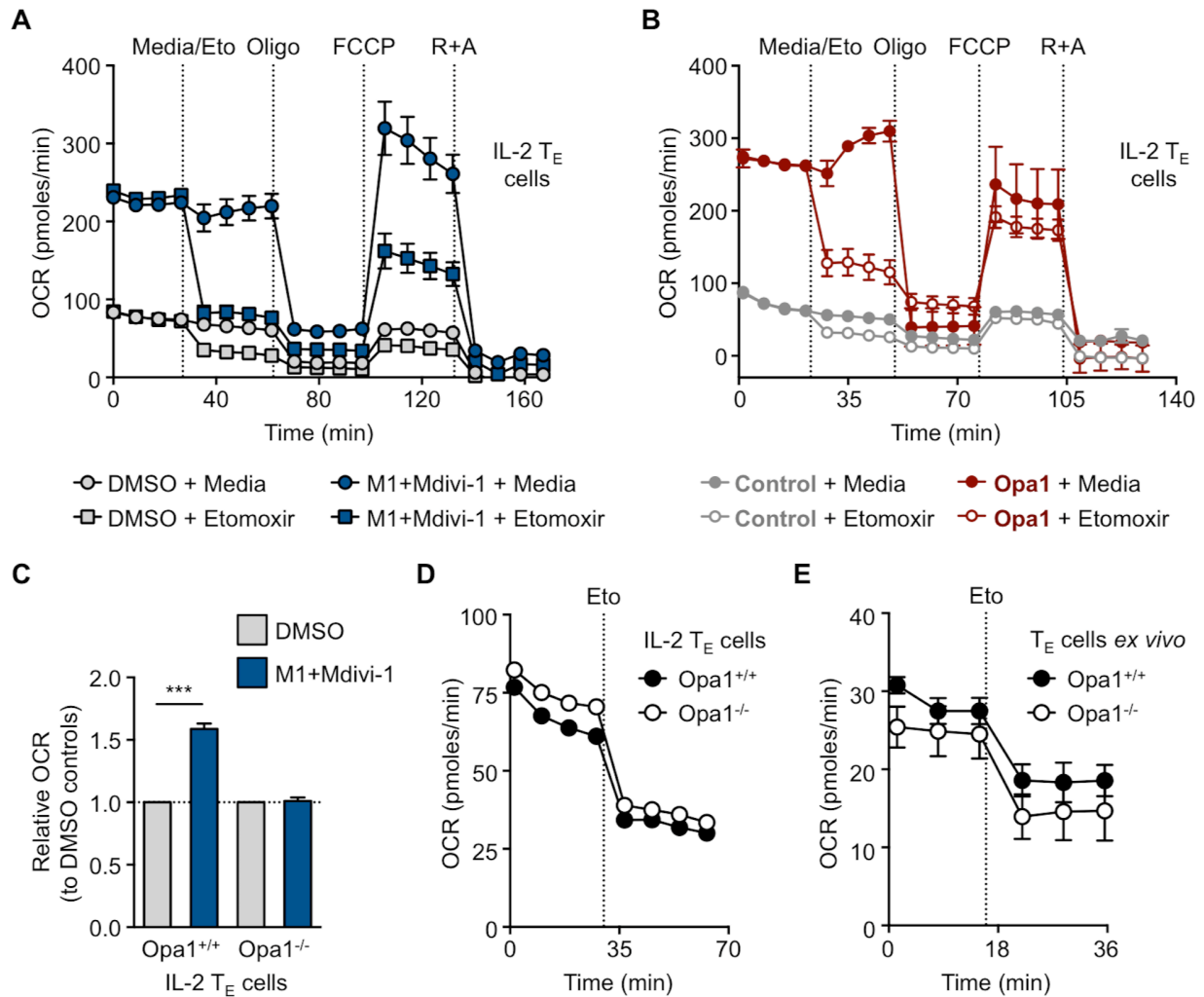
(A-C) Flow cytometry analyses of IL-2 T_E cells previously cultured with DMSO or M1+Mdivi-1 combined from 3 biological replicates. Cells were not subjected to further treatment with DMSO or M1+Mdivi-1 during experiment assays. (A) Cytolysis of EL4-OVA target cells at indicated concentrations. (B) Proliferation after restimulation with α CD3/CD28. (C) Intracellular cytokine staining after 4 hours stimulation with PMA and ionomycin. Relative MFI (left) with mean \pm SEM and representative contour plots (right) with percentage of cytokine positive cells indicated in gated cells and MFI in bold, *p<0.05. Gates based on unstimulated cells (not shown). (D) Human CD8⁺ PBMCs were activated with α CD3/CD28 + IL-2 to generate IL-2 T_E cells and subjected to DMSO or M1+Mdivi-1 treatment. KLRG1, CD127, CD45RA, and CD25 surface marker expression analyzed by flow cytometry shown with representative histograms from 4-6 biological replicates.

Mitochondrial fusion promotes T_M cell metabolism, but Opa1 is not required for FAO

Our data showed that Opa1 was a necessary regulator of T_M cell development, but the question of precisely how Opa1 acted to support T_M cells remained. We hypothesized that mitochondrial fusion, via Opa1 function, was needed for FAO, as the engagement of this pathway is a requirement for T_M cell development and survival (Pearce et al., 2009; van der Windt et al., 2012; van der Windt et al., 2013). This hypothesis was not only based on our observations that these two processes seemed to be linked in T_M cells, but also on a recent study showing that mitochondrial fusion is important for efficient FAO via lipid droplet trafficking under starvation conditions (Rambold et al., 2015). We treated IL-2 T_E and IL-15 T_M cells with M1+Mdivi-1 or vehicle and then measured OCR in response to etomoxir, a specific inhibitor of mitochondrial long chain FAO (Deberardinis et al., 2006), and mitochondrial inhibitors. We found that the increased OCR and SRC evident in these cells after M1+Mdivi-1 treatment was due to augmented FAO (**Figures 14A and 15A**). IL-2 T_E cells transduced with Opa1 also exhibited enhanced OCR that decreased in the presence of etomoxir compared to control cells (**Figure 14B**). Bone marrow derived macrophages (BM-Macs) cultured with M1+Mdivi-1 also increased OCR and SRC to levels similar as M2 polarized macrophages, which engage FAO much like T_M cells do (**Figure 15B**) (Huang et al., 2014). Importantly, M1+Mdivi-1 treatment did not increase OCR (**Figure 14C**) and did not affect ECAR (**Figure 15C**) in Opa1^{-/-} IL-2 T_E cells compared to controls, suggesting a requirement for Opa1 in augmenting OCR and FAO. However, in contrast to what we expected, when we assessed bioenergetics of Opa1^{+/+} and Opa1^{-/-} IL-2 T_E cells (**Figure 14D**) and *ex vivo* isolated T_E cells (**Figure 14E**), we found that both cell types are

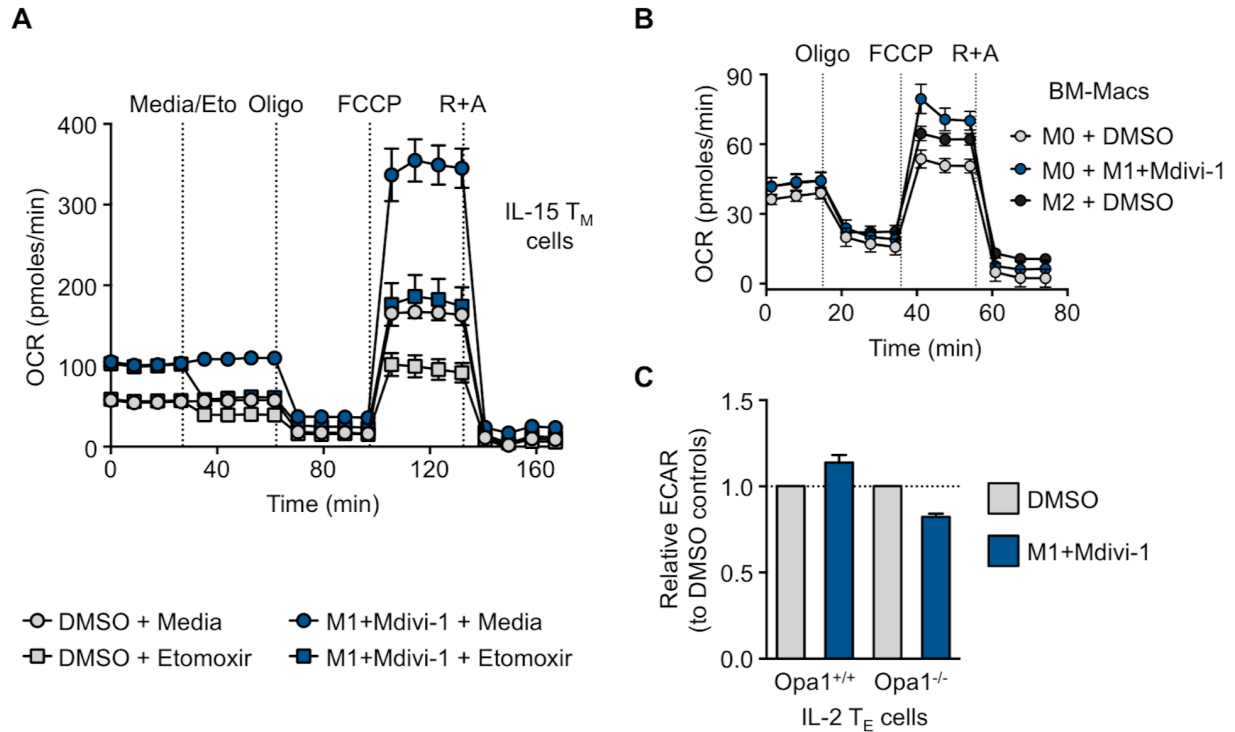
equally responsive to etomoxir. Our results show that while Opa1 could promote FAO in T cells, it was not compulsory for engagement of this metabolic pathway.

Figure 14. Fusion promotes memory T cell metabolism, but Opa1 is not required for FAO.



(A-E) OCR measured at baseline and in response to media, etomoxir (Eto) and other drugs as indicated of (A) IL-2 T_E cells cultured in DMSO or M1+Mdivi-1, (B) control or Opa1 transduced IL-2 T_E cells, (C) Opa1^{+/+} and Opa1^{-/-} IL-2 T_E cells cultured in DMSO or M1+Mdivi-1 (D) or without drugs, and (E) *ex vivo* donor OT-I Opa1^{+/+} and Opa1^{-/-} day 7 T_E cells derived from LmOVA infection. (A-E) Data representative of 2 independent experiments shown as mean ± SEM (***)p<0.0001).

Figure 15. Bioenergetics analysis after promoting mitochondrial fusion in T cells and macrophages.



(A) OCR of IL-15 T_M DMSO or M1+Mdivi-1 treated cells measured at baseline and in response to media, etomoxir (Eto), oligomycin (Oligo), FCCP, and rotenone plus antimycin A (R+A). (B) Bone marrow derived macrophages were cultured overnight with IL-4 (M2) or without (M0) and either DMSO or M1+Mdivi-1 overnight. OCR analyzed at baseline and after injection of mitochondrial inhibitors as indicated. (C) Baseline ECAR relative to DMSO controls of IL-2 T_E Opa1^{+/+} and Opa1^{-/-} cells cultured in DMSO or M1+Mdivi-1. (A-C) Data are shown as mean ± SEM and are representative of 2-3 experiments.

Mitochondrial cristae remodeling signals metabolic adaptations in T_M and T_E cells

Although both Opa1^{+/+} and Opa1^{-/-} IL-2 T_E cells could equally engage FAO (**Figure 14D**), we observed that ECAR was significantly augmented in the Opa1^{-/-} cells both *in vitro* and *ex vivo* (**Figure 16A**). Furthermore, unlike control cells, we observed no additional drop of OCR in Opa1^{-/-} IL-2 T_E cells after the addition of oligomycin (**Figure 16B**), suggesting that in the absence of Opa1, only FAO supports OXPHOS, and that oxidation of other substrates, such as glucose-derived pyruvate, are not utilized for mitochondrial ATP production in this setting. We cultured Opa1^{+/+} and Opa1^{-/-} IL-2 T_E cells with ¹³C-labeled glucose and traced ¹³C into TCA cycle metabolites. We found that while the percent of ¹³C-labeled pyruvate was higher in the Opa1^{-/-} T cells, the frequency of ¹³C-labeled TCA cycle intermediates was significantly reduced in the Opa1^{-/-} T cells compared to controls (**Figures 16C and 17A**), a result that is supported by their higher ECAR (**Figure 16A**). These data suggested that without mitochondrial fusion, pyruvate is preferentially secreted as lactate, rather than oxidized in the mitochondria. Therefore, we questioned whether FAO was a ‘default’ pathway for mitochondria in a resting, or fused state (i.e. Opa1 sufficiency), and that the induction of aerobic glycolysis is a major downstream effect of fission (i.e. Opa1 deficiency). If this were the case, then a balance between fission and fusion, modulated by proteins such as Opa1, could act as a primary signal to dictate the metabolic phenotype of T cells. In support of this idea, T cells from polyclonal T cell-conditional deleted Opa1 animals had higher ECAR and an increased proportion of CD8 T cells with an activated effector phenotype in the basal state based on surface marker expression (**Figure 17B**).

Opa1 is critical for inner mitochondrial membrane fusion, but also for other processes like cristae remodeling (Cogliati et al., 2013; Frezza et al., 2006; Varanita et al., 2015). We observed major changes in cristae morphology in the Opa1^{-/-} T cells (**Figure 8B**). Given the importance of Opa1 function in T_M cell development (**Figure 8**), we further assessed cristae morphology in T_E and T_M cells isolated *ex vivo* after LmOVA infection (**Figure 16D**), as well as in IL-2 T_E and IL-15 T_M cells (**Figure 16E**), and found that T_E cells had many cristae with what appeared to be slightly wider, or more loosely organized intermembrane space, than T_M cells. It has been found that Opa1 overexpression induces cristae tightening and close association of ETC complexes in the inner mitochondrial membrane (Civiletto et al., 2015; Cogliati et al., 2013). Therefore, we surmised that in the absence of Opa1, cristae disorganization leads to dissociation of ETC complexes, and subsequently less efficient ETC activity, in T cells (**Figure 8C**). We assessed OCR after oligomycin in relation to OCR after rotenone plus antimycin A treatment (i.e. proton leak), which indicates the coupling efficiency of OXPHOS with mitochondrial ATP production. Consistent with decreased OXPHOS efficiency, we observed increased proton leak in Opa1^{-/-} T cells compared to control cells (**Figure 16F**). This was also true for *ex vivo* isolated T_E cells when compared to T_M cells (**Figure 16G**), as well as IL-2 T_E and IL-15 T_M cells (**Figure 16H**). Together these data suggest that there are cristae differences between T_E and T_M cells which may contribute to their distinct metabolic phenotypes.

We reasoned that fusion renders tightly configured cristae, which results in closely associated ETC complexes and efficient OXPHOS (Patten et al., 2014), producing conditions that favor the entrance of pyruvate into the TCA cycle. In this situation, NADH generated from the TCA cycle is able to easily donate electrons to complex I, which are passed efficiently along the ETC. Our

data suggested that this predominantly occurs in T_M cells. However, if electron transport across the ETC becomes less efficient, which could be caused by physical separation of the individual complexes due to cristae remodeling via mitochondrial fission, then electrons could linger in the complexes and imbalance redox reactions. NADH levels would build, slowing forward momentum of the TCA cycle. To restore redox balance, cells could augment glycolysis and shunt pyruvate as excreted lactate (i.e. aerobic glycolysis), which would regenerate NAD⁺ from NADH in the cytosol. We speculated that this is what occurs in T_E cells. Correlating with this idea, we previously reported that T_E and T_M cells have different ratios of NAD⁺/NADH (i.e. redox balance), with T_M cells maintaining higher NAD⁺/NADH than T_E cells. We also showed that NADH levels dramatically rise in T_M cells compared to T_E cells when exposed to rotenone/antimycin A, indicating that T_M cells continually consume more NADH for the purpose of donating electrons to the ETC (van der Windt et al., 2012). Together our data suggested that fission and fusion events regulate cristae remodeling, which could alter ETC efficiency and redox balance, ultimately controlling metabolic adaptations in T cells.

To more thoroughly examine this idea, we assessed cristae morphology in T_E and T_M cells by EM following TCR stimulation. We hypothesized that if cristae remodeling acts to induce aerobic glycolysis, changes in cristae structure could be visualized following T cell activation. T_M cells rapidly augment aerobic glycolysis when restimulated (van der Windt et al., 2013). We activated IL-15 T_M cells with α CD3/CD28-conjugated beads (**Figure 16I**), or with PMA and ionomycin (**Figure 17C**), in the presence or absence of Mdivi-1, to modulate activity of the mitochondrial fission protein Drp1 (Cassidy-Stone et al., 2008). We observed dramatic changes to cristae morphology by EM, with the intermembrane space widening over time in control cells

in comparison to drug treated cells. These data are consistent with the hypothesis that fission-induced mitochondrial cristae remodeling supports metabolic reprogramming in T cells.

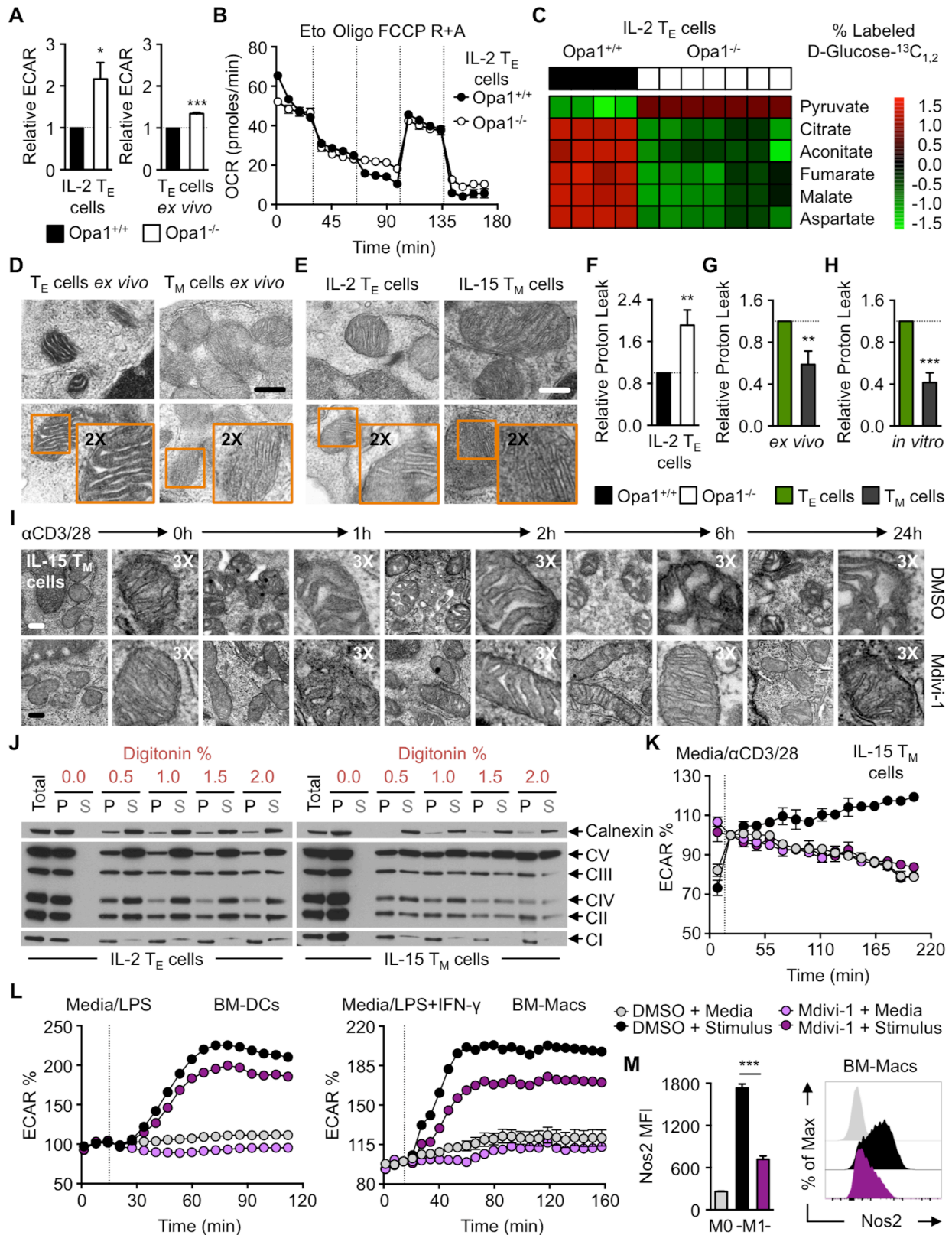
T_M cells maintain tight cristae with closely associated ETC complexes

Our data suggested that unlike T_E cells, T_M cells have tight cristae with close association of the ETC complexes. To investigate this biochemically, we treated native lysates of IL-2 T_E and IL-15 T_M cells with increasing concentrations of digitonin to disrupt all cellular membranes (including mitochondrial). The crude membrane-bound fraction was separated from solubilized proteins by centrifugation. Both pellet and soluble supernatant fractions were loaded on a denaturing reducing gel and then probed for various mitochondrial proteins by western blot. We found that mitochondria in IL-2 T_E cells were susceptible to digitonin disruption, indicated by the fact that ETC complex proteins became less detectable in the pellet, and amplified in the soluble fraction, in 0.5% detergent (**Figures 16J and 17D**). This was in contrast to IL-15 T_M cells, where ETC proteins did not solubilize to the same extent as those in IL-2 T_E cells into the supernatant, even when 2% digitonin was used. To investigate whether this phenomenon was unique to the mitochondrial compartment, we also probed for the ER integral protein calnexin and found that it solubilized similarly in 0.5% digitonin in both cell types. Overall these data suggest that there is more exposed mitochondrial membrane between proteins in IL-2 T_E cells than in IL-15 T_M cells, and correlate with the idea that T_M cells have tight cristae which would yield efficient ETC activity, while T_E cells have looser cristae with less efficient ETC activity, ultimately supporting their distinct metabolic phenotypes.

Mitochondrial fission in activated immune cells facilitates aerobic glycolysis

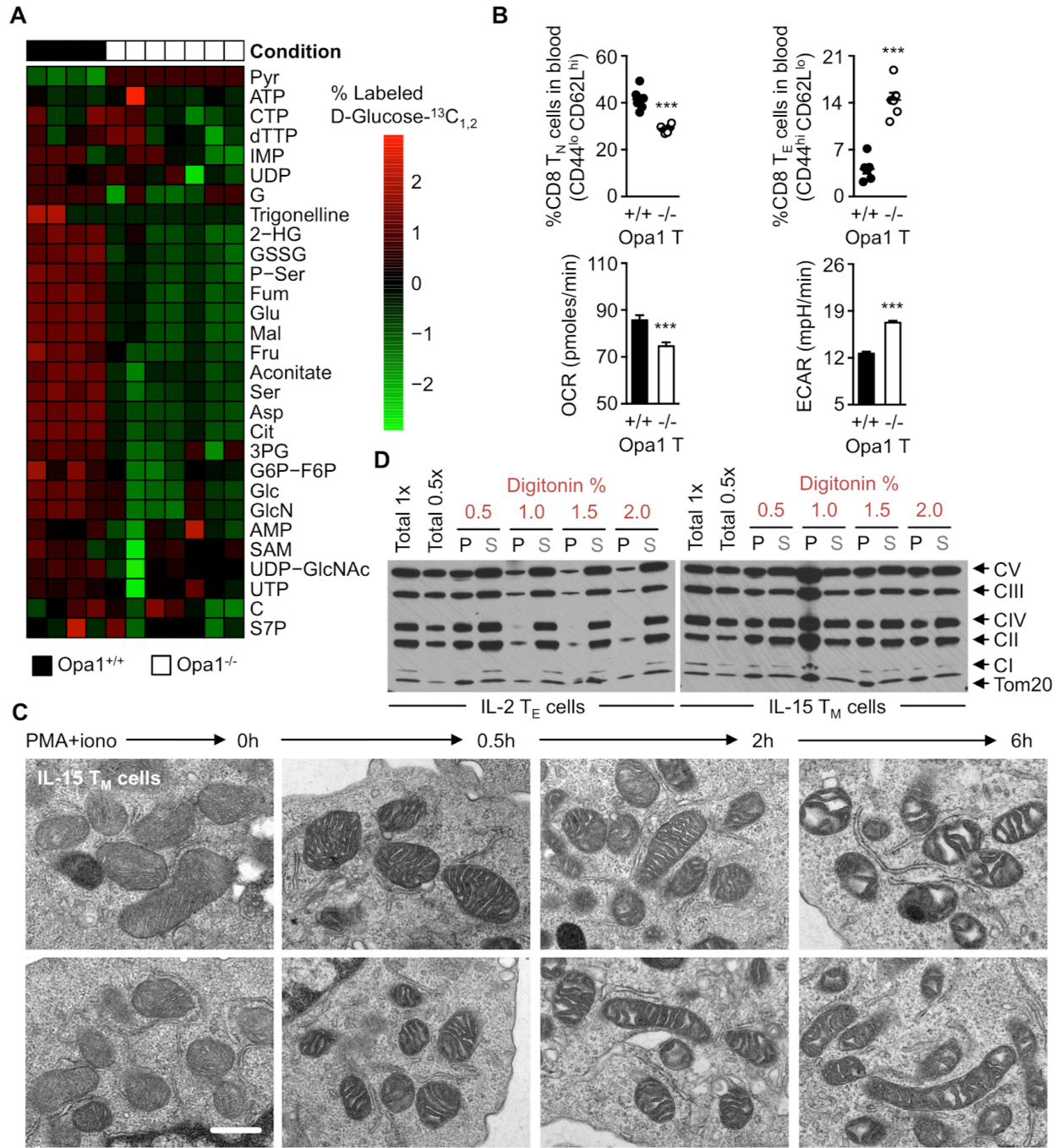
Our data suggested that cristae remodeling, through fission and fusion events, was a mechanism to regulate efficient OXPHOS and FAO in T_M cells, as well as the induction of aerobic glycolysis in T_E cells. To more directly test this idea, we assessed ECAR of IL-15 T_M cells that were stimulated with α CD3/28-conjugated beads in the presence or absence of Mdivi-1. We found that when mitochondrial fission protein Drp1 was inhibited with Mdivi-1, T cell activation did not robustly increase aerobic glycolysis when compared control cells (**Figure 16K**), which correlated with our EM data (**Figure 16I**). Since fission can be associated with cell division (Marsboom et al., 2012; Taguchi et al., 2007), we wanted to test our idea in a non-proliferating cell type that substantially augments aerobic glycolysis upon stimulation (Everts et al., 2014; Everts et al., 2012; Krawczyk et al., 2010). We stimulated bone marrow derived dendritic cells (BM-DCs) and macrophages (BM-Macs) with lipopolysaccharide (LPS) with or without interferon (IFN)- γ in the presence or absence of Mdivi-1 and measured ECAR. Aerobic glycolysis was curtailed in both BM-DCs and BM-Macs following stimulation when Drp1 was inhibited (**Figure 16L**). The blunted ECAR in the Mdivi-1 treated cells correlated with significantly decreased nitric oxide synthase 2 (Nos2) protein expression in the BM-Macs (**Figure 16M**), indicating that their activation was also repressed. These data indicate that cristae remodeling and/or fission acts as a signal to drive the induction of aerobic glycolysis, and subsequent cellular activation via Drp1.

Figure 16. Mitochondrial cristae remodeling signals metabolic pathway engagement.



(A) Basal ECAR of OT-I Opa1^{+/+} and Opa1^{-/-} IL-2 T_E cells (left) and day 7 T_E cells isolated *ex vivo* after adoptive transfer from LmOVA infection (right). Data combined from 2-3 experiments (*p=0.0412, ***p<0.0001). (B) OCR at baseline and after indicated drugs, representative of 2 experiments shown as mean ± SEM, and (C) D-Glucose-¹³C_{1,2} trace analysis of OT-I Opa1^{+/+} and Opa1^{-/-} IL-2 T_E cells. Each lane represents separate mice with a technical replicate. (D) EM analysis of mitochondrial cristae from T_E and T_M cells isolated after LmOVA infection and (E) *in vitro* cultured IL-2 T_E and T_M cells. Data representative of 2 experiments, scale bar = 0.25 μm. Relative proton leak (ΔOCR after oligomycin and subsequent injection of rotenone plus antimycin A) of (F) Opa1^{+/+} and Opa1^{-/-} IL-2 T_E, (G) infection elicited T_E and T_M, and (H) IL-2 T_E and IL-15 T_M cells. (F-H) Data combined from 2-4 experiments shown as mean ± SEM (p**<0.005, ***p<0.0001). (I) EM analysis of IL-15 T_M cell-mitochondrial cristae before and after αCD3/CD28-conjugated bead stimulation over hours, scale bar = 0.2 μm and represents one experiment. (J) Immunoblot analysis of ER protein Calnexin and ETC complexes (CI-NDUFB8, CII-SDHB, CIII-UQCRC2, CIV-MTC01, CV-ATP5A). Equivalent numbers of IL-2 T_E and IL-15 T_M cells were lysed in native lysis buffer followed by digitonin solubilization of intracellular membranes. Pellet (P) and solubilized supernatant (S) fractions were resolved on a denaturing gel. Data representative of 2 experiments. (K) IL-15 T_M cell, (L) bone marrow-derived dendritic cell (BM-DCs) and macrophage (BM-Macs) % ECAR measured at baseline and after media, αCD3/CD28-conjugated bead, LPS, or LPS+IFN-γ injection as indicated. Data are baselined prior to or right after injection with stimuli. (M) BM-Macs stained for intracellular Nos2 protein by flow cytometry with MFI values (left) and representative histogram (right). (K-M) Data shown as mean ± SEM and represent 2-3 experiments (***p<0.0001).

Figure 17. T cell activation induces cristae remodeling that regulates metabolism.



(A) Activated Opa1^{+/+} and Opa1^{-/-} IL-2 T_E cells were cultured overnight with D-Glucose-¹³C_{1,2} and traced for incorporation by mass spectrometry. Heat map representation of % labeled carbons in listed metabolites. (B) Spleens from either polyclonal wild-type (+/+) or Opa1 deficient T cell animals (-/- Opa1 T) were isolated and surface marker expression assessed (CD44, CD62L on CD3⁺ CD8⁺ gates) by flow cytometry (top) or were further purified for CD8 T cells to assess OCR and ECAR at baseline (below) by Seahorse EFA. Data presented with mean ± SEM from n=6 per genotype, ***p<0.0001. (C) EM images of IL-15 T_M cell mitochondria over time before and after PMA and ionomycin stimulation from one experiment. Scale bar = 0.5 μm. (D) Immunoblot analysis of ETC complexes (CI-NDUFB8, CII-SDHB, CIII-UQCRC2, CIV-MTC01, CV-ATP5A) and OMM protein Tom20. Equivalent numbers of IL-2 T_E and IL-15 T_M cells lysed in native lysis buffer followed by digitonin solubilization of intracellular membranes. Pellet (P) and solubilized supernatant (S) fractions were resolved on a denaturing gel. Second experiment represented from **Figure 16J**.

Discussion

Although T_M cells rely on FAO for development and survival, precisely why T_M cells utilize FAO and the signals that drive the induction of aerobic glycolysis in T_E cells remain unclear. Our data suggest that manipulating the structure of a single organelle can have profound consequences that impact metabolic pathway engagement and ultimately, the differentiation of a cell. We found that Opa1 regulated tight cristae organization in T_M cells, which facilitated efficient ETC activity and a favorable redox balance that allowed continued entrance of pyruvate into mitochondria. We originally hypothesized that Opa1 would be an obligate requirement for FAO. However, we found that Opa1^{-/-} IL-2 T_E cells and *ex vivo* T_E cells generated during infection utilized FAO to the same level as cells expressing Opa1. While this was true for T_E cells, this may not be the case for T_M cells, whose survival is severely impaired *in vitro* and *in vivo* when deficient in Opa1. It is possible that Opa1^{-/-} T cells are unable to form T_M cells because they cannot efficiently engage FAO under the metabolic constraints imposed during T_M cell development. Previous studies point to the existence of a ‘futile’ cycle of fatty acid synthesis (FAS) and FAO within T_M cells (Cui et al., 2015; O'Sullivan et al., 2014) whereby carbon derived from glucose oxidation is used to build fat that is subsequently burned by mitochondria for fuel. T_M cells have a lower overall metabolic rate than T_E cells, and tightly configured cristae might be important to ensure that any pyruvate generated will efficiently feed into the TCA cycle not only for reducing equivalents, but also for deriving citrate for FAS. Without tight cristae and efficient ETC activity, electrons may loiter in the complexes, causing more ROS, which could be damaging (Chouchani et al., 2014), but also provide signals that drive cell activation (Sena et al., 2013).

We did not observe a defect in T_M cell survival in Mfn1 and Mfn2 deficient T cells, but this does not exclude the possibility that OMM fusion or additional activities ascribed to each of these proteins are not important. Mfn1 and Mfn2 form both homotypic and heterotypic interactions, suggesting that in the absence of one protein, the other can compensate (Chen et al., 2003). Our preliminary data assessing Mfn1/2 expression in Mfn1^{-/-} and Mfn2^{-/-} T cells indicate that this might be occurring (**data not shown**). However, our results clearly show that unlike Opa1^{-/-} T cells, *in vitro* cultured Mfn1^{-/-} or Mfn2^{-/-} T cells do not have a survival defect when differentiated in IL-15 (**Figure 8A**), even though, like Opa1^{-/-} T cells, they are more glycolytic and OXPHOS-impaired compared to controls (**data not shown**). Further investigation using Mfn1/2 double knockouts is underway in our laboratory to examine whether a lack of both proteins, and presumably total OMM fusion, impairs T_M cell development in similar fashion as deficiency in inner membrane fusion. Our imaging data showed that T_M cells maintained extended fused mitochondrial networks, suggesting that OMM fusion also has a compulsory role in T_M cell development. However, unlike Opa1, retroviral expression of Mfn1 and Mfn2 did not confer a T_M cell phenotype in T_E cells. This could be due to the fact that an increase in OMM fusion, without a concomitant increase in inner membrane fusion, would still yield an overall loose cristae morphology and redox state that by default, results in sustained excretion of lactate.

The question of what signals drive T cell structural remodeling of mitochondria in the first place still remains. In the case of T_M cell development, initial withdrawal of activating signals and growth factors may induce fusion, consistent with previous reports that starvation induces mitochondrial hyperfusion (Rambold et al., 2015; Rambold et al., 2011b), an effect we also

observe in T_E cells after IL-2 withdrawal (**data not shown**). However, pro-survival signals from cytokines such as IL-15 or IL-7 are needed to sustain T_M cell viability and metabolically remodel these cells for FAS and FAO via increased CPT1a (van der Windt et al., 2012) and aquaporin 9 expression (Cui et al., 2015). Factors such as these may enforce the fused state and would be consistent with our observations that activated T cells subsequently cultured in IL-15 become more fused over time (**Figure 6D**). Another possibility is that Opa1 is activated via sirtuin 3 (SIRT3) under metabolically stressful conditions (Samant et al., 2014). Sirtuins are post-translational modifiers that are activated by NAD⁺, directly tying their activity to the metabolic state of the cell (Houtkooper et al., 2012; Wang and Green, 2012). Our previous work demonstrated that the available NAD⁺ pool is higher in T_M cells (van der Windt et al., 2012), which could correlate with this scenario.

In T_E cells we see an immediate activation of Drp1, prior to seeing a fissured phenotype, and inhibition of Drp1 prevents ECAR induction after activation. TCR signals induce Ca²⁺ flux that activates the phosphatase activity of calcineurin (Smith-Garvin et al., 2009), which in turn dephosphorylates Drp1 at Ser637, leading to its activation (Cereghetti et al., 2008). Initial Drp1 activation could facilitate some level of fission and cristae remodeling, tipping off aerobic glycolysis via the initial shunting of pyruvate to lactate. Our data (**Figure 6E**) showed that Drp1 is phosphorylated at its activating site Ser616 at day 1 after activation, which preceded recognizable mitochondrial fragmentation (**Figure 6C**). Our preliminary data did not show overt mitochondrial fragmentation in the initial hours after TLR stimulation of DC or macrophages (**data not shown**), but this does not exclude the possibility that Drp1 is actively mediating more subtle changes to mitochondrial structure that are not discernable by confocal microscopy. For

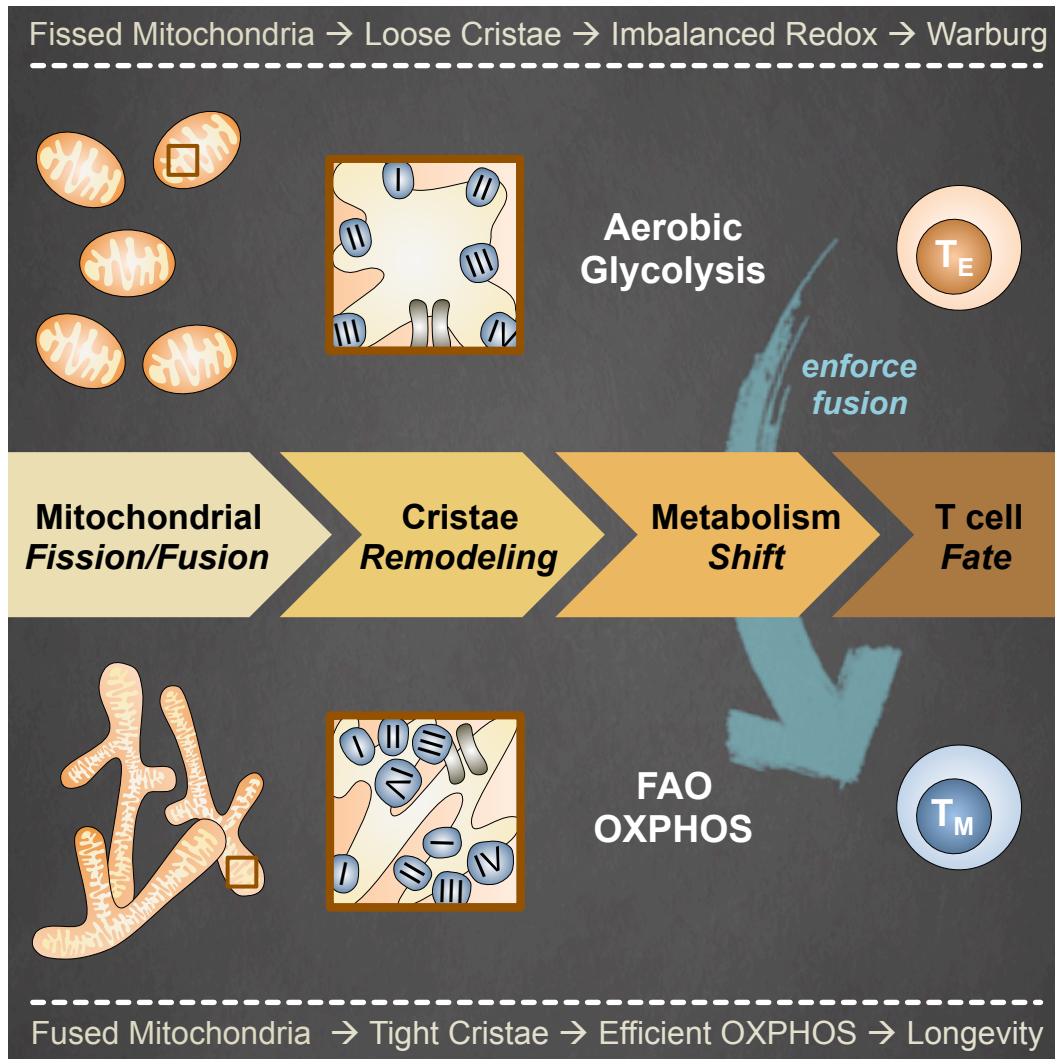
example, Drp1 also has been found to affect cristae structure by altering the fluidity of the mitochondrial membrane (Benard et al., 2007; Benard and Rossignol, 2008). Although Drp1 has been implicated in mitochondrial positioning at the immune synapse (Baixauli et al., 2011), lymphocyte chemotaxis (Campello et al., 2006), and ROS production (Roth et al., 2014) during T cell activation, our data suggest that in addition to these processes, fission underlies the reprogramming of cells to aerobic glycolysis.

We show that IL-2 T_E cells have a mitochondrial structure that is more susceptible to digitonin disruption when compared to IL-15 T_M cells, which suggests a more exposed membrane with less densely packed protein complexes. This relatively enhanced permeability however, does not mean that their mitochondria are damaged, or unable to function. In fact, although T_E cells have less efficient OXPHOS in terms of how it is coupled to ATP synthesis, T_E cells are very metabolically active with high OCR and ECAR (Chang et al., 2013; Sena et al., 2013). Our experiments involving pharmacological enforcement of mitochondrial fusion promoted OCR and SRC (and ECAR, albeit to a lesser extent) in IL-2 T_E cells. The drug modified cells maintained full T_E cell function with no effect on their cytolytic ability or proliferation, but possessed enhanced cytokine expression. Fusion and/or cristae tightening boosted the T_E cells' oxidative capacity, endowing them with longevity and persistence, while their higher aerobic glycolysis supported increased cytokine production, which may explain their superior antitumor function.

Our data suggest a model where morphological changes in mitochondria are a primary signal that shapes metabolic reprogramming during cellular quiescence or activation. We speculate that fission associated expansion of cristae as a result of TCR stimulation physically separates ETC

complexes, decreasing ETC efficiency. With delayed movement of electrons from complex I down the ETC, NADH levels rise in the mitochondria, slowing forward momentum of the TCA cycle and cause an initial drop in ATP. To correct redox balance, cells will export pyruvate to lactate to regenerate NAD^+ in the cytosol, which can enter the mitochondria through various shuttles to restore redox balance (Dawson, 1979) and increase flux through glycolysis to restore ATP levels, all contributing to the Warburg effect in activated T cells. When cristae are tightly configured, the ETC works efficiently and maintains entrance of pyruvate into the mitochondria with a favorable redox balance. In this case, cristae morphology as a result of fusion directs T_M cell formation and retains these cells in a quiescent state. Thus, mitochondrial dynamics control the balance between metabolic pathway engagement and T cell fate.

Figure 18. Mitochondrial dynamics controls T cell fate through metabolic programming.



- T_M cells have fused mitochondria while T_E cells have fissed mitochondria
- Mitochondrial fusion protein Opa1 is required for T_M cells and not T_E cells
- Enforcing fusion improves adoptive cellular immunotherapy against tumors
- Cristae remodeling via fusion/fission signals metabolic adaptations in T cells

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Curriculum Vitae

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Education & Awards

2012-Present: Washington University, Saint Louis, MO USA

PhD Candidate

Overall GPA: 4.00, Expected Graduation: April 2017

Award: National Science Foundation Graduate Research Fellowship 2013-2016

Award: Keystone Symposia Future of Science Fund Scholarship

2005-2009: University of California, San Diego USA

Bachelor of Science in Biochemistry & Cell Biology

Overall GPA: 3.54

Honors: Provost Honors

Research Experience

2012-Present: Washington University, Saint Louis, MO USA

2015-Present: Max Planck Institute of Immunobiology and Epigenetics, Freiburg, Germany

Graduate Student

Principal Investigator: Dr. Erika Pearce, Department of Pathology & Immunology

Thesis: Mitochondrial dynamics controls T cell fate through metabolic programming

2010-2012: La Jolla Institute for Allergy & Immunology, La Jolla, CA USA

Research Technician

Principal Investigator: Dr. Sujan Shresta, Division of Vaccine Discovery

Research Projects: Innate immunity, antiviral efficacy, and mouse models of dengue virus infection

2009-2010: University of California San Diego, La Jolla, CA USA

Laboratory Assistant

Principal Investigator: Dr. Ananda Goldrath, Division of Biological Sciences

Undergraduate Research: Transcriptional regulation of CD8 T-cell memory formation and maintenance

Research Publications

1. **Buck MD**, O'Sullivan D, Klein Geltink RI, Curtis JD, Chang CH, Sanin DE, Qiu J, Kretz O, Braas D, van der Windt GJ, Chen Q, Huang SC, O'Neill CM, Edelson BT, Pearce EJ, Sesaki H, Huber TB, Rambold AS, Pearce EL. Mitochondrial dynamics controls T cell fate through metabolic programming. *Cell* (2016)
2. Monticelli LA, **Buck MD**, Flamar AL, Saenz SA, Tait Wojno ED, Yudanin NA, Osborne LC, Hepworth MR, Tran SV, Rodewald HR, Shah H, Cross JR, Diamond JM, Cantu E, Christie JD, Pearce EL, Artis D. Arginase 1 is an innate lymphoid-cell-intrinsic metabolic checkpoint controlling type 2 inflammation. *Nature Immunology* (2016)
3. Park S, **Buck MD**, Desai C, Zhang X, Loginicheva E, Martinez J, Freeman ML, Saitoh T, Akira S, Guan JL, He YW, Blackman MA, Handley SA, Levine B, Green DR, Reese TA, Artyomov MN, Virgin HW. Autophagy genes enhance murine gammaherpesvirus 68 reactivation from latency by preventing virus-induced systemic inflammation. *Cell Host & Microbe* (2016)
4. Wu D, Sanin DE, Everts B, Chen Q, Qiu J, **Buck MD**, Patterson A, Smith AM, Chang CH, Liu Z, Artyomov MN, Pearce EL, Cella M, Pearce EJ. Type 1 interferons induce changes in core metabolism that are critical for immune function. *Immunity* (2016)
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 7. Plummer E, **Buck MD**, Sanchez M, Greenbaum JA, Turner J, Grewal R, Klose B, Sampath A, Warfield KL, Peters B, Ramstedt U, Shresta S. Dengue viral evolution under a host-targeted antiviral. *Journal of Virology* (2015)
 8. O’Sullivan D, van der Windt GJ, Huang SC, Curtis JD, Chang CH, **Buck MD**, Qiu J, Smith AM, Lam WY, DiPlato LM, Hsu FF, Birnbaum MJ, Pearce EJ, Pearce EL. Memory CD8⁺ T cells use cell-intrinsic lipolysis to support the metabolic programming necessary for development. *Immunity* (2014)
 9. Reese TA, Wakeman BS, Choi HS, Hufford MM, Huang SC, Zhang X, **Buck MD**, Jezewski A, Kambal A, Liu CY, Goel G, Murray PJ, Xavier RJ, Kaplan MH, Renne R, Speck SH, Artyomov MN, Pearce EJ, Virgin HW. Coinfection. Helminth infection reactivates latent γ -herpesvirus via cytokine competition at a viral promoter. *Science* (2014)
 10. van der Windt GJ, O’Sullivan D, Everts B, Huang SC, **Buck MD**, Curtis JD, Chang CH, Smith AM, Ai T, Faubert B, Jones RG, Pearce EJ, Pearce EL. CD8 memory T cells have a bioenergetics advantage that underlies their rapid recall ability. *Proceedings of the National Academy of Sciences USA* (2013)
 11. Perry ST*, **Buck MD***, Plummer EM*, Penmasta RA, Batra H, Stavale EJ, Warfield KL, Dwek RA, Butters TD, Alonzi DS, Lada SM, King K, Klose B, Ramstedt U, Shresta S. An iminosugar with potent inhibition of dengue virus infection in vivo. *Antiviral Research* (2013) | *Authors contributed equally
 12. MakhluF H, **Buck MD**, King K, Perry ST, Henn MR, Shresta S. Tracking the evolution of dengue virus strains D2S10 and D2S20 by 454 Pyrosequencing. *PLoS One* (2012)
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 14. Perry ST, **Buck MD**, Lada SM, Schindler CS, Shresta S. STAT2 mediates innate immunity to dengue virus in the absence of STAT1 via the type I interferon receptor. *PLoS Pathogens* (2011)

Review Articles, Commentaries, and Book Chapters

1. **Buck MD**, Sowell RT, Kaech SM, Pearce EL. Metabolic instruction of immunity. *Cell* (2017) (under review)
2. **Buck MD**, O’Sullivan D, Pearce EL. T cell metabolism drives immunity. *Journal of Experimental Medicine* (2015)
3. **Buck MD**, MakhluF HA, Shresta S. Chapter 23: Animal models of dengue infection and disease. *Dengue and Dengue Hemorrhagic Fever 2nd Edition Book, CABI* (2014)
4. Perry ST, **Buck MD**, Shresta S. Better late than never: antivirals for dengue. *Expert Reviews of Anti-Infective Therapy* (2011)

Patents

- US Patent Application 15/287,294 “Methods of Enhancing T-Cell Longevity and Uses Thereof” (2016)
 US Provisional Patent “Methods of Reducing Immune Cell Activation and Uses Thereof” (2016)

Research Meetings

- Keystone Symposia: Immunometabolism in Immune Function & Inflammatory Diseases (2016 Banff, Canada)
 Presentation and poster: *Promoting CD8 T cell memory through mitochondrial dynamics*

Max Planck Institute of Immunobiology and Epigenetics PhD Student Retreat (2016 Ortenberg, Germany)
Presentation: *Mitochondrial dynamics controls T cell fate through metabolic programming*

Washington University Immunology Program Annual Retreat (2014 Saint Louis, MO USA)
Poster: *Promoting CD8 T cell memory through mitochondrial dynamics*

Dengue Therapeutics Workshop, Oxford Clinical Research Unit (2011 Ho Chi Minh City, Vietnam)
Presentation: *Broad spectrum antiviral UV-4, United Therapeutics*

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