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Translating *in vitro* T cell metabolic findings to *in vivo* tumor models of nutrient competition

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Abstract

Reductionist *in vitro* T cell assays have identified metabolic pathways critical for T cell function within the tumor microenvironment. We discuss the challenges of testing these concepts using *in vivo* tumor models.

Exciting new technologies and conceptual advancements have re-energized the study of the metabolic competition between tumors and the adaptive immune system (Chang et al., 2015; Ho et al., 2015; Sugiura and Rathmell, 2018). Despite these advancements, there remains little discussion in the literature on how investigators should model nutrient depletion in the tumor microenvironment when studying immune cell/tumor cell metabolic interactions and competition. How can we more accurately distinguish and dissociate nutrient deprivation's effects on immune cells from other immunosuppressive components of the tumor microenvironment *in vivo*? Are T cells engineered with altered metabolic programming specifically overcoming a nutrient limitation or are they simply better tumor-specific T cells that function better in all nutrient environments? This essay will lay out some of the critical issues facing the field and hopefully spur the development of improved approaches for testing new immune therapies. We believe that greater mechanistic understanding of immunometabolism will fully harness the clinical potential of immune therapies.

Challenges T cells face in the solid tumor microenvironment

In vivo, tumors alter the abundance of dozens of metabolites in the interstitial fluid relative to healthy tissue (Kamphorst et al., 2015; Pavlova and Thompson, 2016). Most commonly solid tumor environments have been reported to be deprived of oxygen, glucose, glutamine, multiple amino acids (e.g. arginine, tryptophan) and is highly acidic (Fig 1A). Different tumors and even separate regions of the same tumor can host diverse nutrient environments and can experience intermittent blood flow and nutrient supply (DeBerardinis and Chandel, 2016; Vaupel et al., 1987). An abundance of pro-angiogenic factors (like vascular endothelial growth factor-A) promote disturbed vasculature characterized by large and leaky

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vessels, erratic branching, and irregular and slow blood flow which inhibits efficient nutrient delivery (Carmeliet, 2005; Lanitis et al., 2015). In addition metabolites can be altered by elevated expression of enzymes expressed by tumor cells or tumor-associated antigen-presenting cells. For example, high expression of the enzyme indoleamine 2,3-dioxygenase in the tumor microenvironment can lead to local depletion of tryptophan and regulate T cell proliferation and induce apoptosis (Fallarino et al., 2002; Platten et al., 2012; Schafer et al., 2016). Furthermore, the protein kinase, general control nonderepressible 2 (GCN2), also can sense amino acid deprivation in the tumor microenvironment and induce signaling that promotes T cell anergy (Munn et al., 2005), highlighting that signaling pathways induced by low levels of nutrients can be as damaging to the anti-tumor response as the lack of nutrients to support immune cell function and growth. T cells require glucose, glutamine, and mitochondrial pathways for activation, maximal proliferation and/or effector function (Cham and Gajewski, 2005; Macintyre et al., 2014; Procaccini et al., 2016; Ron-Harel et al., 2016; Sena et al., 2013). Aerobic glycolysis serves the increased biosynthetic demands of highly proliferating cells. Glycolytic intermediates can produce nucleotides, lipids, and amino acids necessary for cellular proliferation in both T cells and cancer cells (Vander Heiden et al., 2009). Similar reliance on aerobic glycolysis between proliferating cancer cells and activated T cells intensifies a competition for limited nutrients within the tumor microenvironment (Frauwirth et al., 2002). Competition for glucose also plays a clear role in limiting effective anti-tumor responses *in vivo* (Chang et al., 2015; Ho et al., 2015). Aerobic glycolysis is required for maximal IFN-γ production by effector T cells though at least two independent mechanisms. The glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) can inhibit translation of IFN-γ mRNA, while another study has demonstrated that the glycolytic enzyme, lactate dehydrogenase A (LDHA), promotes histone acetylation along the IFN-γ locus (Chang et al., 2013; Peng et al., 2016). Although some attempts to increase nutrient availability have been successful, we have little understanding how to make T cells more fit in a nutrient limiting environment (Chang et al., 2015; Lanitis et al., 2015).

Due to dysfunctional vasculture and the high glycolytic rate of many tumor cells, the tumor microenvrionment is often acidic because of high lactate concentrations (Fig 1B). Both tumor and T cells rely on monocarboxylate transporters to secrete and uptake lactate generated from glycolysis and pyruvate. High lactate concentrations in the environment prevent T cells from efficiently transporting lactate out of the cell and increase intracellular acidity and inhibit functionality and NFAT activity (Brand et al., 2016; Fischer et al., 2007). Brand et al. demonstrated that melanoma tumor cells engineered to have low lactate dehydrogenase A (LDHA) activity, have greater immune infiltration and activity than tumor cells with normal LDHA expresion. LDHA is the glycolytic enzyme required for conversion of pyruvate to lactate. Furthermore the administration of the proton pump inhibitor, esomeprazole, can normalize tumor pH *in* vivo and when combined with immunotherapy can promote tumor clearance (Calcinotto et al., 2012). While glucose deprivation and high lactate concentrations inhibit anti-tumor responses, they also promote immunosuppressive T regulatory cell and macrophage polarization and function (Angelin et al., 2017; Colegio et al., 2014).

As T cells become exposed to chronic antigen in the tumor microenvironment, they begin to terminally differentiate and become exhausted. As exhaustion becomes more severe, T cells increase expression of multiple inhibitory receptors, while simultaneously inhibiting costimulatory receptor expression (Fig 1C). Inhibitory receptors can inhibit co-stimulation and phosphorylation events downstream of T cell receptor (TCR) activation, and prevent activation signals (Akt and mTORC1) necessary for increasing surface expression of the major glucose transporter, Glut-1, in T cells (Jacobs et al., 2008; Parry et al., 2005; Siska et al., 2016). Without sufficient surface expression of Glut-1, the activated T cells are unable to properly upregulate glycolysis and have inhibited proliferation and effector function.

Upregulation of mitochondrial pathways (oxidative phosphorylation and one-carbon metabolism) are essential for proper T cell proliferation. (Chang et al., 2013; Procaccini et al., 2016; Ron-Harel et al., 2016; Ron-Harel et al., 2015). In addition mitochondria play roles beyond producing energy and biosynthesis, though epigenetic and signaling roles during T cell activation (Minocherhomji et al., 2012; Sena et al., 2013). Chronic antigen exposure affects metabolic pathways beyond glycolysis, by inhibiting mitochondrial functions (Fig 1D). Exhausted T cells in models of cancer or chronic virus infection often have defects in mitochondria number, size, and voltage potential and function (Bengsch et al., 2016; Scharping et al., 2016; Siska et al., 2017). One study has proposed that the tumor microenvironment downregulates mitochondrial biosynthesis by inhibiting expression of PPAR-gamma coactivator 1α (PGC1α), while another has suggested ROS induced damage may induce decreased mitochondrial mass (Scharping et al., 2016; Siska et al., 2017). Scharping et al. has successfully demonstrated that overexpression of PGC1α in tumor-specific T cells is able to prevent downregulation of mitochondrial mass and enhance T cell functionality in the tumor microenvironment.

Tumor infiltrating lymphocytes are exposed to large amounts of reactive oxygen species (ROS, Fig 1E). ROS are highly chemically reactive and can damage cellular structures, and inhibit T cell activation. Production of ROS drastically increases in T cells exposed to hypoxia and is one of the major mechanisms of immune suppression by myeloid derived suppressor cells (Kusmartsev et al., 2004; Tafani et al., 2016). Myeloid derived suppressor cells are a heterogeneous immunosuppressive subset of cells overexpressed in patients and mice with cancer. While ROS are necessary for IL-2 production and proliferation by T cells, it remains unclear the quantities in which ROS will become harmful to T cells (Sena et al., 2013). Exposure to oxidative stress by low doses of exogenous hydrogen peroxide are sufficient to inhibit T cell functionality and survival (Ligtenberg et al., 2016).

Challenges of applying immunometabolism findings to tumor models in vivo.

Given the metabolic challenges tumor-specific T cells face, there have been numerous attempts to mitigate these effects via pharmacological or genetic modifications to improve tumor-specific T cell therapy *in vivo*. However our interpretation of *in vivo* studies has been hindered by numerous technical and biological challenges.

It is incredibly difficult to distinguish whether newly designed immune interventions targeting the metabolic challenges of solid tumors have generated T cells that operate better in nutrient depleted environments or simply have made a better T cell (that operates better in all environments). The key to distinguishing these possibilities are 1) identifying how a modification improves the anti-tumor response and 2) understanding how individual metabolic interactions lead to distinct functional outcomes. For example, enhancing mitochondrial fusion, increasing L-Arginine media concentrations, or inhibiting glycolytic metabolism while T cells are being expanded for adoptive cell therapy all enhance the therapeutic activity of the infused T cells (Buck et al., 2016; Geiger et al., 2016; Sukumar et al., 2013). All of these interventions alter T cell metabolism, but do they enable T cells to function better in the tumor microenvironment? It is challenging to envision a mechanism by which temporarily expanding T cells in low glucose empowers them to function better in the tumor microenvironment. Rather, previous studies demonstrate that T cells expanded in low glucose retain a less differentiated phenotype which enables improved engraftment of the expanded T cells which correlates with improved tumor control (Gattinoni et al., 2011). Expansion in the presence of higher levels of L-arginine also results in T cells that are less differentiated and more reliant on oxidative phosphorylation instead of glycolysis (Geiger et al., 2016). These cells have higher intracellular concentrations of L-arginine, which may make them resistant to low arginine levels in the tumor microenvironment. Likewise, T cells with enhanced mitochondrial fusion are less differentiated in culture and exhibit greater reliance on oxidative phosphorylation (Buck et al., 2016). Enhanced fusion may enhance mitochondrial function and combat mitophagy frequently observed during nutrient deprivation (Rambold et al., 2011). How do we determine to what extent differentiation or improved fitness in the tumor microenvironment results in improved tumor clearance? Furthermore, any attempts to modulate metabolism in the tumor microenvironment is likely going to cascade and affect many of the surrounding cells and may have cell-specific effects. For example, enhancing tumor glycolysis is sufficient to decrease available glucose for T cells, but may also create a better environment for tumor associated macrophages and T regulatory cells (Angelin et al., 2017; Chang et al., 2015; Netea-Maier et al., 2018). In addition, simply increasing T cell activity is sufficient to modulate metabolites in the serum of mice, and the effects of these changes on other cell types is not understood (Miyajima et al., 2017). Future studies must work to better distinguish mechanisms of metabolic immune interventions to provide mechanistic understanding of immunometabolism (Fig 2A).

Unlike standard epigenetic or transcriptional changes that often occur over several hours or days, many metabolic changes occur incredibly quickly in response to different environments. This may even be more pronounced in immune cells, because these cells readily circulate and traffic to diverse areas of the body, and thus must be able to adapt rapidly in diverse metabolic environments. While researchers can observe cell intrinsic differences of different T cell subsets *in vitro*, how much information is lost because of isolation and prolonged *in vitro* expansion (Dimeloe et al., 2016; Pan et al., 2017; Procaccini et al., 2016; van der Windt et al., 2012)? Quantification is further complicated during conventional *in vitro* extraction techniques because of metabolite loss, leakage, or decay (Chen et al., 2016; Van Gulik et al., 2012; Vuckovic et al., 2011; Yang et al., 2017). Thus, during commonly used isolation procedures and before any assay can be performed *in vitro*,

metabolic changes are occurring (Fig 2B). Alternative approaches such as utilizing isotopic tracers are highly compatible with *in vivo* models, can be easily administered, and may better recapitulate *in vivo* biology (Hensley et al., 2016; Sun et al., 2017).

Immune cells isolated for nutrient depletion studies should represent the cells that would traffic to sites of inflammation and nutrient depletion in the tumor microenvironment (Fig 2C). Many *in vitro* T cell studies have examined nutrient depletion on total T cells isolated from the peripheral blood or spleens of humans or mice. However, naïve cells that often represent the majority of T cells in circulation or in the spleen do not travel to sites of inflammation where vasculature is often disturbed or injured (Klebanoff et al., 2006; Thome et al., 2014). Differentiated effector memory T cells or tissue resident memory T cells are the cells that reside in the environments that actually encounter nutrient depletion and have distinct responses to metabolic stress (Dimeloe et al., 2016; Ecker et al., 2018). These studies have suggested that effector memory T cells may prioritize effector functions over proliferation during nutrient limitation. More work is needed to titer nutrient availabilities to better delineate how they affect specific aspects of T cell function and proliferation in different T cell populations. Thus, many previous studies have lacked sufficient resolution to determine whether T cells in the blood mimic the same metabolic and adaptive features of T cells that reside in the tissue. Furthermore, there is on-going discussion on which cell populations represent the best control to tumor infiltrating lymphocytes (TILs). Are cells in the blood or spleen, effector memory T cells, or tissue-resident cells from the tissue of choice the best cells to compare to TILs? While tissue-resident cells are often thought of as the best control because they reside in the same niche and similar effector phenotype, their limited numbers and technically difficult isolation have hindered their wide-spread use.

There remain large gaps in understanding of immune cell localization within hypoxic or glucose depleted regions of tumors. Do we truly observe anti-tumor function and immune cell proliferation only in sites of nutrient availability? Do immune cells have higher rates of death in sites of nutrient depletion or do they simply traffic away from those sites? Which nutrients are the most essential for function *in vivo*? Regions of nutrient depletion can often be examined through florescent analogues like 2-NBDG for glucose distribution, staining with pimonidazole for regions experiencing hypoxia, or through distinct protein expression of hypoxic or nutrient depleted cells (Airley et al., 2001). These are well validated, and compatible with commonly used methods to identify immune cell localization and activity (Bennewith and Durand, 2004; He et al., 2008; Sukumar et al., 2013). Improvements in the resolution of mass spectrometry imaging techniques have also identified tissue-resident immune populations and metabolite gradients in tumors (Dilillo et al., 2017; Holzlechner et al., 2017). Additionally, sensitive reporter constructs could be useful to map the duration and regional location of nutrient limiting milieus. Elucidating the spatial organization of nutrient availability and immune populations will be crucial for greater insight of immune surveillance in the tumor microenvironment (Fig 2D).

The last decade of research has led to incredible findings and a growing interest in immunometabolism. Researchers have only just begun to understand how to translate the basic findings into in vivo tumor models and face many challenges. Future studies designed

to overcome these challenges will be essential for providing mechanistic understanding of *in vivo* immunometabolism and for translating these approaches into the clinical arena.

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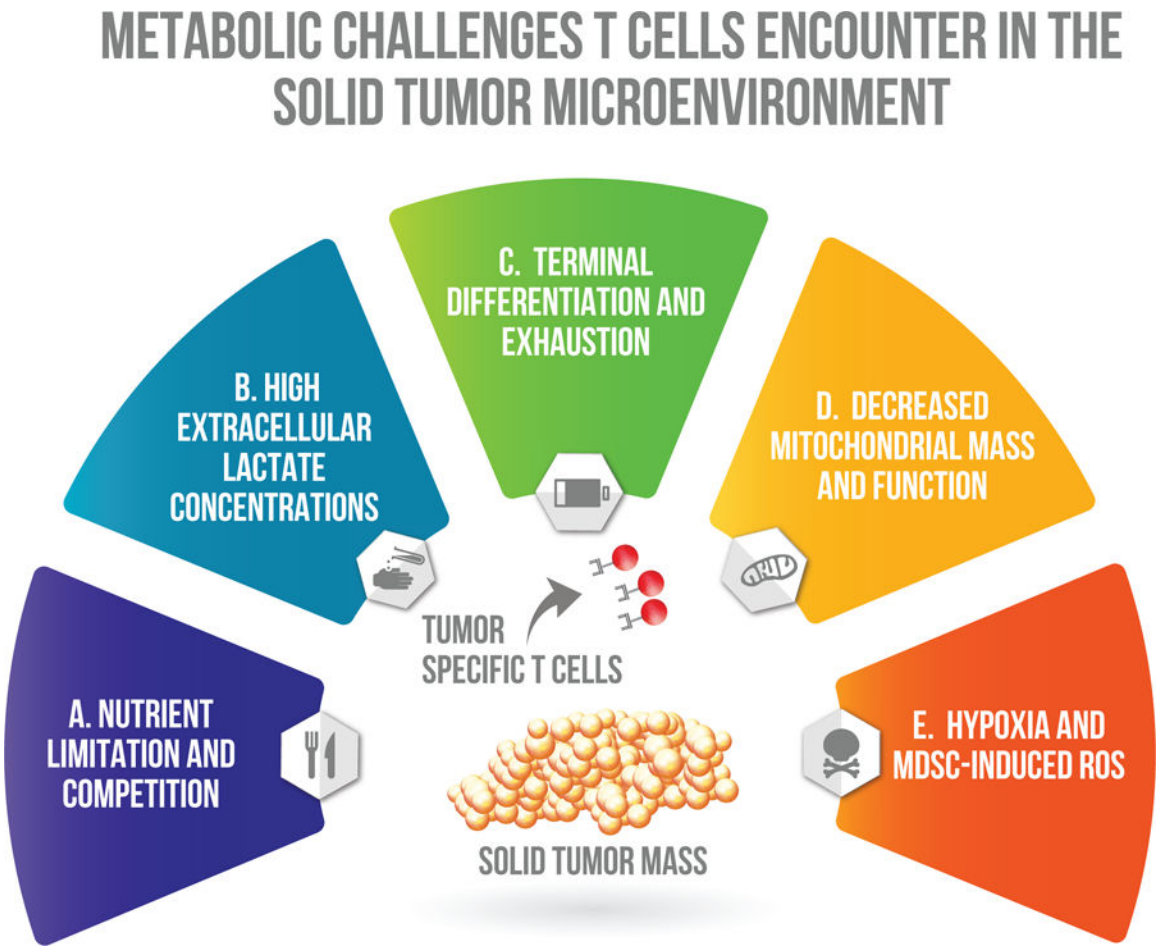

Figure 1: Metabolic challenges T cells encounter in the solid tumor microenvironment.

A. Nutrient limitation and competition. Disturbed vasculature of solid tumors are often unable to provide nutrients critical for T cell activation. Activated T cells and proliferating tumor cells rely on aerobic glycolysis and compete for extracellular glucose. **B. High extracellular lactate concentrations.** Reliance on aerobic glycolysis by tumor cells promotes a large buildup of the waste product lactate in the extracellular milieu of solid tumors. High extracellular concentrations of lactate hinder T cell activation by inhibiting efficient secretion of lactic acid from the cytoplasm and acidifying intracellular pH. **C. Hypoxia and MDSC-induced ROS.** T cells in the solid tumor microenvironment are constantly exposed to reactive oxygen species, which are highly reactive and damage cellular structures. Hypoxia rapidly induces ROS production by T cells and myeloid derived suppressor cells frequently suppress effector T cells by secreting ROS. **D. Decreased mitochondrial mass and function.** Tumor specific T cells have defects in mitochondria biogenesis, size, cristae structure, voltage potential and function. **E. Terminal differentiation and exhaustion.** Chronic antigen exposure causes loss of co-stimulatory receptors while promoting inhibitory co-receptors that inhibit glycolytic metabolism and activation signaling.

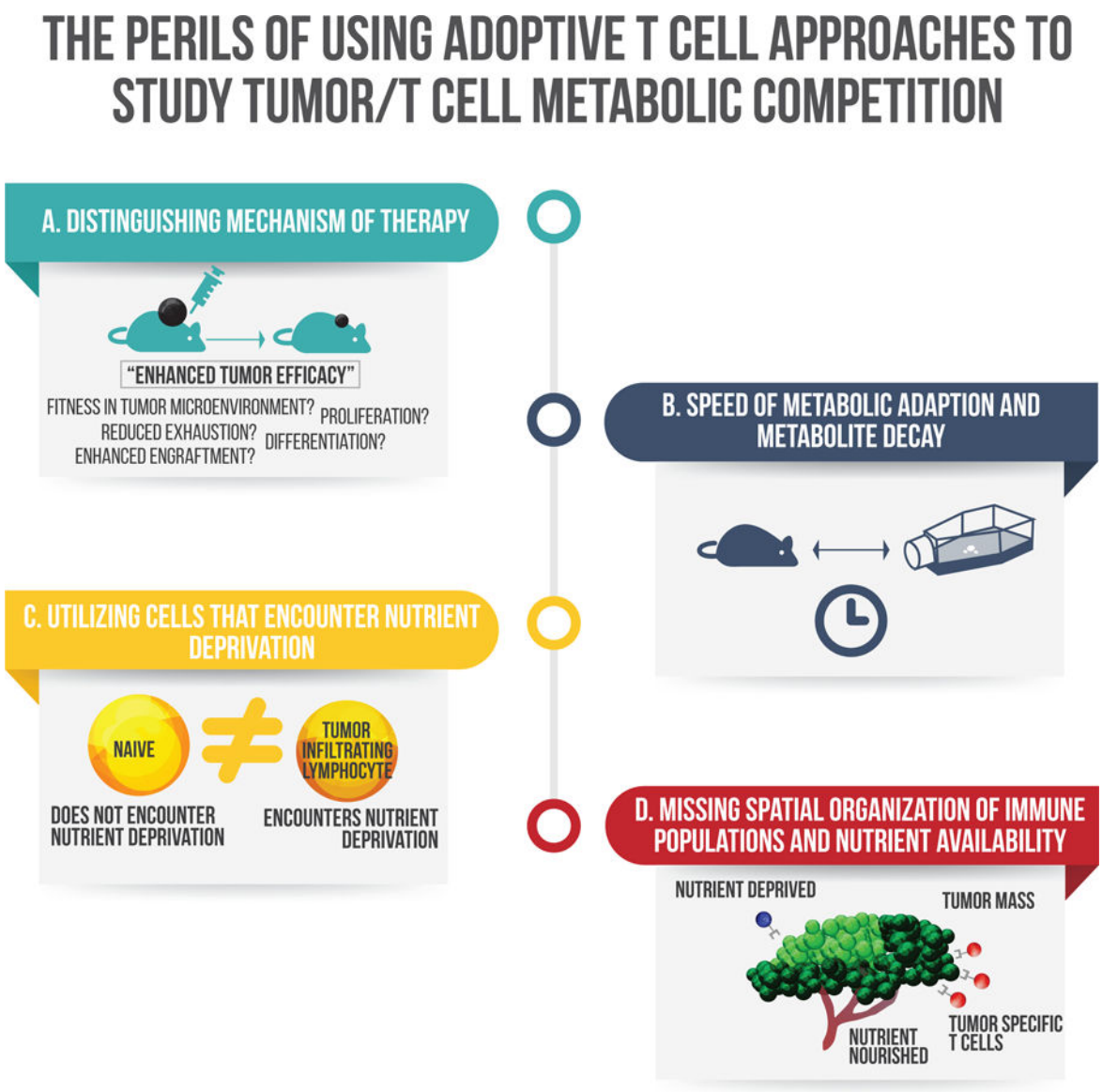

Figure 2: Challenges of applying immunometabolism findings to tumor models *in vivo*.

A. Many researchers have found ways to improve anti-tumor efficacy of adoptively transferred T cells through metabolic manipulation or genetic engineering. Too often though we lack the resolution to distinguish the mechanism by which enhanced tumor efficacy is reached. B. The speed by which cells metabolically adapt to changes in the extracellular environment during extraction procedures, and how quickly some metabolites can decay or be lost in commonly used isolation techniques hinder our understanding of *in vivo* biology. C. The most accessible cells in the blood or spleen (that are commonly used in metabolic studies of nutrient limitation) do not reflect the metabolic traits or adaptive properties of terminally differentiated cells found in most tumors. D. The field has not explored the spatial organization of nutrient depletion and immune cell infiltration in solid tumors.