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Immunometabolic Interplay in the Tumor Microenvironment

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SUMMARY

Immune cells' metabolism influences their differentiation and functions. Given that a complex interplay of environmental factors within the tumor microenvironment (TME) can have a profound impact on the metabolic activities of various immune, stromal, and tumor cell types, there is emerging interest to advance understanding of these diverse metabolic phenotypes in the TME. Discussing various cell-extrinsic contributions to the metabolic activities of immune cells and recent technical advances in experimental systems and metabolic profiling technologies, we propose future directions to better understand how immune cells meet their metabolic demands in the TME, which can be leveraged for therapeutic benefit.

Keywords

metabolism; immunometabolism; tumor microenvironment; immunology; in vitro modeling; physiologic media; metabolomics; stable isotope tracing

INTRODUCTION

Metabolic conditions in the tumor microenvironment (TME) are influenced by many factors, including gradients of nutrient and oxygen levels, tissue vascularization, heterocellular

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Declaration of Interests

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interactions, and systemic metabolism. However, much of the research used to define principles of tumor cell metabolism have relied heavily on cultured cells, which cannot fully recapitulate the metabolic conditions of tumor metabolism in vivo, especially tumor cell-extrinsic factors such as nutrient availability and immune infiltration. To improve the modeling capacity of experimental systems used to study the TME, various cell-intrinsic and environmental inputs should be considered. However, as the TME contains sundry cell types with diverse and flexible metabolic programming, precise investigation of the metabolic demands, adaptations, and interactions among various immune and cancer cells is complex. Recent discussion of such intercellular interactions has been presented in detail by a number of excellent reviews (Bader et al., 2020; Lau and Vander Heiden, 2020). Here, we first highlight how immune cell metabolism and function can be influenced by the TME, and then discuss emerging technical advances in experimental modeling and metabolomics that may be exploited in future studies to further our understanding of immune cell metabolism in the complex context of the TME.

1 ENVIRONMENT SHAPES IMMUNE CELL METABOLISM AND FUNCTION

1.1 Nutrient Availability

Much of the work in immunometabolism to date has focused on how cells concurrently and differentially utilize nutrients to satisfy metabolic demands associated with effector functions and differentiation states. Indeed, the metabolic activities of different immune cell types, particularly lymphocytes, has been extensively reviewed elsewhere (Buck et al., 2017; Lau and Vander Heiden, 2020). The metabolic program of normal resting cells primarily serves to meet the bioenergetic requirements of maintaining homeostatic processes that require ATP. In contrast, proliferating cells—both normal and malignant—not only need to generate energy to support cell growth, but must also meet various anabolic demands of macromolecule biosynthesis (e.g., proteins, nucleotides, lipids) and cellular redox homeostasis (Cantor and Sabatini, 2012; Kong and Chandel, 2018; O'Sullivan et al., 2019; Pearce et al., 2013; Sugiura and Rathmell, 2018). A central metabolic program associated with activation of innate (dendritic cells (DCs), macrophages, neutrophils) and adaptive ($CD8^+$ and $CD4^+$ T cells, B cells) immune cell is aerobic glycolysis, also commonly known as the Warburg effect (Lunt and Vander Heiden, 2011; Warburg, 1956). Increasing glucose uptake and glycolysis not only provides precursors for proliferation, such as glucose-dependent serine production for nucleotide synthesis (Ma et al., 2019), but also supports IFN-γ production (Cham and Gajewski, 2005; Chang et al., 2013a). For instance, T helper (Th) cells are highly glycolytic (Michalek et al., 2011; Shi et al., 2011), relying on the glucose transporter GLUT1 for inflammatory cell (i.e., Th1 and Th17) effector function, and GLUT1-mediated glycolysis in regulatory T (Treg) cells regulates the balance between proliferation and suppressive capacity (Gerriets et al., 2016). However, this may pose a challenge for tumor-infiltrating immune cells given that glucose availability can vary in the TME (Siska et al., 2017; Sullivan et al., 2019). Specifically, there is evidence that glucose limitation in TME can compromise effector functions of tumor infiltrating lymphocytes (TILs) and the antitumor effects of natural killer (NK) cells (Chang et al., 2013b, 2015; Ho et al., 2015).

In addition to glucose, the availability of different amino acids can also influence immune cell metabolism. For example, T cells and macrophages may catabolize glutamine to fuel ATP production (Blagih et al., 2015; Carr et al., 2010; Jha et al., 2015). Yet, blunting glutamine catabolism can promote both increased $CD8^+$ TIL effector function and tumor regression, owing to differential demands for glutamine utilization among tumor cells and TILs (Leone et al., 2019). Given the metabolic variability among cell types in the TME, the targeted inhibition of metabolic enzymes may drive distinct cell-specific consequences. For instance, either genetic or pharmacologic blockade of glutaminase (GLS)—which metabolizes glutamine into glutamate—has been shown to impair proliferation and activation of $CD4^+$ and $CD8^+$ T cells with little effect on cytokine production. The same blockade can also suppress the differentiation of $CD4^+$ Th17 cells (Johnson et al., 2018). In addition, supraphysiologic arginine levels provided by the synthetic cell culture medium RPMI 1640 can blunt cytokine production mediated by T cells but increase their survival (Geiger et al., 2016). Tryptophan depletion also can reportedly hinder T-cell function through activation of the integrated stress response (Munn et al., 2005). Further, the extracellular availability of both serine (Labuschagne et al., 2014; Ma et al., 2017; Ron-Harel et al., 2016) and methionine (Gao et al., 2019; Roy et al., 2020; Sinclair et al., 2019) can influence the proliferation of tumor cells and T lymphocytes through effects linked to one-carbon metabolism. In T cells, as the folate and methionine cycles are uncoupled and serine-derived carbon does not transit to the methionine cycle, methionine may be the exclusive source of the methyl moiety in S-adenosylmethionine (SAM) and, by extension, an essential amino acid for methylation reactions (Roy et al., 2020). In addition, others have recently demonstrated that alanine availability can affect protein synthesis during the early stages of T cell activation (Ron-Harel et al., 2019). Interestingly, genes that encode amino acid transporters are amongst the most upregulated genes upon T cell activation (Howden et al., 2019), suggesting that amino acid exchange is a critical aspect of immune regulation in the TME. Together, these results highlight that amino acid availability can have distinct cell-specific effects on immune cell metabolism and function, perhaps suggesting the potential to target amino acid metabolism of different immune subtypes in the treatment of human disease.

Purported waste products of cellular metabolism—including lactate, kynurenine, and adenosine—can also exert immunomodulatory effects in the TME. For example, whereas lactate can suppress the cytolytic capacity of $CD8^+$ effector cells (Brand et al., 2016; Calcinotto et al., 2012; Fischer et al., 2007), it may instead be used by Treg cells to support metabolic demands (Angelin et al., 2017; Wang et al., 2020). Further, lactate accumulation in a murine model of arthritis induces upregulation of the monocarboxylate transporter *SLC5A12* in $CD4^+$ T cells, a transporter class similarly upregulated in activated T cells. This leads to elevated levels of intracellular acetyl-CoA and citrate, as well as decreased T cell motility (Pucino et al., 2019). Others have also demonstrated that lactate generated by melanoma cells can diminish immune surveillance by T cells and NK cells by suppressing nuclear factor of activated T cells (NFAT)-dependent IFN-γ production (Brand et al., 2016). Further, recent evidence also indicates that lactate can be used to fuel the TCA cycle in both normal tissues and certain lung cancers (Faubert et al., 2017; Hui et al., 2017), suggesting that lactate may promote immune evasion in the TME by supporting demands of tumor

growth (Angelin et al., 2017). In addition, elevated levels of kynurenine, a downstream product of tryptophan catabolism, can induce immunosuppression in T cells (Fallarino et al., 2006; Mezrich et al., 2010; Opitz et al., 2011). Remarkably, Triplett and colleagues recently demonstrated that the enzyme-mediated depletion of systemic kynurenine could increase the tumor infiltration and proliferation of $CD8^+$ lymphocytes (Triplett et al., 2018). Lastly, the accumulation of adenosine, a breakdown product of nucleotide metabolism, effectively reduces the cytotoxic effects mediated by T and NK cells, but enhances the activation of immunoregulatory M2 macrophages (Csóka et al., 2012; Huang et al., 1997).

The impact of free fatty acids on immune cell function in the TME continues to be an active area of investigation. While early models of immune cell metabolic reprogramming proposed that glycolytic and oxidative (fueled by long-chain fatty acid oxidation (FAO)) metabolic programs were associated with distinct immune cell populations (e.g., $CD8^+$ T effector (Teff) versus T memory (Tmem) cells, M1 versus M2 macrophages) (O'Neill and Pearce, 2016; Pearce et al., 2013), the reality has proven to be far more nuanced. Etomoxir, an inhibitor of CPT1A—an enzyme that catalyzes the rate-limiting step in mitochondrial long-chain FAO—has immunomodulatory effects on $CD8^+$ Tmem cell differentiation and M2 macrophage polarization (Huang et al., 2014; van der Windt et al., 2012), reinforcing the notion that FAO-fueled OXPHOS supports these processes. However, genetic evidence has indicated that CPT1A is dispensable for $CD8^+$ Teff function and Tmem generation, as well as for $CD4^+$ Treg suppressive capacity (Raud et al., 2018). Further, the suggested influence of etomoxir on M2 macrophage polarization has been attributed to off-target effects of the compound (Divakaruni et al., 2018). Nonetheless, $CD8^+$ T cells with elevated *Cpt1a* expression display a metabolic advantage *in vivo* (Klein Geltink et al., 2017; van der Windt et al., 2012), underscoring a role for mitochondrial metabolism in supporting optimal T cell function. Long-chain FAO may be more critical for tissue-resident immune cells; recent work has demonstrated that fatty acid import (via FABP4 and FABP5) and CPT1A-dependent lipid oxidation are required for the persistence of tissue-resident $CD8^+$ cells in peripheral tissues (Pan et al., 2017), while the lipid transporter CD36 is required for accumulation of $CD4^+$ Treg cells in the TME (Wang et al., 2020). Interestingly, TILs in B16 melanoma tumors display enhanced fatty acid catabolism, a phenotype attributed to low glucose levels in the TME (Zhang et al., 2017), hinting at a role for FAO in metabolically flexible immune cells in the TME.

Beyond their role as fuel sources, saturated fatty acids can also promote the production of pro-inflammatory cytokines (e.g., IL-23 during Th17 responses), whereas polyunsaturated fatty acids have been linked to anti-inflammatory cytokine production (e.g. IL-10) (Wang et al., 2015). Short chain fatty acids (SCFAs), which are largely byproducts of microbial metabolism, can act as positive regulators of $CD8^+$ Teff function as well (Bachem et al., 2019; Balmer et al., 2016; Trompette et al., 2018). For example, butyrate—which acts as an HDAC inhibitor—can influence T cell function by mediating G-protein-coupled receptor signaling (Bachem et al., 2019). In addition, recent work by Qui and colleagues demonstrated that acetate can enhance Teff cell function by reinforcing permissive epigenetic marks (i.e., H3K27ac) and chromatin accessibility at key effector cytokine loci (e.g., *Gmzc*, *Ifng*, *Tnfa*) (Qiu et al., 2019). These results are consistent with the notion that the composition of the gut microbiome can influence metabolite availability and thus,

patient responses to immunotherapy (reviewed in (Gopalakrishnan et al., 2018)), though specific contributions of microbiome-derived metabolites to immune function in the TME require further research.

1.2 Oxygen, pH, and Ion Concentration

Tissue oxygen (O_2) concentrations are dictated by competing rates of O_2 diffusion and consumption. Most tissues experience oxygen levels of 2–9% (average 40 mm Hg) (Krzywinska and Stockmann, 2018), while cells in the TME may instead experience hypoxic conditions (<2% oxygen) that are driven by disorganized vasculature and the increased metabolic rate of tumor cells (Bertout et al., 2008). Such hypoxic conditions promote the stabilization of hypoxia inducible factor-1α (HIF-1α), whose expression is associated with increased inflammatory potential in both myeloid and lymphoid cells (Palazon et al., 2014). Similar to oxygen availability, extracellular pH varies across the TME—likely in concordance with local accumulation of lactate—and extracellular acidification (pH ~5.8–6.6) can have broad effects on immune cells (Huber et al., 2017; Singer et al., 2018). Notably, small ion availability can also have dramatic effects on T cell function. Elevated sodium levels can drive increased inflammatory potential in $CD4^+$ Th17 cells (Kleinewietfeld et al., 2013; Wu et al., 2013), and increased potassium availability can compromise nutrient uptake and effector function in $CD8^+$ T cells (Vodnala et al., 2019).

Although *Hif1a* deletion has been shown not to affect metabolic reprogramming of activated T cells (Wang et al., 2011), several studies have demonstrated that hypoxic conditions can have immunomodulatory effects (Lim et al., 2020). For example, *Hif1a* expression in $CD8^+$ T cells regulates their proliferation and influences their effector function by promoting a glycolytic phenotype (Finlay et al., 2012; Lum et al., 2007; Palazon et al., 2017). In murine breast tumor models, the deletion of *Hif1a* in $CD8^+$ T cells can result in reduced tumor infiltration and cytotoxic function (Palazon et al., 2017), indicating that HIF-1α can serve a key role in the maintenance of Teff cell function. Further, models of adoptive cell transfer (ACT) have demonstrated that hypoxic pre-conditioning can improve the cytotoxicity and tumor clearance mediated by $CD8^+$ T cells (Gropper et al., 2017). Notably, several studies have shown that oxygen restriction can also have immunosuppressive effects. For example, immunosuppressive ($Arg1^+$) tumor-associated macrophages (TAMs) spatially associate with hypoxic regions in mouse breast tumors (Carmona-Fontaine et al., 2017), while *Hif1a* deletion in macrophages impairs both mobilization and activation (Cramer et al., 2003). In addition, TILs were absent from hypoxic regions in a murine model of fibrosarcoma (Hatfield et al., 2015), and limited oxygen availability has been associated with T cell exhaustion in a murine model of melanoma (Najjar et al., 2019). Further, HIF-1α can promote immunosuppressive characteristics of $CD4^+$ $CD25^+$ Tregs (Ben-Shoshan et al., 2008). Together, these studies illustrate that oxygen availability can strongly impact immune cell function in the TME.

1.3 Intercellular interactions

The diverse milieu of immune, cancer, and stromal cells present within the TME creates a dynamic environment, fostering crosstalk between cancer and immune cells that can be influenced by tumor type (Gentles et al., 2015). Advances in single cell techniques, such as

mass cytometry and single-cell genomics, have revealed considerable complexity among stromal and immune cell subtypes in the TME, as well as heterogeneity in the metabolic profiles of these cells (Hartmann and Bendall, 2020; Li et al., 2017; Xiao et al., 2019). Recent work characterizing metabolic enzyme expression in T cell subsets by mass cytometry has the advantage of characterizing the metabolic potential of rare immune subsets from tumors (Hartmann et al., 2020). The major caveat with any single cell profiling approach is that metabolic enzyme expression only defines how the table is set; nutrient availability in the TME will ultimately define what meal is served. Analysis of The Cancer Genome Atlas (TCGA) datasets revealed six dominant immune signatures—wound healing, IFN-γ dominant, inflammatory, lymphocyte poor, immunologically quiet, and TGF-β dominant—associated with 33 different cancer types (Thorsson et al., 2018). The cell types that contribute to these signatures are not fully understood but likely influence anti-tumor immunity. For instance, potential nutrient competition between distinct cell populations that share overlapping metabolic demands could lead to limitations in the availability of glucose and other cellular fuels. Similarly, stromal and immune cell types can contribute to shaping the metabolic composition within the TME, thus affecting both the nutrient utilization and drug responses of neighboring cancer cells (Halbrook et al., 2018; Sousa et al., 2016).

The potentially competitive environment within the TME can also induce TILs to enter an exhausted state. Exhausted $CD8^+$ T (Tex) cells are a subpopulation of $CD8^+$ Teff cells that exhibit reduced effector function and proliferative capacity, and can arise during chronic infection or cancer (McLane et al., 2019). Tex cells are associated with metabolic insufficiency that may be driven in part by the activation of one (or more) checkpoint inhibitor receptors, such as PD-1, LAG3, or CTLA-4 (Bengsch et al., 2016; Previte et al., 2019). Metabolic characterization of exhausted $CD8^+$ T cells suggests that PD-1 inhibits glycolysis and supports fatty acid oxidation, resulting in the accumulation of $CD4^+$ Tregs that can suppress the effector functions of $CD8^+$ T cells (Bengsch et al., 2016; Patsoukis et al., 2015). Interestingly, PD-1 blockade has been shown to reverse the inhibition of glycolysis in exhausted T cells within a murine sarcoma (Chang et al., 2015). Indeed, therapeutic interventions designed to target checkpoint inhibitors have garnered extensive interest in the treatment of human cancers (Buck et al., 2017). Given the influence of tumor-immune crosstalk on anti-tumor immunity, experimental co-culture models may provide an alternative approach to monoculture strategies in order to model heterocellular interactions within the TME and how these impact immune cell responses and tumor cell sensitivity to small molecule drugs.

Ultimately metabolism in the TME is influenced by numerous factors that impact local nutrient availability, including cell-intrinsic metabolic programs driven by oncogenes (and complicated by genetic heterogeneity within the tumor) and cell-extrinsic factors such as tissue vascularization and nutrient and oxygen gradients within the tumor (Figure 1). Whole-body metabolic fluxes also influence tissue metabolite levels and are heavily influenced by diet and the microbiota (Ang et al., 2020; Biggs et al., 2017; Hui et al., 2020; Rooks and Garrett, 2016). Layered on top of this are tumor-stromal interactions, especially evolving tumor-immune cell interactions, that have the potential to impact the metabolic and inflammatory environment within the TME. Improved modeling of metabolism in the TME

will require being mindful of environmental conditions (nutrient and oxygen levels), knowledge of tumor origin and genetic background, and tumor-immune crosstalk (Figure 1).

2 MODELING METABOLISM IN THE TUMOR MICROENVIRONMENT

Several recent studies have collectively established that metabolic phenotypes may vary by experimental model. For example, the use of stable isotope tracers *in vivo* using either mouse models or human patients has revealed that nutrient utilization by cancer and immune cells can differ from those observed using cell-based culture approaches (Davidson et al., 2016; Fan et al., 2019; Hensley et al., 2016; Ma et al., 2019). Though there are inherent caveats associated with all experimental models, an increased recognition that environmental factors contribute to cell metabolism (DelNero et al., 2018; Lyssiotis and Kimmelman, 2017; Muir et al., 2018; Singer et al., 2018; Wolpaw and Dang, 2018) has rapidly escalated efforts to improve the capacity of *in vitro* culture systems to more closely recapitulate conditions in human circulation or tumor-specific environments (Cantor, 2019).

2.1 Physiological media formulations

The complete medium formulations that remain the workhorses of cell culture studies across all areas of biology typically consist of a synthetic basal medium (e.g., MEM, DMEM, RPMI 1640) that poorly reflects metabolite availability in human biofluids, which is also supplemented with a largely undefined serum component (Cantor, 2019). Such media were primarily designed to promote the rapid growth of specific cell types, rather than to model *in vivo* biochemical conditions. Despite the extensive evidence for how biochemical conditions influence immune cell metabolism (Singer et al., 2018), most cell culture studies (Arora, 2013) are still carried out using these complete media. To address this discrepancy, there has been a recent rise in development of strategies to more closely model biochemical conditions encountered *in vivo* through the use of improved media formulations or systems that dynamically buffer nutrient concentrations (Birsoy et al., 2014). Here we focus on unbiased bottom-up approaches to systematically develop physiologic media that more closely recapitulate the metabolic composition of human blood.

Cantor and colleagues developed human plasma-like medium (HPLM) (Cantor et al., 2017), which contains over 60 metabolites and salt ions at concentrations that represent average reported values for normal adult human plasma (Psychogios et al., 2011; Wishart et al., 2013). Through the use of HPLM, the authors discovered an example of metabolic regulation mediated by a metabolite (uric acid) that, among basal media, was uniquely defined in HPLM, and whose plasma concentrations differ by up to 10-fold between humans and mice. The authors went on to demonstrate that HPLM could influence the relative cytotoxicity induced by 5-Fluorouracil, a classic chemotherapeutic that is widely used. These results provide evidence that the influence of metabolite availability on cell physiology and drug responses need not be restricted to considerations of nutrient utilization alone, and also suggest the potential to identify additional unforeseen metabolite-drug interactions.

Through an independent and parallel approach, Vande Voorde and colleagues developed a comparable basal medium (Plasmax) designed to reflect the polar metabolite composition of

human blood (Ackermann and Tardito, 2019; Voorde et al., 2019). Among the relative metabolic differences described in cultured cells, the authors reported that differential arginine availability between Plasmax and a conventional medium can influence the direction of a reaction catalyzed by argininosuccinate lyase within the urea cycle. The authors also showed that by culturing certain breast cancer cells in Plasmax supplemented with relatively low levels of serum, the availability of selenium (at concentrations defined in Advanced DMEM/F12) could influence the colony-forming capacity of these cells.

Notably, the impact of physiologic media on cultured cells is not restricted to studies of cancer metabolism. Recent work demonstrated that, compared to the historically used medium to culture normal and malignant blood cells (RPMI 1640), HPLM induced markedly different transcriptional responses in human primary T lymphocytes and improved their activation upon antigen stimulation (Leney-Greene et al., 2020). This medium-dependent influence on T cell activation was traced to the differential availability of calcium, which is provided at a 6-fold higher levels in HPLM than RPMI 1640, indicating that small ion availability should be another key consideration in future models of the TME.

The development of physiologic media need not be limited to recapitulating the metabolite composition of human plasma, particularly in considering the environment(s) encountered by cancer and immune cells within a tumor. Through metabolic characterization of tumor interstitial fluid (TIF) isolated from tissue-specific murine tumors, Sullivan and colleagues recently reported that metabolite availability in TIF can differ from matched murine plasma, and that TIF composition may be influenced by additional factors such as cancer type and tumor location (Sullivan et al., 2019). These results suggest the potential to also generate synthetic media guided by non-plasma conditions, though given the anticipated heterogeneity of intratumoral environmental contexts, such approaches will likely require careful consideration in the selection and modeling of these biofluids.

While most efforts toward modeling the defined biochemical conditions of cell-based culture models have focused on polar metabolite availability, the characterization of extracellular lipid composition in different biofluids remains an ongoing consideration. For most complete media, the major source of lipophilic species is a supplement of 10–20% serum from fetal bovine or calf. However, lipid composition can vary between serum sources and lots, thus impacting experimental reproducibility. Charcoal stripping to remove serum lipids and enhance reproducibility can also deplete critical growth factors and hormones, further complicating current strategies to incorporate lipids into culture media at defined concentrations. Given the reported role of free fatty acids as a fuel for certain cancer and immune cell populations (Röhrig and Schulze, 2016), the development of robust and accessible approaches to incorporate at least certain free fatty acids to physiologic media at defined concentrations will be an important future direction to consider.

2.2 Tumor spheroids and organoid models

While physiological culture media systems have sought to recapitulate *in vivo* nutrient availability, tumor spheroid and organoid models have aimed to better model other environmental factors that cells encounter in normal tissues or in the TME. Three-dimensional (3D) organoid culture models, which are designed to more closely mimic the

mechanical properties of the extracellular matrix, can also affect cell metabolism and drug responses (Fujii et al., 2016). For example, colorectal cancer (CRC) spheroids used to model nutrient gradients in the TME revealed p53-dependent metabolic vulnerabilities in response to cholesterol-lowering drugs (i.e., statins), which were not otherwise observed in cells cultured as monolayers (Kaymak et al., 2020). In addition, Neal and colleagues developed an air-liquid interface (ALI) method for culturing patient-derived organoids (PDOs), which enabled the propagation of tumor cells and embedded immune cell populations (including T, B, and NK cells and $CD14^+$ or $CD68^+$ macrophages) for several weeks (Neal et al., 2018). Similarly, others have described the use of: a) co-cultured mouse-derived cancer organoids with autologous immune cells for the study of PD-1:PDL-1 interactions (Chakrabarti et al., 2018); b) heterotypic co-culture of CRC spheroids with immune cells to examine the infiltration and ensuing response of T and NK cells (Courau et al., 2019); c) organotypic tumor spheroids to identify associations between certain cytokines and chemokines and anti-tumor immunity (Jenkins et al., 2018); and d) co-culture of CRC-derived organoids with autologous TILs to predict cellular responses to chemoradiotherapy (Kong et al., 2018). Of note, Kong and colleagues demonstrated that antitumor immunity could be rescued by the addition of antibodies targeting PD-1, highlighting the potential utility of coculturing TILs with tumor organoids to uncover new cancer immunotherapies.

2.3 *In vivo* tumor characterization

One challenge of *in vitro* models is how best to recapitulate both the proportion of immune cell populations and their functional state within the TME. An excellent example of this is $CD8^+$ Tex (for a comprehensive review please see (McLane et al., 2019)). Effectively recapitulating the Tex cell state in cell culture has been challenging, although new chronic stimulation methods developed to study T cell exhaustion *in vitro* show promise (Vardhana et al., 2020). Chronic infection models, such as LCMV Clone 13, and syngeneic tumor models remain the gold-standard for studying this cellular state. Indeed, syngeneic mouse models also remain the current workhorse model system for studying tumor-immune cell interactions, as well as for testing the efficacy of new immunotherapies (e.g., checkpoint inhibitors) (Allard et al., 2016). However, orthotopic xenograft mouse models may better recapitulate the relevant TME context (Sullivan et al., 2019). Interestingly, one recent study demonstrated that tumor infiltration by various immune cells could vary between models that used syngeneic versus orthotopic xenografts, highlighting the potential influence of tumor location on immune cell response (Zhao et al., 2017).

3 EMERGING APPROACHES FOR MODELING TUMOR-IMMUNE METABOLIC INTERACTIONS

In mice and humans, great progress has been made in the study of *in vivo* metabolism through the use of stable isotope-labeled nutrients in tumors or normal tissues. In some cases, these studies have also lent insights into how metabolic activity may vary between tumors in a tissue-dependent manner (Fernández-García et al., 2020; Jang et al., 2018). In contrast to typical *in vitro* methods, *in vivo* tracer studies further enable the consideration of nutrient and oxygen gradients in the context of tissue architecture, intracellular interactions, and systemic metabolism (Figure 2). For instance, recent work by Ma and colleagues used

stable isotope tracer and ^{13}C-glucose infusion techniques to track and compare glucose utilization by CD8$^+$ T cells responding to infection *in vitro* and *in vivo* (Ma et al., 2019). This work revealed that, in contrast to their metabolic phenotypes exhibited *in vitro*, CD8$^+$ T cells *in vivo* displayed an oxidative phenotype and used glucose carbon for anabolic demands such as nucleotide and serine biosynthesis rather than for lactate production. In another recent study, Lercher and colleagues used ^{13}C-arginine infusion to understand how hepatic metabolism is modulated in response to viral infection (Lercher et al., 2019). Together these studies provide a potential foundation for studying the impact of local and systemic environments on T cell metabolism and protective immunity (Figure 1).

Yet, caveats also remain for *in vivo* studies of immunometabolism. One challenge in using ^{13}C tracers to study immune cell metabolism *in vivo* is the natural cellular heterogeneity that exists in different tissues. Given the heterocellular complexity within a tumor, the use of isotopically-labeled infusion techniques typically requires eventual isolation (e.g., via sorting or kit-based purification) of a specific cell subset. Sorting immune cell subsets after isolation takes time (30 minutes to several hours) and often involves culture in minimal buffers that promote changes in metabolite pool sizes (Llufrio et al., 2018). Although this timescale preceding metabolite extraction could be incompatible with retaining the metabolic profile of cells *in vivo* (Cantor, 2019; Llufrio et al., 2018), there is evidence that T cells retain their metabolic programming during short-term culture after sorting (Ma et al., 2019). Taking a different approach, sorting immune cells into distinct cell populations (i.e., Teff versus Tex cells) followed by immediate *ex vivo* ^{13}C tracer analysis represents a feasible way to model immune cell metabolism in the TME, particularly when studying complex cellular heterogeneity or rare cell populations. By further incorporating the use of physiological media and more physiologically relevant oxygen tensions, *ex vivo* tracing experiments could potentially offer a more tangible and accessible approach for mimicking *in vivo* conditions (Figure 2).

Extending beyond metabolomic snapshots of isolated cells, imaging techniques such as matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) mass spectrometry have the potential to further elucidate spatial metabolite composition and nutrient gradients within intact tissues. For example, Fan and colleagues used an *ex vivo* stable isotope-resolved metabolomics (SIRM) technique and coupled histological characterization for samples resected from non-small cell lung cancer patients to investigate tissue metabolism over time (Fan et al., 2016b). Further, Hensley and colleagues infused non-small cell lung cancer patients with ^{13}C-glucose prior to surgical resection, which revealed that tumor areas with high perfusion versus low perfusion exhibited different metabolic phenotypes (Hensley et al., 2016). Others have mapped metabolite clusters in human CRC based on 3D desorption electrospray ionization (DESI)-MS techniques (Inglese et al., 2017). Perhaps the integration of spatial metabolite profiling and high-dimensional immune cell imaging techniques (Hartmann and Bendall, 2020) will bring us closer to achieving single cell resolution of metabolism within complex tissue environments.

4 CONCLUSION

The metabolic networks of different cells are dictated by the sum and interplay of several intertwined cell-intrinsic and environmental factors. By integrating this concept with recent technical advances such as physiologic culture media, culture systems that dynamically buffer the concentrations of certain nutrients, stable isotope tracing, and *in vivo* metabolite imaging, it should be possible to develop new strategies that advance our understanding of immunometabolism in the TME. In addition, co-culture methods that integrate tumor organoids and peripheral blood lymphocytes (Dijkstra et al., 2018) have the potential to support more “personalized” metabolic modeling of patient TME conditions. The culture of *ex vivo* tissue slices (Fan et al., 2016a) or PDOs from patient-derived tumors (Neal et al., 2018) in conditions that more closely recapitulate those in the human body may enable opportunities to more closely model metabolic cross-talk between diverse cell types that reside in the TME. Therefore, contextualizing the study of immunometabolism will be important to improve our understanding of immune cell function and how such knowledge can be leveraged to treat human disease.

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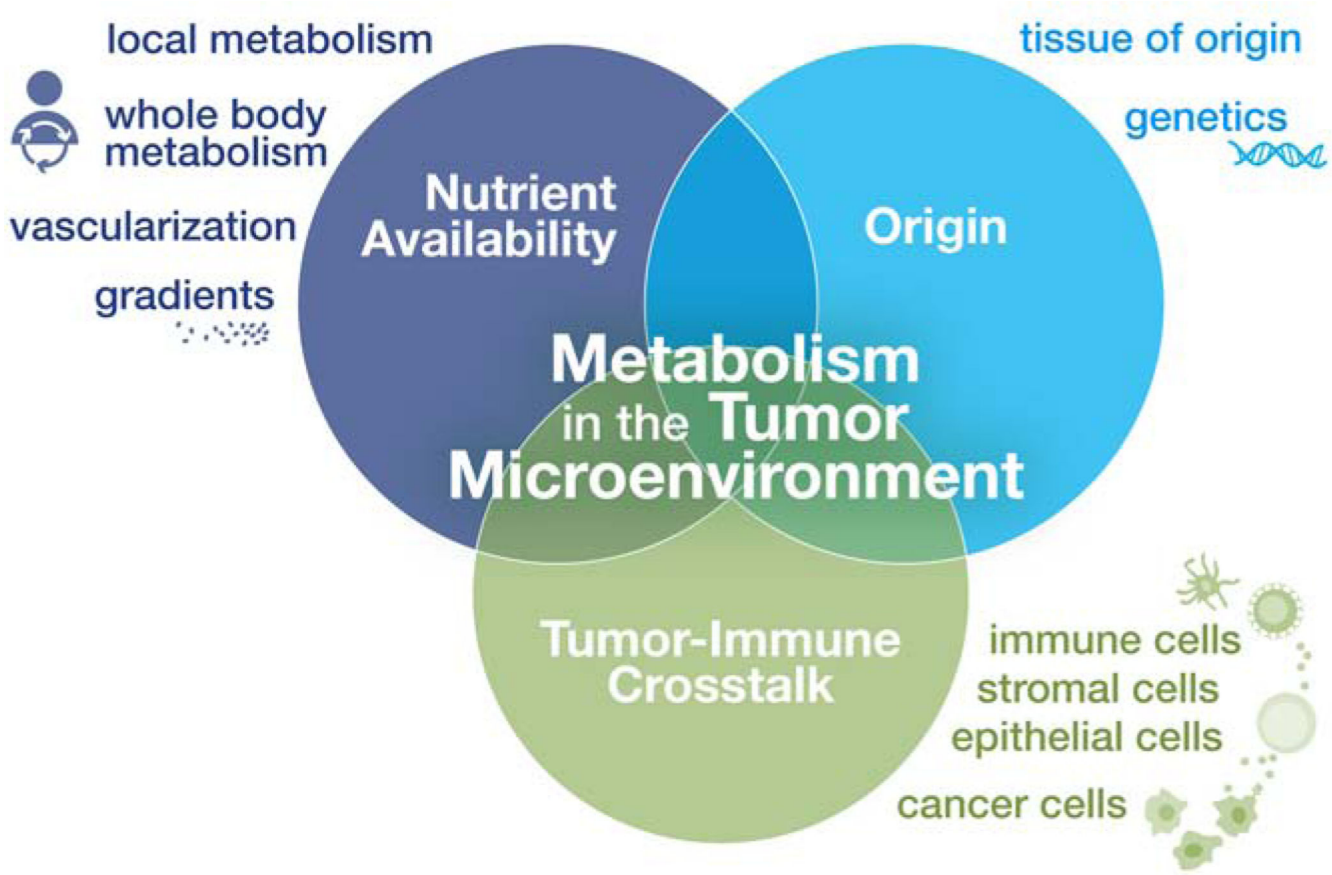

Figure 1: Factors influencing metabolism in the TME.

Metabolic conditions in the TME are influenced by many factors. From the perspective of the tumor, mutations in oncogenes and tumor suppressor genes can promote cell-intrinsic changes in metabolic reprogramming depending on the cancer type (**"Origin"**). Whole body metabolism dictates available nutrients in circulation ("**Nutrient Availability**"), while local nutrient availability in the TME is influenced by tumor vascularization and tissue nutrient uptake. Due in part to the high proliferation of cancer cells, nutrient and oxygen gradients can form which shape the metabolic activities of cells differently across the tumor. Nutrient utilization and signaling by many cell types present (immune, stromal, epithelial, cancer) in the TME can also modulate the metabolic conditions within the growing tumor ("**Tumor-Immune Crosstalk**").

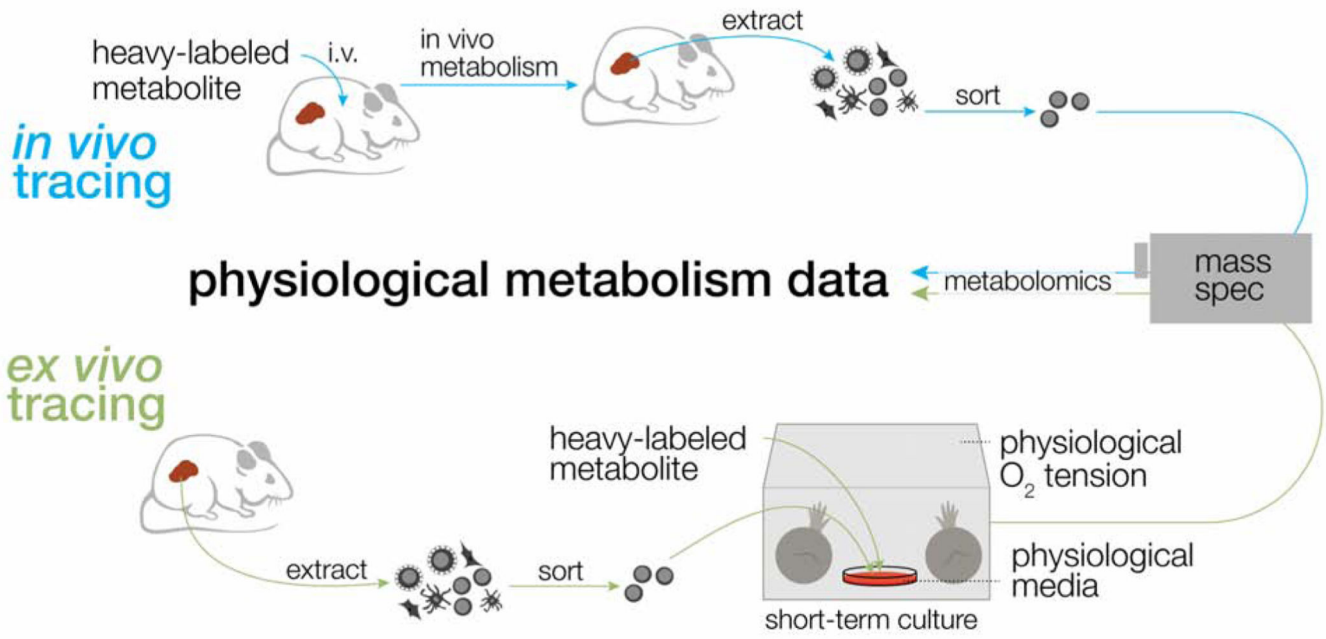

FIGURE 2: Approaches for modeling physiologic immunometabolism using stable isotope tracing.

Isotope tracers, in the form of heavy-labeled metabolites, can be administered intravenously (top) or *ex vivo* to sorted cell populations (bottom) to achieve accurate tracing of *in vivo* metabolic pathways. *Ex vivo* tracing (bottom), in combination with physiologic media and oxygen tension, offers a path for physiological modeling of the metabolic state of specific cell subsets *in vivo* that require extensive sorting after extraction.