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INVITED REVIEW

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The immunological Warburg effect: Can a metabolic-tumor-stroma score (MeTS) guide cancer immunotherapy?

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Abstract

The "glycolytic switch" also known as the "Warburg effect" is a key feature of tumor cells and leads to the accumulation of lactate and protons in the tumor environment. Intriguingly, non-malignant lymphocytes or stromal cells such as tumor-associated macrophages and cancer-associated fibroblasts contribute to the lactate accumulation in the tumor environment, a phenomenon described as the "Reverse Warburg effect." Localized lactic acidosis has a strong immunosuppressive effect and mediates an immune escape of tumors. However, some tumors do not display the Warburg phenotype and either rely on respiration or appear as a mosaic of cells with different metabolic properties. Based on these findings and on the knowledge that T cell infiltration is predictive for patient outcome, we suggest a metabolic-tumor-stroma score to determine the likelihood of a successful anti-tumor immune response: (a) a respiring tumor with high T cell infiltration ("hot"); (b) a reverse Warburg type with respiring tumor cells but glycolytic stromal cells; (c) a mixed type with glycolytic and respiring compartments; and (d) a glycolytic (Warburg) tumor with low T cell infiltration ("cold"). Here, we provide evidence that these types can be independent of the organ of origin, prognostically relevant and might help select the appropriate immunotherapy approach.

KEYWORDS

acidification, GLUT, immunotherapies, lactate, T cell, Warburg

1 | HISTORICAL TIMELINE—THE WARBURG EFFECT THEN AND NOW

In the early 20s of the last century, Warburg, Posener, and Negelein[1] discovered a unique behavior of tumor tissue in vitro. They examined respiration and glycolysis of different tissue sections and found that tumors exhibit an unusually high glycolytic activity and production of lactic acid from glucose when compared with normal tissues. Surprisingly, glycolysis was "aerobic" and was not inhibited by oxygen in malignant cells meaning that cancer cells lack the "Pasteur effect." Carl F. Cori and Gerty Cori confirmed this finding in living animals. Interestingly, the lactic acid content of tumors was low in starving animals with low-glucose levels and the authors concluded "up to a certain limit an

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excess of lactic acid can be completely eliminated into the blood stream." In another set of experiments, the Cori laboratory compared blood that had passed through a tumor with normal venous blood in chicken and observed decreased sugar and increased lactic acid in blood from the tumor-bearing wing.[2,3] In 1926, Otto Warburg further analyzed the energy metabolism of tumors and discussed possible ways to kill tumor cells through a "lack of energy".[4] Warburg measured glucose and lactic acid in tumor arteries and veins and found greater differences than the Coris. His results showed that the concentration of glucose falls by about 57% after passing through a tumor, whereas normal tissues consume about 2%-18% of the arterial glucose. Tumor veins contained clearly more lactic acid than arteries, but this was not the case for normal tissues. He stated that tumor cells obtain energy in two ways, by respiration and fermentation and calculated that about 66% of the glucose is used in fermentation and the rest for respiration, but this may vary in different parts of the tumor based on heterogeneity in glucose and oxygen availability. He concluded that "it is necessary to stop both respiration and fermentation, if cells are to be killed for want of energy".[5] Later in his life, he changed his side of view and claimed that tumor cells are characterized by a damaged respiration.[6,7]

In contrast to these observations and numerous studies since, two recent publications stated that the tumor environment contains no elevated lactate levels in the interstitial fluid,[8,9] and lactate may accumulate preferentially in tumor cells. In this case, the tumor stroma, for example, immune cells, would not face high-lactate concentrations. However, Sullivan and colleagues examined pancreatic ductal adenocarcinoma (PDAC), a tumor with high stromal/fibroblast content. A possible explanation for low lactate levels in this tumor model is a metabolic symbiosis in heterogeneous tumors consisting of fibroblasts and highly glycolytic tumor cells. Here, tumor cells produce and secrete lactate which is consumed and used as a fuel for respiration by adjacent stromal cells.[10,11] Alternatively, it has been shown by Sonveaux and colleagues that respiring tumor cells in oxygenated regions take up lactate and metabolize it.[12] Low lactate levels may also occur in tumors where lactate is eliminated by the blood stream in well-perfused tumor regions. But compromised blood perfusion is a typical feature of tumors and may help to build up extracellular levels of lactate and protons in the tumor environment especially in tumors with highly accelerated glycolysis. In tumors with extremely high glycolytic activity, elevated lactate levels can also be detected in sera of tumor patients.[13,14] Accordingly, lactate levels have been suggested as a prognostic biomarker in high-grade primary brain tumors and metastatic lung cancer where elevated pretreatment serum lactate levels were associated with worse progression free survival.[15,16] Bringing an additional layer of complexity, in the "Reverse Warburg Effect" stromal cells such as fibroblasts or macrophages undergo "aerobic glycolysis" and produce lactic acid which can either accumulate or be utilized by cancer cells for mitochondrial oxidative phosphorylation (OXPHOS).[17-19] Thus, not only malignant cells but also stromal cells contribute to tumor lactic acidosis. Furthermore, acidosis is not only associated with lactic acid secretion, as another major source of protons is CO_2, produced in more oxygenated tumor areas which is hydrated into $HCO3^-$ and H^+ ions by carbonic anhydrases leading to acidification.[20]

Today, we know that tumors often represent a mosaic of tumor cells with different metabolic properties. While some tumors rely more on oxygen, others can be more glycolytic. Metabolic heterogeneity regarding glucose metabolism within and between human lung tumors was nicely demonstrated by the group of DeBerardinis using intraoperative 13C-glucose infusions in patients.[21] The authors concluded that enhanced glucose uptake by lung tumors supplies glucose oxidation rather than enhanced lactate fermentation. On the other hand, in vivo isotope tracing in human clear cell renal cell carcinomas (ccRCC) revealed enhanced glycolysis and minimal glucose oxidation compared to adjacent kidney consistent with the classical Warburg phenotype.[22] A metabolic heterogeneity among ccRCC tumors was demonstrated by Brooks et al with low or no 18F fluoro-deoxy-glucose (FDG) uptake in some tumors but uniformly high uptake in other tumors.[23] We studied T cell infiltration in relation to tumor glucose transporter 1 (GLUT1) expression in ccRCC and identified two major tumor types. Classical Warburg tumors with high GLUT1 expression and low T cell infiltration, and tumors with low tumor GLUT1 expression and high T cell infiltration in the tumor (Figures 1 and 2).[24] In line a recent publication described a negative correlation between increased glycolysis and CD8 T cell infiltration in colon cancer.[25] What is more, GLUT1 expression inversely correlated with numbers of $CD3^+$ T cells in HNSCC and lung SCC.[26]

Two molecular subgroups with low- and high OXPHOS were also identified in high-grade serous ovarian cancer. While low-OXPHOS tumors mainly exhibit a glycolytic metabolism, high-OXPHOS tumors rely on oxidative phosphorylation supported by glutamine and fatty acid oxidation. Furthermore, distinct metabolic subtypes were described in PDAC cell line models. Here, lipogenic tumor cell lines showed higher oxygen consumption and greater mitochondrial content compared with glycolytic tumor lines. In primary pancreatic tumor samples, the lipid subtype was strongly associated with an epithelial phenotype, whereas the glycolytic subtype was associated with a mesenchymal phenotype.[27] Based on the negative correlation between glycolytic activity, GLUT expression and T cell infiltration, these data suggest a stronger immune cell infiltration in high-OXPHOS tumors compared to tumors with glycolytic activity in ovarian and pancreatic cancer (Figure 1).

These data also implicate that the "Warburg phenotype" is not characteristic for all tumor entities and not even for all tumor cells within one given tumor. Nevertheless, the work of Otto Warburg and colleagues has an enormous impact on current cancer research and diagnostics. Uptake of the positron-labeled glucose analogue 18F-fluoro-deoxy-glucose determined with positron emission tomography (18F-FDG-PET) is a well-established method for tumor diagnosis and staging. Even disseminated and hematologic malignancies, such as lymphoma can be imaged by FDG-PET,[28,29]

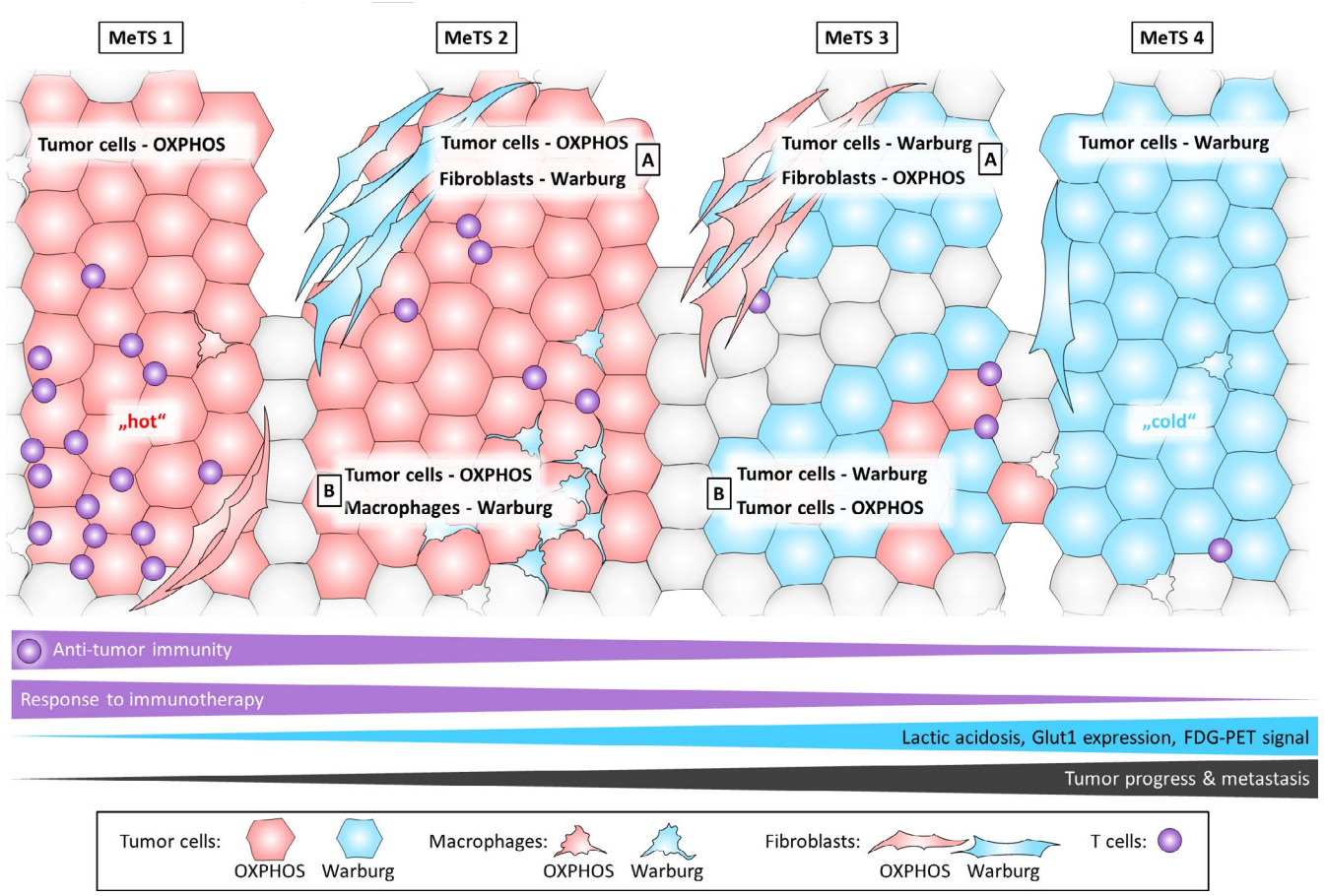

FIGURE 1 A metabolic-tumor-stroma score (MeTS) to describe the tumor metabolic and cellular heterogeneity and to assess the quality of anti-tumor immune response

demonstrating that not only solid tumors but also leukemia and lymphoma cells of different origin display an accelerated glucose metabolism, which was confirmed by gene expression and functional analysis.[30-32] Moreover, the discovery that oncogenes and tumor suppressor genes are closely linked to and regulate the Warburg effect has led to a renewed interest in tumor metabolism which is now regarded as a hallmark of cancer.[33]

2 | WARBURG EFFECT IN TUMOR STROMAL CELLS

The observed composition of tumors regarding both a cellular and a metabolic heterogeneity lead us to propose the metabolic-tumor-stroma score (MeTS) with following subtypes: (MeTS1) a respiring tumor type; (MeTS2) a reverse Warburg type with respiring tumor cells but glycolytic stromal cells; (MeTS3) a mixed type with both glycolytic and respiring cells; and (MeTS4) a glycolytic (Warburg) tumor (Figure 1). High metabolic heterogeneity puts into question whether the PET signal is related to glucose metabolism of tumor cells, stromal cells or both and to what extent in which tumor entity. A shift in energy metabolism toward aerobic glycolysis is not only a hallmark of cancer cells but also of activated immune cells. Here, aerobic glycolysis is discussed as a prerequisite for T and NK cell effector functions.[34-39] Moreover, high glycolytic activity is associated with myeloid cell activation as shown for monocytes, macrophages and dendritic cells (DCs).[40-42] TLR signaling promotes glycolysis and leads to a decline in OXPHOS.[43,44] Thus, 18F-FDG can be taken up by glycolytic inflammatory cells and has been reported to accumulate in acute and chronic inflammatory lesions, granulomatous diseases, and autoimmune diseases. Accordingly, tumor-infiltrating immune cells are also able to take up 18F-FDG and it has been shown that high uptake of 18F-FDG by inflammatory cells is a frequent cause of false positive results in 18F-FDG-PET.[45] However, it is difficult to differentiate viable tumor cells from tumor-infiltrating immune cells and similarly, to distinguish lymph node metastases from immune activation in reactive lymph nodes.[46,47] A clinical correlate of increased activity of immune cells in tumors is the phenomenon of pseudo-progression. Tumor pseudo-progression describes an apparent tumor growth after immunotherapy and might be explained by increased tumoral inflammation after immune cell re-activation. Even though challenging for patients and clinicians, tumor pseudo-progression in a computer tomography (CT) scan or 18F-FDG-PET might precede a good clinical response to immunotherapy.[48] Importantly, the immune activity can emit PET signals similar to those from tumors, and therefore, 18F-FDG-PET cannot differentiate a true progression from a

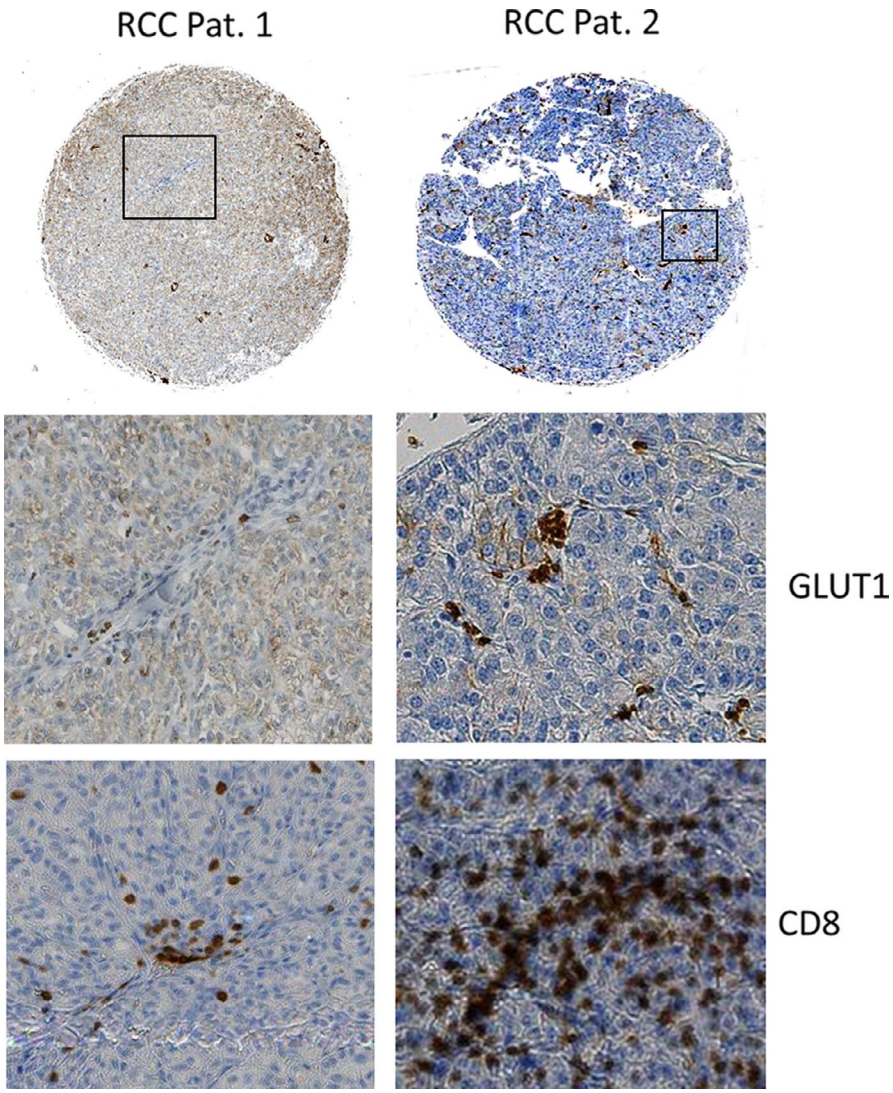

FIGURE 2 GLUT1 expression and CD8 T cell infiltration in clear cell renal cell carcinoma (ccRCC). Shown are tissue microarrays of two ccRCC patients. Patient 1 displays ubiquitous GLUT1 expression in the tumor tissue while lacking CD8 T cell infiltration. Patient 2 shows low tumor GLUT1 expression and high CD8 T cell infiltration. Figure adapted from (Singer et al 2011[24]) with a permission from the publisher

pseudo-progression.[49,50] Cancer-associated fibroblasts (CAFs) can account for a high proportion of the tumor stroma and can contribute to the uptake of 18F-FDG. Shangguan et al[51] analyzed the association of CAFs with 18F-FDG signals in colon cancer and showed that CAFs density correlated with the standardized uptake value.

In a small cohort of patients, we analyzed stroma-rich tumors for GLUT1 and CD3 expression by immunohistochemistry and detected GLUT1 positive CAFs in a pancreatic carcinoma patient, however, only in a subset of stromal cells (MeTS2a, Figure 3). In colon carcinoma and mammary carcinoma tissues, CAFs were mainly GLUT1 negative and these areas were often highly infiltrated by CD3-positive T cells (MeTS3a, Figure 3).

In light of these observations, high 18F-FDG uptake can also reflect an intense ongoing host reaction, such as after radiation therapy, where 18F-FDG uptake by tumor tissues is often increased despite the decreased viability of tumor cells. In an interesting study, Mamede and colleagues analyzed the distribution of 18F-FDG in immunocompetent and immunodeficient mice bearing the same murine ovarian squamous cell carcinoma. Despite comparable tumor growth and histology in both mice strains, the 18F-FDG signals were significantly higher in tumors in immunocompetent than in immunodeficient mice.[46] Thus, it was unlikely the tumor metabolic activity, but rather an immune activation that led to the increased 18F-FDG uptake.[46,52,53] In a murine mammary carcinoma model, 18F-FDG accumulation was even higher in macrophages in the outer zones of necrosis than in tumor cells,[54] in line with data from Seth et al who demonstrated that tumor-associated macrophages (TAMs) produce high amounts of lactate which in turn support tumor growth.[17] These tumors would classify as a reverse Warburg (MeTS2) type with respiring tumor cells but glycolytic stromal cells. Recently, Jeong et al nicely showed a positive correlation between $CD68^+$ TAMs and 18F-FDG uptake in NSCLC patients. Depletion of TAMs in a murine model diminished 18F-FDG uptake of tumors and improved T cell infiltration.[55] These data strongly indicate that high glycolytic activity not only in tumor but also in stromal cells such as TAMs can impair immune cell function.

The metabolic heterogeneity of tumors extends beyond different populations having different metabolic requirements and tumor cells show various metabolic interactions with stromal cells. For example, it has been shown in pancreas cancer, that tumor cell-produced

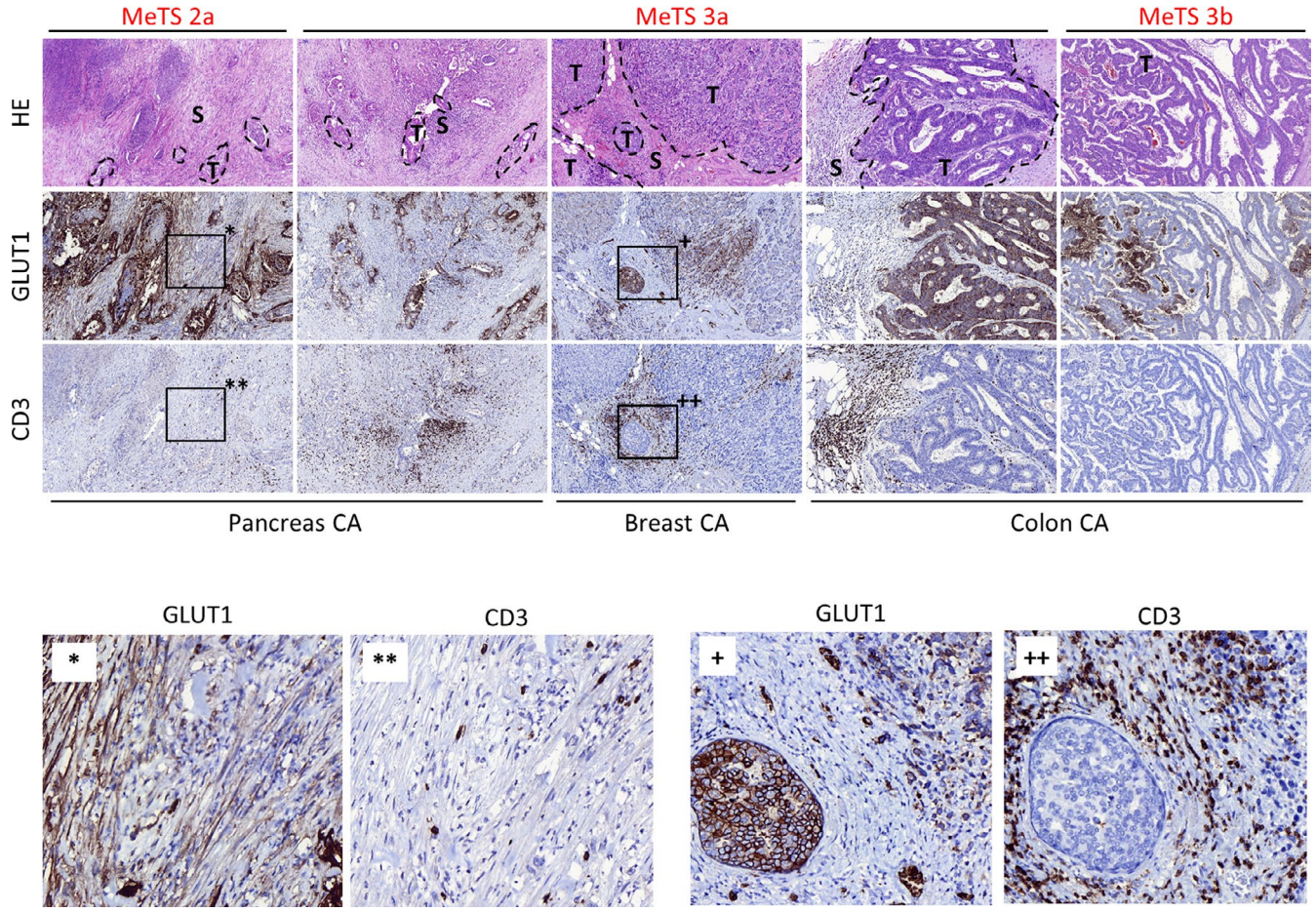

FIGURE 3 Representative images from three tumor entities correlating high glucose metabolism in tumor or stromal cells with T cell infiltration. HE staining of primary resected carcinoma of the pancreas (column 1,2), the breast (column 3), and the colon (column 4,5) without any neoadjuvant treatment (original magnification column 1: 133x, column 2, 3: 114x, column 4: 153x, column 5: 91x) and the corresponding immunhistochemical staining of GLUT1 (middle row) and CD3 (lower row). Higher magnification of the marked areas is shown in the lower part. The cancer-associated fibroblasts in pancreatic carcinoma are GLUT1-positive (*) with almost no CD3-positive T cells (**) in the stroma. The breast cancer sample shows a strong staining for GLUT1 (+), while CD3-positive T cells are exclusively located in the GLUT1-negative stroma (++). MeTS 2a,3a,3b: suggested scoring of the tumor-stroma metabolic interaction with infiltrating immune cells

lactate can be taken up by CAFs, which in turn produce alpha-ketoglutarate, leading to TET activation and subsequent decreased cytosine methylation and overexpression of CAFs inflammatory genes.[56] In breast cancer, tumor cell-produced lactate can be taken up and used by mesenchymal stroma cells and CAFs as an energy source.[57] On the other hand, CAFs are able to drive glycolysis in tumor cells—Kumar et al showed that CAF-secreted hepatocyte growth factor (HGF) induces tumor cell glycolysis and secretion of basic fibroblast growth factor (bFGF). Interestingly, bFGF induces OXPHOS in CAFs, leading to HGF production, thus closing the vicious circle.[58] In a clinical study, breast cancer patients with stage 0/1 tumors were treated with N-acetylcysteine (NAC) to reduce oxidative stress in stromal cells and decrease tumor growth. Interestingly, post-treatment biopsies showed that stromal (but not tumor) cells decreased expression of the lactate exporter MCT4 as a result of NAC treatment, while the proliferative index of tumor cells was reduced.[59]

These studies confirm that tumors are heterogeneous, and high glucose uptake is not necessarily a proxy for tumor cell viability and proliferation but activated immune cells and other stromal cells such as fibroblasts and macrophages can contribute. Therefore, glucose and lactate levels in the tumor microenvironment can depend (a) on the tumor metabolic type, (b) on the relative percentage of tumor-infiltrating cells such as immune cells, their type, and activation status, and (c) on the type and quantity of stromal cells, such as fibroblasts (Figure 1).

3 | MOLECULAR BACKGROUND OF THE WARBURG EFFECT IN TUMOR AND STROMAL CELLS

Warburg proposed that cancer is caused by a metabolic "switch" from mitochondrial respiration to aerobic glycolysis. However, in following years numerous groups provided evidence of the important role of respiration for tumor growth in several preclinical models and human tumor entities. We know that cancer development is caused by genetic alterations and signaling networks downstream

of oncogenes and tumor suppressors converge to adapt tumor cell metabolism.[60,61]

Mutations in the tricarboxylic citrate acid cycle enzymes succinate dehydrogenase and fumarate hydratase have been identified in a limited number of cancers. As a consequence, a dysfunction of OXPHOS can induce the Warburg effect in tumor cells. Due to these mutations, succinate and fumarate accumulate in mitochondria and also leak out to the cytosol. They inhibit prolyl hydroxylase enzymes leading to a pseudo-hypoxic response conveyed by hypoxia-inducible factors (HIF) that enhance glycolysis.[62] In addition, alterations in mitochondrial DNA have been reported in some types of cancer, but their functional relevance and contribution to carcinogenesis is discussed controversially. Mitochondria are needed as synthetic organelles to supply components for the generation of nucleic acids, proteins and phospholipids; thus, tumor cells might not be able to tolerate defective mitochondria in the long run.[63] In line, Baker et al[64] demonstrated that mitochondrial DNA mutations are increased in number in early colon dysplasia but seem to be negatively selected in the further course of cancer development.

The catalytic subunit of the mitochondrial H^+-ATP complex (β-F1-ATPase) is central for ATP synthesis during OXPHOS and integrates bioenergetic and death-signaling functions of mitochondria. In line with the established role of mitochondrial dysfunction in cancer, β-F1-ATPase is often downregulated in tumors compared to healthy tissues. The group of Cuezva reported a repression of β-F1-ATPase in kidney, colon and breast carcinomas.[65,66] Moreover, some carcinomas upregulate the ATPase inhibitory factor 1, which inhibits the activity of the H^+-ATP synthase.[67] This is of special importance for chemotherapy as downregulation of mitochondrial ATP synthase is associated with drug resistance linking the success of anti-cancer therapy to the (dys)function of mitochondria.[68]

Genetic alteration or loss of p53, one of the most frequently mutated genes in cancer, also modulates the balance between respiration and glycolysis. p53-deficient cells show decreased oxygen consumption and increased lactate production.[69] Oncogenic transformation does not only decrease the mitochondrial activity of tumor cells but can directly accelerate glycolysis. For example, cells lacking the tumor suppressor PTEN exhibit a glycolytic phenotype reminiscent of the Warburg effect. This has been attributed to the hyperactivation of PI3K/AKT signaling as constitutive AKT activity accelerates glucose uptake and stimulates aerobic glycolysis in transformed cells.[70] In addition, the loss of PTEN leads to a stabilization of 6-phosphofructo-2-kinase (PFKFB3) and increases synthesis of its product fructose-2,6-bisphosphate.[71] PFKFB3 has been shown to be highly expressed and active in human cancer cells and fructose-2,6-bisphosphate is a key regulator of the glycolytic enzyme phosphofructokinase-1. Selective inhibition of PFKFB3 in HCT-116 colon adenocarcinoma cells causes a marked decrease in glucose uptake simultaneously with an increase in autophagy.[72] Chronic inflammation can promote tumorigenesis and it has been shown that pro-inflammatory cytokines such as TNFα and IL-17 stimulate glycolysis in colorectal cancer cells.[73]

Dysregulated expression of the MYC oncogene occurs in about 30% of human cancers and MYC overexpression induces mitochondrial glutaminolysis and triggers upregulation of glycolytic enzymes, like lactate dehydrogenase (LDH) A.[74] Oncogenic MYC also interacts with HIFs. Hypoxia is characteristic for the tumor milieu as a result of decreased tissue microcirculation. HIFs are stabilized in response to hypoxia and induce the transcription of more than 70 genes via hypoxia response elements, among them genes involved in glucose metabolism like GLUT1, LDH or the monocarboxylate transporter 4 (MCT4) involved in lactate transport.[75,76] But even under normoxic conditions accumulation of the glycolytic products lactate and pyruvate can promote HIF-1 protein stability and regulate gene expression.[77] Acidosis enables HIF to evade destruction in the presence of oxygen as low pH elicits a transient and reversible loss of von Hippel-Lindau (VHL) function by promoting its nucleolar sequestration.[78] In summary, HIF activation and stabilization is not only a result of hypoxic conditions but is also achieved under normoxia through low pH, lactate or pyruvate.

Hypoxia and acidification also suppress the circadian clock through diminished translation of clock constituents,[79] adding another level of complexity to the metabolic regulation. Clock genes control the circadian rhythm in mammalian cells and epidemiological studies indicate that disruption of the circadian clock and decreased expression of the clock components BMAL1 and PER2 contribute to tumorigenesis. PER2 promotes p53 function, while BMAL1 expression is suppressed by MYC, suggesting bidirectional interactions between clock proteins and key oncogenic regulators of tumor metabolism.[80]

Overall, a complex network of transcriptional regulators controls tumorigenesis and metabolism. Even though metabolic reprogramming may result from different driver mutations, it appears that recurrent master regulators ultimately lead to common metabolic phenotypes across multiple tumor entities. Accordingly, Peng and colleagues analyzed TCGA samples across 33 cancer types and characterized metabolic tumor subtypes. Master regulators of carbohydrate and nucleotide metabolism showed consistently poor prognostic patterns across all analyzed cancer types whereas lipid metabolism showed the opposite association.[81] Accordingly, the expression of enzymes and transporters involved in glucose metabolism has been linked to unfavorable patient outcome in different tumor entities such as hepatocellular carcinoma,[82,83] melanoma[84,85] or urothelial carcinoma.[86] Moreover, in oral squamous cell carcinoma a high PET signal, thus a high glucose uptake, correlates with worse prognosis[83] and similarly, high intra-tumoral lactate concentration predicts the occurrence of metastases and patient survival.[87,88] Thus, the phenomenon of lactate accumulation and acidification in solid tumors is not simply a surrogate for hypoxia but is the result of an interplay of different transcriptional master regulators and, importantly, determines patient outcome.

Similar transcriptional networks and key players such as HIFs and MYC described in cancer cells regulate metabolism in immune cells and CAFs. Specific deletion of HIF-1α in CAFs reduced VEGF production, angiogenesis and reduced myeloid infiltration which in

turn restricted tumor growth.[89] Moreover, HIF alters the function of myeloid derived suppressor cells (MDSCs) in the tumor microenvironment and redirects their differentiation to TAMs hence providing a mechanistic link between different myeloid suppressive cells in the tumor microenvironment.[90] In addition, HIF-1α selectively upregulates PD-L1 on MDSCs, but not other B7 family members and blockade of PD-L1 abrogates MDSC-mediated T cell suppression.[91] In macrophages, tumor-conditioned media with high-lactate levels stabilized HIF-1α protein under normoxic conditions. HIF induced M2-polarization in TAMs and increased VEGF and arginase 1 expression, which play an important role in regulating tumor growth. Collectively, these findings identify a mechanism of communication between macrophages and tumor cells via lactate which may have evolved to promote tumor growth.[92] These data also suggest that tumor-derived lactate can determine the suppressive phenotype of different myeloid tumor-associated cell populations. A role for HIF proteins has also been discussed in lymphoid cells as their activation stabilizes HIF proteins.[93,94] Cho et al show an important role for both, HIF-1α and HIF-2α, in triggering cytokine response and for the induction of antigen specific B cells by CD4 T cells. Moreover, HIF-1α is involved in CD4 Th17 polarization[95] and important for the cytolytic function of CD8 T cells.[96,97] In contrast, Wang et al[94] described no apparent phenotype deleting HIF-1α, but the deletion of c-MYC impaired T cell activation. In line, c-MYC increased the expression of genes related to glucose and glutamine metabolism in T cells.[98] Furthermore, MYC upregulation has been reported in macrophages associated with a M2-like phenotype and was detected in human TAMs. In line, in vitro alternative polarization of macrophages requires the transcription factor c-MYC.[99]

In summary, the same signaling pathways are essential for the metabolic regulation in immune cells and tumor cells. However, in tumor cells oncogenic regulation of these pathways results in a stable phenotype, whereas immune cells show a transient metabolic reprogramming in response to activation or environmental factors.

4 | EVOLUTION OF THE WARBURG EFFECT—BENEFITS FOR THE TUMOR

In 2004, Gatenby and Gillies asked "why do cancers have high aerobic glycolysis?"[100] and proposed that "cell populations with upregulated glycolysis and acid resistance have a powerful growth advantage, which promotes proliferation and invasion." However, the metabolism of glucose to lactate is less efficient compared to oxidative phosphorylation, at least in terms of ATP production per mol of glucose. Why would a proliferating cell use a less efficient metabolism? A possible explanation is that proliferating cells have requirements that extend beyond ATP as they need to replicate all of its cellular contents such as nucleotides, amino acids, and lipids. Vander Heiden and colleagues proposed that "the metabolism of cancer cells, and indeed all proliferating cells, is adapted to facilitate the uptake and incorporation of nutrients into the biomass needed to produce a new cell".[101] Would this explain why the Warburg effect provides an evolutionary benefit for tumor cells? The excess lactate secretion that accompanies the Warburg effect leads to a loss of three carbons that could be utilized for building blocks or ATP production—an inefficient use of resources. In a more recent publication, the Vander Heiden group investigated the fraction of carbon mass in cells derived from different nutrients and found that the majority of carbon mass is not derived from glucose but rather from glutamine indicating that high glycolysis supports cell proliferation through mechanisms beyond providing carbon for biosynthesis.[102]

Alongside its role in providing carbon for building blocks and energy generation, aerobic glycolysis results in a high rate of lactate production. The maintenance of the glycolytic flux requires a continuous export of lactate and protons from the cancer cell, which is carried out by MCTs. As a result, lactate and protons accumulate in the tumor environment resulting in acidification of the extracellular space. Acidification is a well-known feature of the tumor environment and can sustain tumor growth by promoting local invasion, metastasis, and inhibiting anti-tumor immunity. Gatenby et al[100] proposed an "acid-mediated tumor invasion model" where an altered glucose metabolism leads to acidification of the tumor milieu which in turn allows tumor cells to form invasive cancers. In line, low extracellular pH promotes metastasis in a human melanoma model[103] and buffering with bicarbonate prevented acidosis and reduced the formation of spontaneous metastasis.[104] Accordingly, Mueller-Klieser and colleagues have nicely shown that high-lactate levels in the primary lesion of human tumors correlate with the incidence of distant metastases.[87,88] Therefore, the glycolytic phenotype of tumor cells appears to facilitate tumor invasion and metastatic spread.

Another integral factor of sustained tumor growth and metastasis is angiogenesis. Tumor-derived lactate can induce vessel formation through stimulation of VEGF production by endothelial cells.[105] Vegran et al[106] have shown, that endothelial cells take up lactate through MCT1, resulting in nuclear factor κB activation, expression of IL-8, induction of IL-8-dependent angiogenesis and tumor growth. In addition to the generally accepted mechanism of tumor vascularization through sprouting of endothelial cells from pre-existing vessels, some studies suggest a contribution of stem cell-derived endothelial progenitors as well as cells from the myeloid lineage. We found that incubation of tumor-associated DCs with pro-angiogenic factors, such as vascular endothelial growth factor and oncostatin M, led to trans-differentiation of DCs into endothelial-like cells.[107]

5 | IMMUNOLOGICAL CONSEQUENCES OF THE WARBURG EFFECT—A METABOLIC IMMUNE CHECKPOINT

Several publications underline the importance of immune cell infiltration for patient outcome. Galon and colleagues suggested in 2006 that the type, density, and location of immune cells within colorectal tumor samples are a better predictor of patient survival than classical histopathological methods.[108] We and others have shown that tumor-derived lactate strongly inhibits both T cell and NK cell

function[14,84,109] and the differentiation and activation of myeloid cells.[110,111] This indicates that the tumor-promoting effect of lactate and acidification may in part be related to its immunosuppressive function and the metabolic phenotype of tumors may be decisive for T cell activity and thereby for patient prognosis.

Myeloid cells such as MDSCs and TAMs are regarded as important regulators of the tumor environment and perform key homeostatic functions that allow tumor maintenance and growth. Concordant with the hypothesis that lactate modulates myeloid effector functions, Husain et al[112] described a decrease in the frequency of MDSCs in the spleens of mice carrying Ldh-a depleted tumors indicating that lactate promotes MDSC differentiation. Furthermore, Shime et al[113] demonstrated that lactate increases the transcription and secretion of IL-23, a tumor-promoting cytokine involved in the generation of Th17 cells, in human monocytes/macrophages. In addition, acidification of the tumor microenvironment can be sensed by TAMs via GPR43 resulting in macrophage polarization toward a tumor-promoting phenotype.[114] Interestingly, TAMs are not only a target of the tumor-induced lactate acidosis but are also important producers of lactate. Seth and colleagues showed that TAMs express LDH-A, secrete lactate in the tumor microenvironment and myeloid specific deletion of LDH-A supported T cell anti-tumor response and reduced tumor growth.[17] Yet another player might contribute to tumor lactate acidosis, as it has been demonstrated that glycolytic CAFs release lactate which reduced the percentage of the anti-tumoral Th1 subset, and increased regulatory T cells (Treg) cells in a prostate cancer model, indicating an important immunosuppressive role of the reverse Warburg effect.[115]

Therefore, targeting the Warburg effect in cancer and stromal cells, such as via LDH-A inhibition could be an effective strategy to reactivate the adaptive anti-tumor immune response.[17] It is widely established that tumor-derived lactic acid and acidification impair T and NK cell function.[14,84,112,116,117] NK cells from Ldha-depleted tumors show improved cytolytic function and lactate treatment of NK cells inhibits cytolytic function in vitro. Furthermore, LDH-A expression in melanoma biopsies correlated with T cell activity and LDH-A associated lactic acid production and acidification impaired IFNγ expression in tumor-infiltrating T cells and NK cells, thereby inhibiting tumor immunosurveillance and promoting tumor growth.[84] A relation of the tumor metabolic status with an anti-tumor T cell response has been observed in several entities and prominent examples are summarized in Table 1.

But what is the underlying mechanism for the profound impairment of immune cell function through lactic acidosis? Stimulation of monocytes and lymphoid cells results in accelerated glycolysis which is crucial for their function. Glucose transporters, glycolytic enzymes, and lactate transporters such as MCT1 and MCT4 are upregulated to support glycolytic activity in immune cells.[36,39,118-120] To assure the continuation of glycolysis, protons and lactate molecules have to be exported by MCTs following a concentration gradient. An inauspicious lactic acid gradient between the extracellular tumor milieu and the cytoplasm of immune cells leads to uptake of lactate and protons in immune cells, lowering the intracellular pH and limiting the glycolytic flux which results in impaired monocyte and T cell function.[14,40,121] On the molecular level, treatment of T cells with lactic acid prevented TCR-triggered phosphorylation of JNK, c-Jun, p38, and NFAT activation and lowered ATP levels causing a metabolic catastrophe.[84,122] A very recent study in tumor cell lines and murine macrophages shows that intracellular lactate, either endogenously produced or taken up, can directly bind to histone lysine residues and trigger epigenetic modifications that guide macrophage polarization.[123] This study raises the question, whether lactate has yet unknown direct effects on gene expression or post-translational modifications of proteins. Besides lactate production and acidification, accelerated glucose metabolism of malignant and stromal cells can result in a nutrient competition in the tumor environment.

TABLE 1 The relation of tumor glucose metabolism and anti-tumor T cell response

	Metabolic feature			
Tumor entity	**High tumor glucose uptake/ metabolism (MeTS 3/4)**	**High tumor-stroma glucose metabolism (MeTS 2)**	**OXPHOS-dominant tumor (MeTS 1)**	**Ref.**
Melanoma	Decreased T cell activity, inferior response to checkpoint blockade		Good response to checkpoint blockade[a]	84,119,173,175
Renal cell carcinoma	Lower T cell infiltration, inferior response to checkpoint blockade			24,172
Colon cancer	Lower CD8 T cell infiltration			25
Head and neck squamous cell carcinoma	Lower CD8 T cell infiltration			26
Lung squamous cell carcinoma	Lower CD8 T cell infiltration			26
Lung adenocarcinoma		Lower T cell infiltration		55
Lung carcinoma		Impaired anti-tumor T cell response		17
Prostate cancer		Reduced anti-tumoral Th1 cells		115

[a]A contradictory finding by Najjar et al[174] who found high tumor OXPHOS as a barrier to checkpoint blockade.

A metabolic interplay between tumors and immune cells has been demonstrated in murine models.[34,124] These studies suggested that a glucose poor environment limits glycolysis of tumor-infiltrating T cells which suppresses effector functions. In contrast, Tregs seem to be more resistant to the metabolic challenges in the tumor environment. Angelin et al[125] reported that Tregs can maintain their immunosuppressive function even in low-glucose, high-lactate environments based on their metabolic status and reduced glycolytic activity. The transcription factor Foxp3 downregulates MYC and glycolysis and induces oxidative phosphorylation. In addition, enforced glycolysis decreases the immunosuppressive function of Tregs and conversely, expression of Foxp3 opposes mTORC1 mediated metabolic switch toward glycolysis.[44] These metabolic adaptations in Tregs could be a physiological mechanism of peripheral tolerance in tissues with low-glucose and high-lactate concentrations, such as the intestinal tract or ischemic tissues. These sites require a certain level of immune tolerance, avoiding unwanted reactions against self-antigens or commensal bacteria. Even though glucose levels are decreased in multiple tumor types analyzed up to now, the average glucose level rarely fall below 1 mmol/L.[84,126] Intriguingly, these levels will most likely not limit key T cell effector functions.[127-129] In rectal carcinomas and ovarian peritoneal metastases, however, Walenta et al[126] reported glucose levels below 1 mmol/L in over 65% and 40%, respectively, of all tumors, indicating that specifically in these tumor entities nutrient competition may play an important role.

In conclusion, these data clearly demonstrate that tumor-derived lactate and acidification have a broad spectrum of effects and regulate tumor growth and metastasis at different levels, for example, via promotion of cell motility and evasion, modulation of tumor-associated stromal fibroblasts, stimulation of endothelial cells and suppression of anti-tumor immune cells.

6 | LACTATE ACCUMULATION AND ACIDIFICATION—FUNDAMENTAL IMMUNOSUPPRESSIVE STRATEGIES

Apart from their role in the tumor environment, lactate accumulation and acidification are evolutionary conserved phenomena that occur in localized inflammation. It has been known for decades that the synovial fluid of patients with arthritis contains high-lactate concentrations and that synovial fluid acidosis positively correlates with joint destruction.[130] Gobelet et al described lactate concentrations up to 23 mmol/L (a more than 20-fold increase compared to serum) in non-gonococcal septic arthritis. Lactate levels in synovial fluid from patients with a chronic inflammation, such as rheumatoid arthritis (RA) were around 6 mmol/L in this study.[131] Three major factors have been proposed to induce a local lactic acidosis in inflamed tissues: (a) tissue hypoxia through damaged small blood vessels, (b) increased metabolic activity of leukocytes, and (c) the accumulation of short-chain fatty acids produced by bacteria.[132,133] Moreover, high LDH activity has been described in the synovial fluid of RA patients with a shift from LDH1 and LDH2 isoforms to the glycolysis-associated isoforms LDH4 and LDH5.[134] Lactate is then taken up via the sodium-lactate transporters in T cells thereby leading to entrapment and functional changes that drive chronic inflammation.[135] Lactate uptake also results in increased IL-17 production via PKM2/STAT3 signaling and enhanced fatty acid synthesis.[136] Interestingly, the presence of synovial lactate has also been proposed as a fast clinical diagnostic tool to identify patients with septic arthritis.[137,138]

Long known and widely accepted, the acidification of the skin represents a pillar of its barrier function. It has been observed that low pH of the skin regulates its permeability, improves the integrity and cohesion of stratum corneum (SC), and increases anti-microbial defenses.[139] Interestingly, Hatano et al reported that acidification inhibits infiltration of Th2 cells into the skin. Furthermore, low pH prevents the production of IL-1α and TNF, indicating immunosuppression.[140] These observations lead to preclinical and clinical trials that aimed to lower skin pH and induced acidification of SC was able to delay progression of atopic dermatitis.[141] Furthermore, systemic acidosis can affect the inflammatory milieu of the skin; Tzeng et al[142] induced systemic acidosis through hypercapnia, leading to an improved survival of skin allografts in mice. This was mediated through a reduction of pro-inflammatory cytokines, decreased immune cell infiltration and NF-kB activation.

Thus, lactate accumulation and local lactic acidosis are evolutionary conserved mechanisms that both prevent an infection and accompany the inflammation. High lactate and low pH in inflamed tissues might be beneficial, such as through being a natural "antiseptic" or through a confinement of T cells at the inflammatory site, thus supporting a clearance of the pathogen. At the same time, they might do harm by inhibiting cytolytic function of CD8 T cells or inducing a Th17 phenotype in CD4 T cells.

7 | TARGETING THE WARBURG EFFECT FOR TUMOR THERAPY AND IMMUNE ACTIVATION

It appears that tumors exploit metabolic pathways that are universal and can be beneficial for the resolution of inflammation in a non-malignant setting, as discussed above. The dramatic increase in glycolytic activity in MeTS4 tumors as well as their dependency on the Warburg effect appears to provide an ideal target for therapy. Even though a direct metabolic modulation of tumors is challenging, several preclinical and clinical studies addressed the metabolic vulnerability of tumors and identified promising targets.

Highly accelerated glycolytic activity was regarded a unique feature of tumor cells for a long time, but strategies to target the Warburg phenotype were sparse. Beside some early trials administering the glucose analogue 2-deoxy-glucose to patients,[143,144] no major efforts were made. The situation, however, has changed and in the meantime there is a myriad of drugs available either to block the glycolytic pathway itself or to reverse acidity in the tumor microenvironment, extensively reviewed by Feichtinger

et al.[145] Among those drugs, 3-bromo-pyruvate, inhibiting glyceraldehyde 3-phosphate dehydrogenase,[146] gained great attention due to convincing results in preclinical studies, but enthusiasm was dampened as specificity and safety concerns were raised.[147] One of the most common structures to target the Warburg effect is the LDH-A. More than 1000 FDA approved drugs against LDH-A are now available; however, only a few of them are tested in preclinical or clinical trials. Some of them lack specificity, show side effects (reviewed in [148]), or their clinical use is hampered by a low potency. Some examples include gossypol,[149-151] its analogue FX11,[152] oxamate or quinolone-3-sulfonamide.[153] Others showed promising results in preclinical studies, but are not yet tested in clinical trials, such as the pyrazol-based inhibitor GNE-140.[154,155] Surprisingly, recent studies show anti-glycolytic properties of vitamin C.[156,157] Nevertheless, targeting LDH-A alone might not be sufficient to significantly reduce lactate secretion in certain cancers as only a double knock out of both isoforms LDH-A and LDH-B resulted in a complete block in lactate secretion.[158] Finally, drugs targeting MCT1 and MCT4 are currently investigated in preclinical studies[159,160] with the MCT1 inhibitor AZD3956 being tested in a clinical trial (NCT01791595). Beside these more specific inhibitors, the well-known non-steroidal anti-inflammatory drug diclofenac and its derivative lumiracoxib block both MCT1 and MCT4[119,161] and reduce lactate secretion. In addition, immune-modulatory drugs such as lenalidomide act on these transporters by disrupting the MCT-CD147 axis, which is essential for their membrane expression.[162]

A highly glycolytic tumor metabolism is also associated with resistance to conventional therapies. Such Warburg effect-mediated therapy resistance were observed with the proteasome inhibitor bortezomib in multiple myeloma cells, carboplatin in non-small-cell lung[163] and paclitaxel in lung cancer cells.[164] Furthermore, a study by Zhao et al revealed that heat shock factor 1 and LDH-A drive glycolysis and induce resistance to trastuzumab, an anti-HER2 receptor antibody in breast cancer cells.[165] Mechanisms of how tumor glycolysis mediates therapy resistance are not completely elucidated, but this phenomenon has repeatedly been linked to the activity of P-glycoprotein (P-gp), which actively pumps cytotoxic drugs such as doxorubicin and paclitaxel out of the cell and the activity of P-gp increases in hypoxia and acidosis.[166] Another mechanism how tumors benefit from acidosis, and which is frequently neglected, is the "ion trapping"—a process where charged compounds such as chemotherapeutics cannot pass a cellular membrane due to decreased permeability.[167] Therefore, a combination of anti-glycolytic therapy and acidification reversal might strengthen the therapeutic outcome and this approach is being currently tested in clinical trials (NCT01748500, NCT01069081, NCT01163903). Whether using conventional or experimental approaches to target cancer cells, tumor stroma should not be neglected; in several entities, the role of stromal cells, such as CAFs, has been established in therapy resistance. Several approaches have been proposed to target CAFs. For example, pharmacological targeting by sibrotuzumab and pirfenidonen was able to prevent CAFs activation and thus tumor growth.[168,169] Other strategies for stromal cell targeting were summarized by Dykes et al.[170]

Given that the accumulation of lactate and acidification block the anti-tumor function of T and NK cells and foster the differentiation and activity of immune cell populations supporting tumor growth such as Tregs or MDSCs, the Warburg effect limits the success of immunotherapeutic approaches. In line, the efficacy of adoptive T cell transfer can be limited due to increased tumor glycolytic activity.[171] Furthermore, first studies in humans show a correlation between a high glycolytic activity in tumors and a low response rate to checkpoint blockade.[119,172] Accordingly, good response to immunotherapy was associated with enriched mitochondrial metabolism in melanoma patients.[173] Surprisingly, the opposite finding was reported regarding PD-1 blockade in a melanoma study showing that tumor oxygen consumption was associated with T cell exhaustion and progression under checkpoint blockade.[174] Nevertheless, the efficacy of checkpoint blockade was improved by LDH suppression in murine tumors.[119,175,176] Glycolytic tumor stroma (equivalent to MeTS2a) might drive cancer progression even in the presence of non-glycolytic tumor cells (Figure 1). In line with the work of Seth et al[17] inhibition of pyruvate dehydrogenase kinase by dichloroacetate targeted macrophages and decreased T cell and NK cell suppression in tumors.[177]

As the Warburg phenotype is not specific for tumor cells or tumor-promoting stromal cells, but a common feature of proliferating and activated immune cells, such as effector T cells and NK cells,[34,35,37,38,178,179] the application of glycolytic inhibitors could exert side effects on the immune system. Buffering, therefore, might be an alternative strategy to reduce the negative effects of tumor acidification not interfering with immune cell activation.[104,180] Accordingly, different buffering approaches such as the administration of bicarbonate or proton pump inhibitors (PPIs) promoted immunotherapy.[181-183] Moreover, Vishvakarma and collegues showed that the application of PPIs resulted in an enhanced recruitment of M1 macrophages and shifted the cytokine profile toward tumor cytotoxic cytokines. Finally, this study showed that macrophages isolated from PPI treated tumors, which were adoptively transferred in tumor-bearing mice, showed superior capacity to control tumor growth.[184]

Glycolytic restriction might not affect all immune cell populations to the same extent; it has been shown that under low-glucose conditions, T cells show a remarkable flexibility and while proliferation decreases, effector functions are preserved.[127-129,185] Therefore, glycolytic inhibitors might be beneficial even in a tumor setting. In line, lenalidomide has been shown to promote IL-2 expression in T cells.[186] Notably, the response to adoptive T cell transfer was improved by the application of the LDH-A inhibitor GNE-140.[171] Finally, we investigated the effect of diclofenac and lumiracoxib in vitro and in vivo. Lowering glycolytic activity reduced proliferation of T cells but effector functions were not altered and NSAIDs supported checkpoint inhibition in vitro and in vivo. Furthermore, it has been proposed by different studies that lowering glycolytic activity but supporting mitochondrial fitness

and enhancing fatty acid metabolism in T cells might strengthen their anti-tumor immune response.[187-191] This view is further supported by a recent study of Ma et al showing that in contrast to in vitro activated T cells which display hallmarks of Warburg metabolism, physiologically activated CD8 T cells displayed greater rates of oxidative metabolism in vivo, as assessed by ^{13}C-glucose infusion techniques in an infection model.[192]

8 | CONCLUSION AND OUTLOOK

Almost 100 years after its first description, many new tumor-promoting aspects of the Warburg effect have been discovered beside its classical role in energy generation. The "waste products" lactate and acidification have profound impact on tumor angiogenesis, migration and last but not least on the anti-tumor immune response. Importantly, not only tumor cells but also activated stromal cells such as fibroblasts and macrophages can produce lactate in the so called "Reverse Warburg effect," contributing to tumor lactic acidosis and shaping the immune response even in tumors with oxidative metabolism in malignant cells. Therefore, the immunosuppressive microenvironment does not solely depend on the metabolic phenotype of tumor cells but also on the composition and activation status of stromal cells. Based on the important role of T cell infiltration for patient outcome, we suggest a metabolic-tumor-stroma score (MeTS) to determine the likelihood of a successful anti-tumor immune response. We define four major metabolic tumor types (Figure 1): (MeTS1) OXPHOS tumors, (MeTS2) a reverse Warburg type with OXPHOS in tumor cells but high glycolysis in stromal cells, (MeTS3) a mixed type with glycolysis and OXPHOS, and (MeTS4) a highly glycolytic Warburg type. MeTS1 tumors may show the best response to immunotherapy without further intervention, MeTS2 tumors could benefit from stroma-targeted approaches, while in MeTS3-MeTS4 tumors with increasing levels of lactate and acidification, targeting glycolysis and/or stromal cells might be essential to allow an effective immune response, which is of special importance in the context of cancer immunotherapy.

CONFLICT OF INTEREST

None.

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