Targeting tumor-associated acidity in cancer immunotherapy

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REVIEW

Targeting tumor-associated acidity in cancer immunotherapy

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Abstract

Checkpoint inhibitors, such as cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) and programmed cell death-1 (PD-1) monoclonal antibodies have changed profoundly the treatment of melanoma, renal cell carcinoma, non-small cell lung cancer, Hodgkin lymphoma, and bladder cancer. Currently, they are tested in various tumor entities as monotherapy or in combination with chemotherapies or targeted therapies. However, only a subgroup of patients benefit from checkpoint blockade (combinations). This raises the question, which all mechanisms inhibit T cell function in the tumor environment, restricting the efficacy of these immunotherapeutic approaches. Serum activity of lactate dehydrogenase, likely reflecting the glycolytic activity of the tumor cells and thus acidity within the tumor microenvironment, turned out to be one of the strongest markers predicting response to checkpoint inhibition. In this review, we discuss the impact of tumor-associated acidity on the efficacy of T cell-mediated cancer immunotherapy and possible approaches to break this barrier.

Keywords Cancer · Immune therapy · Checkpoint blockade · Acidity · Lactic acid · Metabolism

Abbreviations

CA	Carbonic anhydrase
CEST-MRI	Chemical exchange saturation transfer-MRI
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DCA	Dichloroacetate
DNP-MRSI	Dynamic nuclear polarization-MRSI
MCT	Monocarboxylate transporter
NHE	Sodium proton exchanger
NKT cell	Natural killer T cell
PET	Positron emission tomography
PPI	Proton pump inhibitor
TME	Tumor microenvironment
T_{reg}	Regulatory T cells
V-ATPase	Vacuolar-type H^+-ATPase

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Introduction

Cancer is a relentless disease capable of adapting to a multitude of therapies; therefore, we are urgently in need of novel treatment strategies. One angle is to exploit the power of the immune system. Its role in tumor control was already proposed over a century ago [1]. Yet, findings from the last decades conclusively show its involvement in tumor control, with the discovery of neo-antigen specific immune cells in patients cementing its importance [2]. However, tumors use a myriad of strategies to circumvent immune pressure; accordingly, "avoiding immune destruction" is acknowledged as a hallmark of cancer [3]. Currently, the most successful immunotherapeutic strategy against cancer is to target immune checkpoints. These are "switches" that can either promote or inhibit the activity of immune cells. Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), an inhibitory surface receptor, was first therapeutically exploited. A monoclonal antibody against this protein (Ipilimumab) changed profoundly systemic treatment of late stage melanoma, leading to long-term survival in a portion of patients [4]. Targeting the Programmed Cell Death Protein (PD-1)/PD-1 ligand 1 (PD-L1) pathway with monoclonal antibodies (nivolumab, pembrolizumab) further enhanced prognosis for melanoma patients [5–7]. First trials combining PD-1 and CTLA-4 blockade revealed additional clinical benefit [8–10]. The success of immune checkpoint targeting

in melanoma is being translated to other tumor types such as non-small cell lung cancer, renal cell carcinoma, bladder cancer and breast cancer with varying success [11–15].

Still, immunotherapy is successful only in a subset of patients. This is due to the many mechanisms that tumors adopt to blunt anti-tumor immunity. These can be tumor cell intrinsic such as loss of antigen presentation or impaired responsiveness to interferons (IFN) [16–18]. While others are imposed by the tumor microenvironment (TME) such as stromal barriers [19], insufficient vascularization [20], hypoxia [21], nutrient shortage [22, 23], and lactate accumulation and concomitant acidification [24].

Acidification of the TME has been shown to promote angiogenesis [25–28], invasion [29–33] and metastasis [34–38]. Not surprisingly, tumor acidity is strongly linked to a poor prognosis [39–44]. Furthermore, acidity is associated with resistance to chemotherapy [45–48], radiation therapy [49, 50], and reduced survival after surgery [51, 52]. More recently, it has been recognized that the acidic nature of the tumor limits the efficacy of checkpoint therapies [53–55]. Thus, it is likely that the low intra-tumoral pH also downregulates anti-tumor immune responses. Median extracellular pH inside patients' tumors is between 6.9 and 7.0 (compared to 7.3–7.4 in normal tissue) [56]. At the same time, intracellular tumor pH remains unaffected [57]. In murine cancer models, the extracellular pH is reported to be even lower (6.2–6.9) [58]. Acidity is a consequence of tumor metabolism. It predominately originates from the fermentation of glucose to lactate, which is secreted in co-transport with a proton, below referred to as lactic acid [59–61]. Hypoxia as a consequence of inadequate vascularization can force tumor cells to anaerobic glycolysis. However, hypoxia and acidity have been established as independent parameters [62, 63]. Aerobic glycolysis appears to be favored also by oxygenated tumor cells. This metabolic preference, the so-called "Warburg effect", is named after Otto Warburg, the first scientist who observed aberrant glucose metabolism in tumors [64]. Glycolysis is hypothesized to rapidly supply both the energy and the carbon source obligate for proliferation and adaptation to an ever-changing tumor microenvironment [65–67]. Tumor cells were shown to import exogenous lactate. Moreover, inhibiting lactate uptake debilitated tumor growth, testifying to the importance of this metabolic reprogramming [68–71]. Lactate uptake by tumor cells was recently demonstrated in vivo in patients with lung cancer [72]. Oncogenic mutations are at the root of this metabolic inclination [73–79]. As aerobic glycolysis is considerably less energy efficient compared to oxidative phosphorylation, an accelerated glycolytic flux is required. This necessitates a high rate of glucose uptake and lactic acid secretion that can be up to 30-fold the rate found in healthy tissues [80]. As a consequence, lactic acid accumulates inside the TME (mean 6.0 mM for non-metastatic lesions; 12.5 mM for metastatic lesions [44]) resulting in a low pH. Recently, the combination of 2-deoxy-2-[fluorine-18]fluoro-D-glucose Positron emission tomography (^{18}F-FDG PET) and acid-sensitive MRI was used to elegantly demonstrate the overlap between areas of high glucose uptake and areas of acidity [81]. Still, glycolysis deficient tumors were, despite lacking lactic acid production, able to acidify their surroundings [82–84]. An alternative source of acidity is CO_2 production by oxidative phosphorylation. The importance of the export of this molecule is indicated by reduced tumor growth when membrane carbonic anhydrases or bicarbonate transporters were inhibited [85, 86]. Considering both, the physical and the financial toxicities of checkpoint modulating therapies, one might want to exclude patients harboring acidic tumors or pre-treat them with anti-acidic therapies to ensure efficacy. Therefore, it is imperative to be able to diagnose tumor acidity. Here, we will discuss methods of establishing tumor acidity, followed by reviewing negative effects of acidity on the anti-tumor immune response and outcome of immune-based therapies. Finally, we outline therapeutic options that can counteract acidity and could promote the efficacy of immune therapies.

Techniques to diagnose tumor acidity

Tumor acidity can be characterized in situ by technical adaptations of magnetic resonance imaging (MRI) and positron emission tomography (PET). Chemical exchange saturation transfer MRI (CEST-MRI) is a highly sensitive technique based on saturation of exchangeable protons on a molecule of interest at a frequency different from water protons. A detectable signal is generated when a proton on the molecule is transferred to the surrounding water. The molecule of interest can be one of the clinically approved pH-sensitive agents to allow for pH measurement. Murine studies revealed the potency of CEST-MRI. For example, tumor pH was mapped with ultra-high resolution (< 0.6 mm^2) in a broad spectrum of murine models [81, 87–92] and confirmed by pH electrode measurements [90]. CEST-MRI makes use of clinical grade equipment, facilitating its introduction into the clinic. A substantial step was recently taken when CEST-MRI was used in a clinical setting to accurately determine the pH of urine inside the bladder of a human volunteer [93]. In addition, PET can be modified to approximate tumor acidity. Advantages over MR-based methods are the high signal-to-noise ratio and the wide availability. One modification that allows PET to be used for pH estimation utilizes ^{18}F-FDG derivatives that can only be transported inside the cell under acidic conditions [94]. Another adaptation makes use of radiolabeled peptides that preferentially insert in cell membranes in acidic environments. The latter method was used to map acidity in a variety of murine tumors [95, 96].

Alternative methods for determining tumor acidity focus on the detection of lactate as it is a reliable surrogate for acidity [59–61]. Lactate can be detected in cryopreserved tumor material with enzymatic assays [97]. Additionally, bioluminescence imaging can be employed to visualize lactate distribution [98]. However, the latter technique necessitates skill and specialized equipment. Implementation of enzymatic assays into clinical routine is further complicated by the requirement to freeze samples instantly after the biopsy is taken. In addition, tumors are known to be heterogeneous, and as a result the probed area may not be representative of the whole tumor. To overcome these problems, tumor-wide lactate can be detected in situ with MRI. Quantification of lactate with 1^{H}-MRI has been tested in patients with brain malignancies [99, 100], revealing that lactate is a marker of progression, inversely correlated with response to a combination of radiotherapy, temozolomide and enzastaurin [101, 102]. A limitation of 1^{H}-MRI is the low specificity for lactate, preventing its use in tumors originating from tissues other than the brain [103, 104]. CEST-MRI grants detection of lactate with improved sensitivity [105]. As a proof of concept, CEST-MRI was recently used to detect lactate in muscle tissue of human volunteers [106]. Dynamic nuclear polarization-MRSI (DNP- MRSI) is a different MRI technique that can be used to quantify lactate. It derives its sensitivity from the injection of substrates with hyperpolarized nuclear spins. An advantage of using DNP-MRSI is that the faith of the labeled substrates can be followed.

Lactate measurements obtained with this method could be used to monitor development, progression, and response to therapy in murine models of cancer [107–114]. Currently, the technique is being prepared for entry into clinical routine [115]. A major hurdle was taken with the development of hyperpolarization equipment compatible with clinically available MRI scanners [116, 117]. Additionally, a recent study with the aim of detecting lactate levels in prostate cancer patients demonstrated safety, feasibility and sensitivity of DNP-MRSI [118]. Alternatively, lactate dehydrogenase (LDH), the enzyme responsible for the conversion of pyruvate to lactate, can be taken as a surrogate for the presence of lactate. LDH can be detected with immunohistochemistry if there is a biopsy available [119]. Due to accessibility it is more common in the clinic to measure the activity of LDH in patient blood. Studies indicate a correlation between serum and tumor LDH [120–123]. It is generally assumed that tumor LDH corresponds to intra-tumor lactate levels, yet, only anecdotal evidence exists for this [36, 124, 125]. On the other hand, serum LDH is an indicator of cell death [126, 127]. Therefore, it is conceivable that LDH release reflects tumor necrosis, which is expected to be higher in larger tumors [128]. However, recent evidence showed that there is no correlation between tumor burden and serum LDH [129, 130]. Notwithstanding, both tumor lactate and blood LDH activity share prognostic value in cancer [37, 43, 44, 131].

Which of these techniques should be used? Lactate detection in biopsies or LDH activity in the serum is both good starting points for a basic understanding of tumor acidity. Yet, more insight can be gained using advanced techniques. DNP-MRSI can be employed to measure lactate and other metabolites to monitor tumor progression or response to anti-glycolytic drugs. However, this method requires introduction of new machinery into the clinic. On the other hand, pH measurements with CEST-MRI, a technique which relies on clinically available resources and contrast agents, could be used to select patients for anti-acidic drugs in the near future.

Tumor acidity as a predictive and prognostic marker for IT

Analysis of tumor acidity in patients treated with checkpoint inhibitors has not yet been established in clinical research. All studies, so far, are restricted to analyzing LDH activity in peripheral blood. In melanoma, LDH activity is an established prognostic marker for survival and is embedded in the AJCC-staging criteria [132]. Our group was one of the first showing that high LDH activity in peripheral blood correlated strongly with a negative outcome (in terms of objective response rate, progression free survival and overall survival) upon immunotherapy with ipilimumab in advanced melanoma patients [133]. The same was found later for melanoma patients treated with pembrolizumab [55], and for the combination of CTLA-4 and PD-1 blockade [134]. Furthermore, LDH correlates with outcome upon CTLA-4 and PD-1 blockade in uveal melanoma [135] and PD-1 blockade in non-small cell lung cancer [136]. Interestingly, high LDH activity in melanoma patients also impairs progression free survival and overall survival upon chemotherapy, and single BRAF or combined BRAF + MEK inhibition [129, 137, 138]. In contrast to checkpoint inhibitors, patients with elevated LDH activity initially do respond to BRAF + MEK inhibition [138]. Moreover, targeted therapy rapidly lowers LDH levels [139] and increased.

T-cell infiltration into the tumor [140–142]. Based on these data LDH has been incorporated into the Cancer Immunogram, a concept summarizing the requirements for an efficient anti-tumor immune response [143]. Thus, targeted therapy might be a promising combination partner for checkpoint inhibition by lowering tumor acidity, which is currently tested in several clinical trials (NCT02968303, NCT02631447, NTC02902029).

Effects of acidity on the immune system

Tumor acidity and high LDH activity have been established as negative prognostic factors long ago [39–44]. Yet, only recently, studies have been uncovering the effects of acidity on blunting the anti-tumor immune response. T cells are thought to be crucial for effecting the anti-tumor immune response. Their importance is reflected by the prognostic power of CD8+ T cell infiltration in patients [144–146]. Furthermore, increased frequency of tumor infiltrating T cells (TIL) is associated with improved response to immune-based therapies [147]. One of the earliest studies showing the effect of low pH on T cells in vitro was performed by Bosticardo et al. in 2001. Their pioneering work showed that at pH 6.6, which is close to the physiological range of tumors [56], CD3+ T cell proliferation, cytokine production and cytotoxicity were impaired [148]. Subsequent studies which explored both CD4+ and CD8+ T cell function in medium acidified with either lactic acid or hydrochloric acid confirmed these original findings [24, 149–152].

These studies suggest that the effects of lactic acid are a consequence of the concomitant acidification rather than of the lactate molecule itself. This claim is further supported by the fact that buffering lactic acid to neutral pH abrogates its negative effects [24, 150]. Moreover, equal concentrations of sodium lactate had no negative impact on CD8+ T cells [24, 149]. Interestingly, subsequent culturing in fresh medium for 24 h reversed functional impairment [24, 149–152]. Whether reduced CD3+ T cell function is a consequence of increased cell death is under discussion. Several works argue that survival is initially unaffected [149, 151, 152, Lacroix and Blank, unpublished], but reduced after long-time culture in acidic medium [24, 153]. Comparable to T cells, natural killer (NK) and natural killer T (NKT) cells displayed reduced function when cultured at low pH in vitro [153–156]. Impaired viability of NK cells cultured in lactic acid was also reported [153]. The effects of lactic acid on lymphocytes are increasingly studied in physiological settings. This line of research started with Dröge et al. who showed in the late eighties that systemic injection of lactate did not impair priming of lymphocytes [157]. Our group (Kreutz lab) was one of the first focusing on the consequences on distinct immune subsets. We demonstrated, by applying an inhibitor of LDH-A in a tumor-T cell co culture, that tumor-derived lactic acid can inhibit CD8+ T cell function [24]. Subsequently, we demonstrated the negative impact of tumor-derived lactic acid on anti-tumor immunity in vivo [153]. LDH-A deficient tumors grew markedly slower than control tumors in immunocompetent mice, but not in mice lacking T and NK cells. Reduced interferon gamma (IFNγ) and Granzyme B production by NK cells and both CD4+ and CD8+ T cells were identified as mechanisms of the adverse effect of lactic acid. In line with these data, increased NK cell activity and extended survival were observed in a murine B cell lymphoma model when tumor acidity was counteracted with sodium bicarbonate [158]. Expression of LDH-A in human melanomas negatively correlates with survival and expression of T cell activation markers, suggesting the relevance of these findings for immunotherapy in humans [153]. In contrast to conventional T cells, regulatory T cells (T_{reg}) thrive in high lactate environments. The master T_{reg} transcription factor forkhead box P3 (FoxP3) skews cellular metabolism away from glycolysis towards oxidative phosphorylation, negating the need to export lactate for their function [159]. Vice versa, inhibition of T cell glycolysis, as is proposed to be a consequence of lactic acid, leads to FoxP3 expression and induction of regulatory T cells [160–162]. Recent findings propose that lactate uptake might even be essential for immune suppression by T_{reg} [163]. Adaptation of T_{reg} to function in high concentrations of lactic acid could be understood from the fact that tumors and sites of trauma share the abundance of this molecule.

Monocytes and macrophages produced less pro-inflammatory cytokines when cultured in the presence of lactic acid [164–166], instead factors that promote tumor progression were being produced such as interleukin-17 (IL-17), interleukin 23 (IL-23), arginase (ARG1) and vascular endothelial growth factor (VEGF) [167–175]. Furthermore, priming of T cells was impaired in vitro [170]. No cell death was reported in vitro. The consequences of lactic acid on myeloid cells in vivo are subjected to increased study. Lactate prevented inflammation in a murine colitis model [176], presumably partly due to its anti-inflammatory effect on Macrophages [177]. Lewis lung carcinomas developed more rapidly in immunocompetent mice when cells were co-injected with macrophages cultured in medium with lactic acid [169]. This experiment showed for the first time that lactic acid can skew macrophages towards a *bona fide* pro-tumor phenotype. Lower frequencies of myeloid cells were found in glycolysis deficient tumors [153, 154]. While LDH-A deletion in myeloid cells themselves decreased tumor angiogenesis and boosted levels of intra tumor $PD1^+$ T cells. An explanation for the latter findings might be deduced from the in vitro observation that LDH-A deficient macrophages were more readily skewed towards an anti-tumor phenotype [178]. It was recently found in biopsies from patients with head and neck cancers that lactic acid was correlated with the levels of pro-tumor ($CD163^+$) macrophages [97]. Altogether, these data firmly establish the importance of lactic acid in skewing macrophages towards a pro-tumor role.

In dendritic cells (DC), lactic acid impaired induction of monocyte-derived DCs [179], cytokine production and priming of T cells [177, 180, 181]. DCs extracted from murine gliomas were unable to produce interleukin 12 (IL-12) upon Toll-like receptor stimulation ex vivo. Functionality could be restored by treating mice with the glycolytic inhibitor Diclofenac [182]. Interestingly, and in contrast to its many inhibitory effects, DC antigen uptake, processing and expression of costimulatory molecules are reported to be higher in acidic conditions [183, 184].

Altered activity of immune cells in acidic conditions is partially a consequence of impaired metabolism. T cells [185, 186], NK cells [187, 188], monocytes [165], macrophages [166, 189] and DCs [189, 190] increase glycolytic rates after activation to support their function. Conversely, inhibiting glycolysis leads to decreased or alternative functionality of these immune cells [165, 166, 187, 188, 190–192]. Efficient disposal of lactic acid is a prerequisite for sustaining high glycolytic rates [193]. Immune cells unable to export lactic acid displayed decreased functionality [194, 195]. Reduced export of endogenous lactic acid is a consequence of high concentrations of extracellular lactic acid [24, 153, 165]. In line with these findings, reduced (glycolytic) metabolism is observed in immune cells cultured with lactic acid [152, 153, 165, 166]. These sequences of events may act as a common mechanism of metabolic inhibition by lactic acid preceding impaired function. The molecular consequences of lactic acidosis remain to be studied in detail. Lactate was shown to activate extracellular signal–regulated kinases (ERK)/Signal transducer and activator of transcription 3 (STAT3) signaling in bone marrow-derived macrophages [167]. Yet, in T cells no activation of ERK signaling was observed. Neither was the phosphorylation of mitogen-activated protein kinase kinase (MEK), Protein kinase B (AKT/PKB) and proteins downstream of the T Cell receptor (TCR) affected. Rather, lactic acid led to a rapid reduction in phosphorylation of mitogen-activated protein kinase 8 (JNK), c-Jun, and p38 MAP kinase [149], proteins that are linked to cytokine production [196]. In contrast to these data, we showed that of the latter proteins only phosphorylation of p38 was reduced. Instead, we showed reduced upregulation of the transcription factor nuclear factor of activated T-cells (NFAT) upon culturing with lactic acid. NFAT is upregulated upon T cell activation and involved in IFNγ signaling [197]. It is well known that acidity downregulates protein synthesis via the mechanistic target of rapamycin (mTOR) pathway [198, 199]. This pathway links metabolism to immune cell function and is a pivotal regulator of the immune response [200, 201], suggesting that acidity could impair immune cell function via mTOR signaling. Indeed, impaired NKT cell functionality as a consequence of acidity was mediated via the mTOR pathway [156]. An overview of the effects of lactic acid on immune cells is given in Table 1.

Anti-acidic interventions combined with immune therapy

Acidity (indirectly measured by LDH levels) is associated with poor response to immune-based therapies in cancer [55, 133–136]. This might be in part due to the inhibitory effect on immune effectors as described afore. Thus, there is a clear rationale for combining immune therapies with compounds that prevent or counteract acidity. One option is to prevent (lactic) acid from being deposited in the tumor

Table 1 Functional and metabolic consequences of acidity on immune cell populations (OxPhos: oxidative phosphorylation)

Immune population	Functional effects	Metabolic effects	Pathways affected
Tumor cell	↑ PD-L1 [178]		HIF-1a [202]
Effector T cell	↓ Proliferation [153] ↓ Effector cytokines [153] ↓ Cytotoxicity [153] ↑ FoxP3 (induction of T_{reg}) [160–162]	↓ Glycolysis [153]	↓ NFAT [153]
Regulatory T cell	↑ Immune suppression [163]	↓ Glycolysis [159] ↑ OxPhos [159]	↓ Myc [159]
NKT cell	↓ Effector cytokines [156]		↓ mTOR [156]
NK cell	↓ Effector cytokines [153–155] ↑ Apoptosis [153]	↓ metabolism [153]	↓ NFAT [153]
Monocyte	↓ Inflammatory cytokines (CCL2, TNFα) [164, 165] ↑ Pro-tumoral factors (IL-23) [168]	↓ Glycolysis [165]	↓ AKT/NFκB [164]
Macrophage	↓ Inflammatory cytokines (IL-1b, IL-6, IL-12) [166] ↓ T cell priming [170] ↑ Pro-tumoral factors (IL-17, IL-23, ARG1, VEGF) [169]	↓ Glycolysis [166]	↓ ERK/STAT3
Dendritic cell	↓ T cell priming [180, 182] ↑ Pro-tumoral factors (IL-10) [181]		

microenvironment; production and export could be blocked by inhibiting glycolysis or monocarboxylate transporters (MCT's), respectively. A second opportunity is to inhibit transporters used by tumors to transfer excess protons out of the cell such as proton pumps and carbonic anhydrases. Alternatively, systemic alkaline supplementation can buffer tumor acidity. These strategies demonstrated efficacy in combination with conventional anti-cancer treatments in clinical trials. For instance, high-dose omeprazole improved efficacy of chemotherapy in breast cancer [203]. Whereas buffering the tumor with sodium carbonate increased the impact of chemoembolization in hepatocellular carcinoma [204]. A myriad of other combination therapies containing anti-acidic interventions is currently being evaluated in clinical trials (among others: NCT01791595, NCT01748500, NCT01069081, NCT01163903). A concise review covering the current state of de-acidifying drugs in preclinical and clinical studies was recently published by Tomas Koltai [205]. The impact of anti-acidic drugs on immune therapies is currently being subjected to intense study.

Glycolysis inhibitors

Tumor acidity can be prevented by blocking glycolysis. Compounds that inhibit key players in this metabolic pathway are being investigated as anti-tumor drugs. For example, dichloroacetate (DCA) shifts metabolism away from glycolysis, thereby reducing the production of lactic acid and increasing tumor pH [110, 206, 207]. DCA showed potent anti-tumor effects in preclinical studies [208–210]. Opposing to earlier claims made that glycolysis inhibition affects macrophage function; it was shown that DCA rescued lactic acid-induced impairment of priming by murine macrophages in vitro. Furthermore, lower arginase expression was detected inside tumors from mice treated with DCA. Treatment with DCA synergized with poly I:C TLR stimulation to control the growth of subcutaneous tumors. Contrary to expectations, intra-tumor levels of lactic acid did not change upon DCA treatment, suggesting alternative immune potentiating mechanisms of the compound [170]. Altogether these data led to DCA being tested in cancer patients [211]. Exposing T cells to DCA in vitro did not reduce survival and proliferation. However, within human CD4+ T cells, DCA led to induction of regulatory T cells and interleukin 10 (IL-10) production at the expense of IFNγ production [160–162]. Thus, in our view, this molecule might be counterproductive in cancer therapy. The widely used drug diclofenac was recently shown to reduce lactate production by tumor cells in vitro and in vivo [182, 212]. Furthermore, diclofenac generated a more immune permissive environment in murine gliomas characterized by increased DC function and lower levels and activation of T_{reg}. Still, combining diclofenac with an immune potentiating TLR stimulus did not increase survival of glioma-bearing mice [182]. In contrast, diclofenac exerted a positive impact on the response to anti-CTLA-4 and anti-PD1 therapy in vivo in a 4T1 model (Renner et al., data submitted). Yet, direct incubation of murine T cells with diclofenac dose-dependently reduced proliferation and cytokine secretion [182]. A variety of studies show a dependence of murine T cells on glycolysis. For instance, LDH-A knock-out murine $CD4^+$ T cells exhibited reduced cytokine production [213]. However, the importance of glycolysis for effector functions is questioned in human T cells, as effector functions are preserved in low glucose conditions [214, 215]. In our opinion, diclofenac or derivatives deserve further studying in combination with immune based therapies.

Lactate transport inhibitors

Molecules that block the export of lactic acid by tumor cells can prevent tumors from becoming acidic. Export of lactic acid occurs mainly via the monocarboxylate transporters (MCTs). The importance of these proteins for tumor cells is illustrated by accumulation of intracellular lactate and growth reduction in vivo following impairment of MCT or its essential subunit CD147/BASIGIN [193, 216–220]. A MCT-1/MCT-2 inhibitor (AZD3965) is currently tested in phase I clinical trials for advanced solid tumors and diffuse large B cell lymphomas (NCT01791595). One concern might be that MCT inhibitors impair the functionality of T cells. Indeed, intracellular acidification and reduced proliferation was observed in T cells after inhibition of MCT1 and 2 with AR-C155858. Yet interleukin 2 (IL-2) production was conserved [195]. One study showed that anti-PD1 combined with an inhibitor specific for lactate uptake (7-ACC) generated superior tumor control compared to the single agents in the murine B16 melanoma model. Of note, this intervention did not reduce tumor acidity. Instead its efficacy is based on reducing the suppressive capability of T_{reg}'s. Conventional T cells will not be affected by this intervention [163].

There might be a window where MCT blockade reduces T cell proliferation without affecting cytokine production. In that way, lactate transport inhibitors might become effective combination partners of checkpoint inhibition.

Proton transport inhibitors

Besides MCT's, tumor cells use Vacuolar-type H^+-ATPase's (V-ATPase), sodium proton exchangers (NHE's) and carbonic anhydrases (CA's) to dispose of excess protons. Compounds that inhibit V-ATPase's (PPI's) such as esomeprazole and pantoprazole are currently widely used in the clinic for gastric protection. Inhibiting V-ATPase's demonstrated anti-tumor effects in vitro [221–223]. ^{31}P-MRSI in combination with the cell impermeable pH reporter 3-aminopropyl

phosphonate (3-APP) was used to show that a single dose of esomeprazole rapidly increased the pH of engrafted murine (12.5 mg/kg; pH_e 6.5–7) and human (2.5 mg/kg; pH_e 6.55–6.85) melanomas. The increase in pH was maintained for several hours [151, 223]. Esomeprazole was combined with adoptive cell transfer (ACT), a therapy based on transfer of tumor reactive immune cells, in mice carrying B16 melanomas. Addition of the PPI resulted in increased frequency of IFNγ producing TIL's. Of note, both transferred and endogenous T cells profited from the PPI treatment. The combination of ACT and PPI improved survival of tumor-bearing mice [151].

A different series of studies focused on the application of PPI's in T cell lymphoma. The tumor used in these studies strongly suppresses myelopoiesis. Dosing the mice with Pantoprazole rescued myelopoesis and led to increased infiltration of anti-tumor macrophages in the tumor. Notably, a general shift away from immunosuppressive cytokines was observed in the TME, suggesting also other immune populations could benefit from PPI therapy [224, 225]. Although some studies dating back to the late 1990s suggest that direct inhibition of V-ATPase's in T cells might inhibit function and viability [226, 227]. Carbonic anhydrases (CA's) allow tumor cells to dispose of protons. Genetic knock down of these enzymes markedly reduced outgrowth of murine tumors [85, 86]. Acetazolamide is a clinically approved pan-CA inhibitor that showed anti-tumor activity in vitro, but remains to be tested in vivo [228–230]. A different class of proton exporters used by tumors is the sodium proton exchangers (NHE's). The relevance of these transporters for tumor cells is demonstrated by impaired tumor progression after genetic ablation or small molecule inhibition [86, 231–234]. Cariporide is a NHE inhibitor with reported anti-tumor activity in vitro [235, 236]. This inhibitor never made it to the clinic after failed clinical trials for ischemic cardiac events [237]. The current knowledge on inhibitors of proton pumps, carbonic anhydrases and sodium proton exchangers certainly supports further research on these molecules as partners for immune therapies.

Buffer therapies

A straight forward approach to counter tumor acidity is systemic buffering. Sodium bicarbonate is a non-toxic compound widely used to neutralize stomach acid. It was demonstrated in mouse models that bicarbonate increased intra-tumoral pH and suppressed tumor progression [35]. A concise summary of the impact of systemic buffers on tumors was published recently by Faes and Dormond [238]. Currently, the consequences of systemic buffering on immunity are being scrutinized in preclinical studies.

It was shown that raising tissue pH with sodium bicarbonate led to increased function of NK cells and a significant survival benefit in an endogenous model of murine B cell lymphoma. This advantage was abrogated when either NK cells or T cells were depleted, showing that the effects of systemic buffering were immune mediated, and may not be confined to a single immune population [158]. Bicarbonate improved both $CD8^+$ T cell infiltration and tumor control in the $BRAF^{V600E}/PTEN^{ko}/\beta$ catenin-driven Yumm 1.1 melanoma model but not in $BRAF^{wt}$ B16 melanomas. Therefore, the authors combined sodium bicarbonate with immune therapies in B16 melanoma. Neither addition of bicarbonate to anti-CTLA-4 nor to the combination of CTLA-4 and PD-1 blockade led to a significant reduction in tumor size. However, buffering improved tumor control by PD-1 blockade to the level of the dual checkpoint blockade. The anti-PD-1/anti-CTLA-4 combination is approved for the treatment of melanoma, but is accompanied by severe adverse effects. Testing of anti-PD1 in combination with bicarbonate might therefore be clinically relevant. In a subsequent experiment, buffering substances were given together with adoptive T cell transfer. Systemic buffering increased persistence of transferred cells and resulted in an increased percentage of long-term surviving mice in comparison to mice that received adoptive cell transfer alone [152]. Furthermore, buffer therapy increased infiltration of transferred immune cells into the tumor in a xenograft model of hepatocellular carcinoma. Moreover, these TILs stained more positive for the cytotoxic marker perforin. In addtion here, the combination of cell transfer and buffer therapy showed superior tumor control [239].

The question remains if systemic buffering is a clinically applicable strategy. In all studies, mice received drinking water with sodium bicarbonate at a concentration of 200 mM (16.8 g/l), leading to an intake of ~ 3.5 g/kg [35]. Only a single study reported toxicity at this concentration [240]. Murine data was used to simulate the sodium carbonate intake required for a human to achieve similar results. They concluded that a dosing of 1.1–1.7 g/kg was needed, far beyond the 0.5 g/kg that their model deemed save [241]. High bicarbonate intake has been evaluated with athletes to counteract the negative effects of acidosis on performance. The tested doses were between 0.2 and 0.5 g/kg and were maintained for 5–6 days without side effects [242, 243]. A phase I clinical trial was recently completed where the participants received 0.5 g/kg bicarbonate daily for 90 days [NCT02531919], unfortunately no results were published. Side effects of systemic alkalization could be reduced by selecting a buffer substance with a more suitable acid dissociation constant (pK_a) [241, 244]. While the undesirable effects of large sodium intake could be circumvented by choosing a buffer without a counter ion, such as 2-imidazole-1-yl-3-ethoxycarbonylpropionic acid (IEPA) or tris(hydroxymethyl)aminomethane (THAM, Tris). Both molecules prevented cancer metastasis in murine models

[245, 246]. Alternatively, novel means of administration could reduce systemic consequences. For instance, calcium carbonate nanoparticles were synthesized that, when dosed intravenously, release carbonate proportionate to the surrounding acidity, increasing the pH to a maximum of 7.4 [247]. Buffering therapies show promising results in preclinical cancer studies, while reports from other fields demonstrated clinical feasibility. Additionally, novel compounds and ways of administration could make buffering safer and easier. Hence, the combination of buffering agents and immune therapies especially deserves further investigation, having in our view a realistic chance to translate to the clinic. An overview of the discussed anti-acidic strategies is given in Table 1 and graphically represented in Fig. 1.

Conclusion

Characteristic for tumors is their high rate of aerobic glycolysis. As a consequence, high amounts of lactic acid are produced, resulting in an acidic tumor environment. Acidity, correlates with both impaired prognosis and lower response rates to immune-based therapies such as checkpoint inhibitors in cancer.

This might be in part due to the detrimental effects acidity has on immune effector cells. On the other hand, subpopulations of immune cells that do well in acidic conditions have acquired immune suppressive or tissue regenerating properties (Table 1). Targeting tumor acidity might thus be a promising approach to improve efficacy of immunotherapies. Several strategies are discussed in this review (Fig. 1; Table 2). As T cells rely on aerobic glycolysis and subsequent export of lactic acid to exert their function, caution is warranted when glycolysis inhibitors are to be combined with immune therapies. Immune cells appear less dependent on proton transporters to maintain their intracellular pH. PPI's for example might therefore be a promising partner for immune therapies. A different strategy to reduce tumor acidity without apparent negative effects on immune cells is systemic buffering. This intervention has been shown to improve endogenous anti-tumor immune responses, the effect of checkpoint inhibition and the efficacy of adoptive T cell therapies. As knowledge on tumor acidity is coming of age, its relevance in tumor immune evasion becomes clearer. We propose, therefore, that tumor acidity needs to

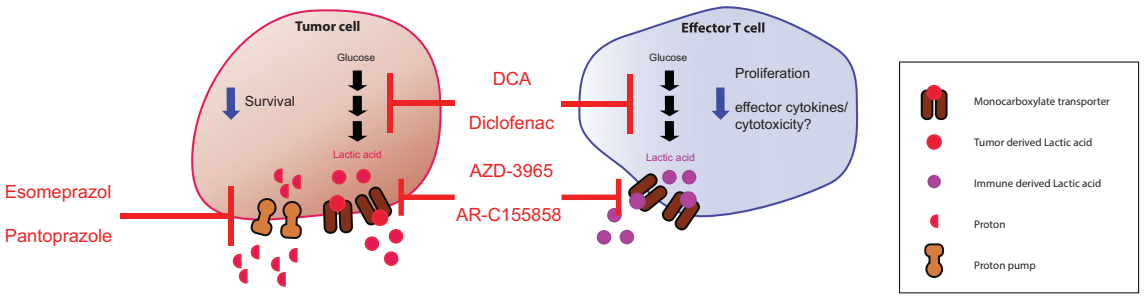

Fig. 1 The effects of anti-acidic strategies on tumor and T cells. While targeting mediators of tumor acidity could lead to increased effect of immune based therapies, direct detrimental effects on T cell function are reported as well. Effects of anti-acidic interventions are denoted inside the cell. Downwards facing arrows indicate a decrease. Systemic buffer therapies are omitted since there are no negative consequences for T cell function reported

Table 2 Summary of tested combinations of anti-acidic partners with immune therapies

Compound	Effect on tumor acidity	Used in combination with which type of Immune therapy	Effect on immune cells	Effect on tumor control
DCA [170]	No difference in lactate	Poly I:C	↓ Arginase activity	Reduction in outgrowth of murine B16 and EG7
Esomeprazole (PPI) [151]	↑ pH	Adoptive cell transfer	↑ Persistence of transferred cells ↑ Immune cell function	Extended survival in murine B16.OVA
Sodium bicarbonate [152]	↑ pH	Anti-PD1 Adoptive T Cell transfer	↑ $CD8^+$ infiltration	Extended survival in murine B16 and Panc02
Sodium bicarbonate [239]	↑ pH	Adoptive T and NK cell transfer	↑ Persistence of transferred cells ↑ Immune cell function	Reduction in outgrowth of murine HepG2

be considered as biomarker and that it should be targeted in combination with checkpoint inhibition or cellular therapies. We envision that tumors from patients with increased LDH activity will be examined with cutting-edge magnetic resonance-based imaging techniques to confirm tumor acidity. Subsequently, these patients will receive treatment regiments incorporating anti-acidic compounds. We hope that the diagnostic and therapeutic options proposed here will pave the way towards personalized immunotherapy and improve so the patients' outcome.

Author contributions Ruben Lacroix wrote the majority of the review and processed writing and suggestions by the co-authors. Elisa A Rozeman wrote the chapter "Tumor acidity as a predictive and prognostic marker for IT" and provided comments for the rest of the review. Kathrin Renner contributed considerable to the chapters "Anti-acidic interventions combined with immune therapy" and "Effects of acidity on the mmune system" and provided input for other parts. The whole was done under the supervision of and final inspection by Marina Kreutz and Christian Blank.

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Compliance with ethical standards

Conflict of interest Christian U. Blank receives grants and/or research support from Novartis and BMS, and has received honoraria or consultation fees for MSD, BMS, Roche, Novartis, GSK, Pfizer and Lilly. The other authors declare that they have no conflict of interest.

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