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Cancer acidity: an ultimate frontier of tumor immune escape and a novel target of immunomodulation

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Abstract
The link between cancer metabolism and immunosuppression, inflammation and immune escape has generated major interest in investigating the effects of low pH on tumor immunity. Indeed, microenvironmental acidity may differentially impact on diverse components of tumor immune surveillance, eventually contributing to immune escape and cancer progression. Although the molecular pathways underlying acidity-related immune dysfunctions are just emerging, initial evidence indicates that antitumor effectors such as T and NK cells tend to lose their function and undergo a state of mostly reversible anergy followed by apoptosis, when exposed to low pH environment. At opposite, immunosuppressive components such as myeloid cells and regulatory T cells are engaged by tumor acidity to sustain tumor growth while blocking antitumor immune responses. Local acidity could also profoundly influence bioactivity and distribution of antibodies, thus potentially interfering with the clinical efficacy of therapeutic antibodies including immune checkpoint inhibitors. Hence tumor acidity is a central regulator of cancer immunity that orchestrates both local and systemic immunosuppression and that may offer a broad panel of therapeutic targets. This review outlines the fundamental pathways of acidity-driven immune dysfunctions and sheds light on the potential strategies that could be envisaged to potentiate immune-mediated tumor control in cancer patients.

Abbreviations: Ab, antibody; BM, bone marrow; CA, carbonic anhydrase; CCL, C-C motif chemokine ligand; CTL, cytotoxic T lymphocyte; DC, dendritic cell; FAO, fatty acid oxidation; GPCR, G-protein-coupled receptors; ICI, immune checkpoint inhibitor; IL, interleukin; mAbs, monoclonal antibodies; MDSC, myeloid-derived suppressor cell; MCT, monocarboxylate transporter; NK, natural killer; PPI, proton pump inhibitor; TAM, tumor-associated macrophage; TAN, tumor-associated neutrophil; TCR, T cell receptor; TDAC, tumor-associated dendritic cell; TIL, tumor-infiltrating lymphocyte; TLR, toll-like receptor; TME, tumor microenvironment; Treg, regulatory T cell

Keywords: acidity, immunity, cancer, hypoxia, lactate, pH, glycolysis, tumor microenvironment, myeloid-derived suppressor cells, regulatory T cells, immunotherapy, immune checkpoints, therapeutic antibodies, immunosurveillance

1. Introduction on cancer immunity

Tumor immunity is emerging as crucial factor in cancer control and therapy. Spontaneous immune responses arising in cancer patients have been proved to condition disease course and positively impact prognosis. Although rather neglected for decades, immunotherapy is now acknowledged as a valuable tool to improve survival in patients with most cancer types. This rebirth is linked to the discovery of “immune checkpoints” and the development of therapeutic tools to abrogate the blocking effect of these regulators on tumor immunity, as in the case of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein-1 (PD-1) inhibitors [1,2]. Nevertheless, despite the extraordinary recent success, cancer immunotherapy is still effective in a minority of patients, and strategies to broaden the population benefitting from this novel treatment are urgently needed.

If tumor cells have intrinsic antigenicity, meaning that they cannot avoid being recognized by specific immune cell subsets, they do learn quite rapidly how to escape immune recognition. Developing sophisticated mechanisms to shut down immunological responses, cancer cells not only survive in an immune competent host, but they proliferate, progress locally and disseminate systemically, often overcoming the control attempts of immune defense. Hence, the study of these escape pathways represents one of the most promising areas of research and a rich pool of potential targets for more effective avenues of cancer immunotherapy.

One of the driving forces that render tumor microenvironment (TME) a hostile milieu for antitumor immune cells stems from hypoxia and the cascade of biochemical reactions leading to local acidity. Even if diverse immune cell subsets are differentially sensitive to low pH and adopt heterogeneous solutions to survive acidic conditions, metabolic dysfunctions featuring tumor site are undoubtedly an obstacle to effective immune responses and an appealing target for cancer immunomodulation.

In this review we will provide an overview of the mechanisms leading to tumor immune recognition and escape, the effects that local acidity may exert on the different players of cancer-immune cross talk, and the possible therapeutic targets offered by these biochemical features of TME.

Albeit the immune system can rely on a large armamentarium of cellular and molecular components to fight cancer, preclinical and clinical evidence points to $CD8^+$ T cells as key players in immune-mediated tumor rejection. Spontaneous triggering of specific $CD8^+$ T cells, resulting in tumor infiltration, is indeed an independent positive prognostic factor in most solid cancers, recently validated as immunohistochemical "immunoscore" in colorectal carcinoma by the pivotal studies of Jerome Galon and collaborators [3]. $CD8^+$ T cells recognize antigenic determinants of tumor proteins quantitatively or qualitatively altered in their expression as a consequence of neoplastic transformation. Among them, a dominant role is being attributed to the so called "neoantigens", stemming from non-synonymous mutations occurring due to the intrinsic genetic instability of cancer cells [4]. A multistep process starting from the killing of tumor cells by innate effectors, particularly Natural Killer (NK) cells, triggers $CD8^+$ T cells. Resident phagocytes, including dendritic cells (DCs), clear the antigen-containing cell debris released into the TME. Then DCs migrate to regional lymph nodes, where they present the antigens to $CD8^+$ T lymphocytes bearing the cognate T cell receptors, and prime them thanks to the proper costimulation and cytokine contexture there provided. From this encounter, activated $CD8^+$ T cells are driven to clonal expansion and to enter the blood, for eventually homing to the tumor site through defined adhesion pathways. Here, after docking to antigen-expressing cancer cells, lymphocytes release their cytolytic granules causing apoptosis in their targets. This process, recently defined as "tumor immunity cycle", may eradicate nascent tumor foci and contributes to cancer immunosurveillance [5,6]. However, in most cancer patients, cancer cells survive the immune attack but, retaining their immunogenicity, they give rise to chronic immune stimulation and the onset of a complex network of associated immune dysfunctions, that are responsible for further and progressive immune escape (Figure 1). As in chronic viral infections, tumor antigen persistency forces T cells to enter a well defined immunological state, known as "exhaustion", in which lymphocytes lose most of their effector properties and survive in a sort of functional paralysis. Exhausted T cells upregulate immune checkpoints such as CTLA-4, PD-1, lymphocyte-activation gene-3 (LAG-3) and others, rendering them hyporesponsive to antigenic stimuli with the final aim of preserving tissue immune homeostasis [7,8]. Concomitantly, as part of the physiological process controlling autoimmunity and wound healing, additional immune cells, namely regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs) and neutrophils (TANs) are recruited to tumor site [9-11]. Together, they attempt to further control immune overstimulation but unintentionally sustain tumor progression and dissemination. As a result, the TME soon changes into a potently immunosuppressed milieu, where the ability of antitumor immunity to eradicate tumor cells is nullified or in the best case heavily reduced [12]. In such a scenario, the biochemical outcome of tumor metabolism, driven by oncogenic pathways, plays a central role. It majorly contributes to T cell exhaustion and favors the accumulation of immunosuppressive cell components.

Figure 1 should be placed here.

2. Immunological outcome of metabolic reprogramming in cancer

Growing tumors are characterized by insufficient blood perfusion, hypoxia, inflammation, enhanced fatty acid metabolism, nucleotide synthesis and glutaminolysis. Transformed

cells undergo a metabolic reprogramming characterized by the switch of their energy metabolism into glycolysis, even under aerobic conditions. In contrast to mitochondrial oxidative phosphorylation (OXPHOS), the glycolytic metabolism of cancer cells employs lactate dehydrogenase (LDH) to convert pyruvate into lactate [13,14]. Accumulating pyruvate inhibits the formation of 2-oxoglutarate, an enzyme responsible of hypoxia-inducible factor (HIF) -1α ubiquitination and degradation in the proteosome [15]. Hence, stabilization of HIF-1α by lactic acid and reduced HIF-1α degradation by mutated or inactivated von Hippel-Lindau protein induces an increase of HIF-1α levels [15,16]. HIF-1α is regulated by oxygen tension and plays a critical role in several processes, cooperating with other transcription factors or oncogenes such as c-myc, p53 or Oct1 to induce the expression of glycolytic genes and suppressors of the tricarboxylic acid (TCA) or Krebs cycle [17].

HIF-1α is also targeted by activated mammalian target of rapamycin (mTOR), a cell growth regulator, downstream of phosphatidylinositol 3-kinase/Akt (PI3K/Akt), a driver of the metabolic shift from oxidative phosphorylation (OXPHOS) to glycolysis. Activated PI3K/Akt in fact, leads to enhanced expression of glucose transporters, such as GLUT-1, and glycolytic enzymes such as hexokinase 2 (HK2), 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) and pyruvate kinase muscle isozyme M2 (PKM2) [18]. The accumulation of lactate and protons, products of cancer metabolism, causes a decrease of intracellular pH. To neutralize metabolic acid loading, cancer cells exploit several proton pumps and transporters whose activity is tightly controlled by intra-cytoplasmatic sensors. These pumps or transporters include the family of carbonic anhydrases (CAs), such as CA II, CA XI, and CA XII [19], V-ATPase [20], anion exchangers such as AE1 (also termed SLC4A1) and AE2 (also termed SLC4A2) [21], $Na^+/HCO3^-$ co-transporters [22], Na^+/H^+ exchanger 1, NHE1 (also known as SLC9A1) [23] and monocarboxylate transporters (MCTs) that cotransport lactate and protons [24]. Through pH sensing proteins tumor cells promote their own survival in the acidic environment. G-protein coupled receptors (GPCRs) including G protein-coupled receptor 4 (GPR4), ovarian cancer GPR 1 (OGR1), T cell death-associated gene 8 (TDAG8) or acid-sensitive ion channels (ASICs) are pH sensors localized on the plasma membrane. Upon activation by acidic extracellular pH they induce cAMP production and ultimately cancer cell survival, acting on PI3K/Akt signaling pathway, or enhanced proliferation and glycolysis and influencing protein kinase-A/extracellular signal-regulated kinase (PKA/ERK) pathway [25] (Figure 2).

The discovery of an acidic pH in inflammatory loci and at sites of immune activity aroused interest in potential effects of low extracellular pH on the immune functions. As summarized and discussed by Anne Lardner, early studies already pointed to the predominance of inhibitory effects on different immune cell types and their functions. Polymorphonuclear leukocytes (PMNs) displayed impaired chemotaxis, respiratory activity and bactericidal capacity. Lymphocyte cytotoxicity and proliferation appeared inhibited at reduced pH and, more generally, clinical acidosis stemming from inflammatory processes seemed associated with a state of immunodeficiency [26]. More recently, the link between cancer metabolism and immunosuppression, inflammation and immune escape has generated major interest among scientists [27].

One main driver of the metabolic reprogramming occurring in the TME is undoubtedly represented by hypoxia. Cells of both innate and adaptive immunity are highly sensitive to

the hypoxic microenvironment featuring most solid cancers, sensing low oxygen availability mainly by the HIF transcriptional system and *via* other molecular mechanisms. Hypoxia has been shown to regulate aggregation, invasion and motility of macrophages and neutrophils, favoring local inflammation and immunosuppression. Low oxygen tension can also affect T cell differentiation and function, possibly tilting the balance toward a T helper (Th)17/Treg phenotype, and impair cytotoxic properties of NK cells. Of note, hypoxia upregulates immune checkpoints in the TME, promoting tumor immune escape but also increasing the expression of molecular targets for cancer immunotherapy. The multiple and complex hypoxia-driven pathways affecting tumor immune surveillance, that are recently gaining special attention as potential targets of immunomodulation in cancer, have been elegantly and extensively addressed in dedicated reviews [28-31].
Here we will focus on studies specifically investigating how acidic pH prevailing in the TME interferes with different cell components of cancer immunity. Interestingly, initial evidence suggests that cancer acidity could drive immune escape not only by blunting antitumor effectors (*i.e.* T and NK cells), but also by promoting recruitment and activity of immunosuppressive protumor immune cells including MDSCs and Tregs. The molecular pathways underlying this opposite effect, eventually resulting in disease progression, are presently under investigation. However, it could be speculated that differential expression levels and patterns of pH sensors including CAs, MCTs, NHE, V-ATPases, GPCRs and non-GPCRs (suppl. Table 1), could play a role. Indeed, while pH regulators are selectively detected in T cells, they are instead broadly expressed in tumor associated myeloid cells, indicating a possible application in counterbalancing local acidic pH conditions to survive in the hostile TME milieu (suppl. Table 1).
Figure 2 should be placed here.

3. Dysfunctional T lymphocytes driven by tumor low pH

T cells are key players of tumor immunity, thanks to their ability of selectively recognizing and killing cancer cells. Upon priming from naïve precursors, activated T lymphocytes, particularly $CD8^+$ effectors, sense tumor cells through the interaction of the cognate T cell receptor (TCR) with the HLA/antigen complex expressed on the target. This docking, strengthened by the interaction of the costimulatory molecule CD28 with CD80/CD86 expressed on target cells, establishes the so called “immunological synapse” and the subsequent local release of perforin-containing granules, forming pores on the tumor cell membrane and causing its death by osmotic unbalance and apoptosis *via* DNA-damaging molecules (*i.e.* granzyme). T cell activation is also associated with clonal expansion and production of immunostimulating cytokines including the T cell growth factor interleukin (IL)-2, interferon (IFN)γ, tumor necrosis factor (TNF)α and others, whose release is instrumental to sustain antitumor immunity, and develop long-term immunological memory. Similarly to tumors, primed T cells exploit aerobic glycolysis to maintain their high proliferative rate, thus applying the Warburg effect to keep glycolytic intermediates at high levels in support of anabolic reactions at the intracellular level [32]. Indeed, the conversion of glucose into lactate in the presence of oxygen, i.e. aerobic glycolysis, has been demonstrated to be a characteristic feature of the metabolic switch of naïve into effector T cells, thereby promoting the proliferation and differentiation process. The adoption of this much less efficient energy metabolism is specifically required for effector functions, as

demonstrated by compromised IFNγ production of T cells blocked from engaging glycolysis. Conversely, proliferation and survival appeared to be independent from this pathway [33]. Recently, Sukumar *et al.* demonstrated that this metabolic switch contributes to shortened lifespan of $CD8^+$ memory T cells associated with increased lactate production, decreased mitochondrial respiration and oxygen consumption. Accordingly, inhibition of glycolysis enhanced the formation of memory $CD8^+$ T cells together with their antitumor functions [34]. T cells use glycolysis during activation and export lactate through MCT-1. However, disposing of lactate into the extracellular milieu depends on the gradient between cytoplasmic and extracellular lactic acid concentrations. Thus, the high lactic acid concentration characterizing the TME blocks MCT-1, resulting in impaired cytotoxic T lymphocyte (CTL) function, as demonstrated by increased apoptosis rate, reduced IL-2, IFNγ, perforin and granzyme production. Interestingly, sodium lactate (at neutral pH) does not exert such inhibitory effects on CTLs. Considering that MCTs cotransport lactate and protons following their respective concentration gradient, both lactate and the low pH of the TME seems required for the metabolic block of CTLs [35]. Additionally to MCT-1, T cells express also MCT-2 and MCT-4 involved in the uptake and export of lactate, suppression of proliferation and cytokine production in CTLs [35-39].

The direct consequences of pH alterations on T cells have been investigated by studies involving *in vitro* culture and stimulation of immune cells at low pH. In 2001, Bosticardo *et al.* demonstrated that pH as low as 6.6 led to impaired activation and proliferation, shown by impaired expression of the high-affinity IL-2 receptor CD25, as well as diminished cytokine secretion and cell cycle progression, in response to TCR triggering or phytohemagglutinin (PHA) stimulation. Providing stronger T cell activation, by for instance engaging the costimulatory CD28 signal, restored complete function, indicating that acidity might raise the activation threshold in T cells. Of note, these authors also detected an upregulation of IFNγ-R2 and CTLA-4 in low pH conditions, rendering T cells sensitive to negative regulatory signaling and suggesting that the acidity-induced upregulation of targetable immune checkpoints such as CTLA-4 should warrant further investigation [40]. Our group also investigated the effect of extracellular decreased pH on the *in vitro* activity of tumor-infiltrating T cells (TILs). A pH of 6.5 had detrimental but reversible effects, including decreased expression of TCR components such as ζ chain and CD3 expression accompanied by impaired IL-2, TNFα and IFNγ secretion, on TILs from melanoma patients [41]. Antigen-specific T cell cytotoxicity appeared to be highly sensitive to low pH and lactic acid addition to culture medium, as shown in a recent study with murine CTL lines. In contrast, hypoxia enhanced the induction of CTLs *in vitro*, an effect that was markedly inhibited in low pH conditions [42]. The inhibitory effect of acidic pH on IFNγ production was found to take place at post-transcriptional level, potentially involving acid-sensing receptors or inhibition of signaling pathways including nuclear factor of activated T cells (NFAT) by altered lactate levels [41,43,44].

Of the diverse proton-sensing molecules, T cells selectively express ASIC-1 and 4, (transient receptor potential vanilloid) TRPV, OGR1 and TDAG8 [43]. However, the functional role of these receptors, particularly in the context of cancer acidity, is still elusive. Upon activation, TDAG8 can reduce T cell activation by regulating cytokine production through a sort of negative feedback loop [45-47]. GPCRs are transmembrane receptors and, among their different roles, they can sense mild variations in extracellular

pH (range 6.0-8.0), which activates them to exert a variety of different actions. In contrast, TRPV and ASICs are activated by severe acidic pH ranging from 4 to 7 [48]. The complete inhibition of cytokine production and the only partial blockade of granule exocytosis observed during lactic acidosis of CTLs could be explained by impaired JNK, c-Jun and p38 phosphorylation after TCR-triggering. In contrast, MEK1 and ERK, involved in granule exocytosis, were not affected [49]. The products of cell metabolism can also impact T cell motility. Haas *et al.* showed in a recent study how lactate accumulated in the synovia of rheumatoid arthritis patients can inhibit T cell migration and thereby contributes to entrapping T cells at inflammatory sites [50], a setting that can be foreseen to occur in cancer as well. A detailed description of the effects of pH on T cells is reported in Table 1.
Much of the contribution in the understanding the impact of tumor acidity on T cell functions comes from studies in which pH buffering has been applied either *in vitro* or *in vivo*, in preclinical models. As abovementioned, our analyses in melanoma setting showed that TILs enter a remarkable anergic state when exposed to subtle pH changes, and that these alterations can be fully corrected if pH buffering is applied *in vitro*. To parallel this evidence in melanoma-bearing mice, high-dose administration of esomeprazole, a proton pump inhibitor (PPI) largely used in clinical setting for gastric protection and dyspeptic disease, has been applied [41]. Notably, treatment was associated with a increase of tumor pH, paralleled by a boost of T cell infiltration and anergy reversion that could be selectively detected at tumor site but not in tumor-free organs. This specificity was likely due to the prodrug nature of esomeprazole, which needs an acidic milieu (as occurring in cancer lesions) to display its inhibitory activity on V-ATPase proton pump function. Interestingly, recovered T cell activity was associated with improved tumor control particularly in combination with DC-based vaccine or immunotherapy with adoptively transferred antigen-specific T cells [41].
In a recent preclinical study, the effect of pH buffering in the context of cancer immunotherapy has been evaluated applying bicarbonate administration to treatment with anti-PD-1 antibodies (Abs). This combination led to improved antitumor responses in animals challenged with different tumors, suggesting that reversing tumor acidity contributes to higher response rates during immune checkpoint blockade [43]. Although molecular pathways underlying this synergism were not dissected, a more evident T cell homing at tumor site was reported, indicating that acidity-reversing drugs may potentiate the therapeutic activity of immune checkpoint inhibitors (ICIs) by allowing an increased tumor infiltration by activated T lymphocytes. At this regard, it is worth mentioning that reversion of acidity by bicarbonate appears to be associated with normalization in tumor vasculature [43], which would obviously contribute to improve T cell homing at tumor site.
Further support to a potential combination between pH buffering and ICIs comes from a recent study by Patsoukis *et al.,* who found a metabolic explanation for the anergic state induced by PD-1 ligation. These authors observed that PD-1 binding on activated T cells rendered T cells unable to engage in glycolysis or amino acid metabolism but instead led to an increase of fatty acid β-oxidation (FAO). Since the differentiation into effector T cells involves glycolysis, the enhancement of FAO represents a new metabolic mechanism for PD1-mediated effector T cell differentiation. FAO could also be responsible for the longevity of PD-1 expressing T cells found in cancer patients [51].
Table 1 should be placed here.

4. Effects of extracellular acidity on dendritic cells

Tumor associated dendritic cells (TADCs) play an ambiguous role in cancer progression. TADCs contribute to antitumor immune surveillance, since they can present tumor antigens and initiate specific antitumor T cell responses. Indeed, infiltration of DCs in solid tumors positively correlates with prolonged patient survival, reduced incidence of metastasis, delay in tumor progression, and a favorable prognosis [52]. Nevertheless, tumor infiltrating DCs and DCs from tumor-draining lymph nodes often exhibit an immature or dysfunctional phenotype. The TME can alter the differentiation of DCs from hematopoietic precursors and impair DC functions at multiple levels by inhibiting their maturation and differentiation or by inducing immune-suppressive DCs [53]. TADCs from several tumors are characterized by low expression of the costimulatory molecules CD80, CD86 and CD40, impaired production of IL-12 and proinflammatory cytokines, increased IL-10 production, reduced antigen-presentation capacity, and reduced ability to induce specific T cell responses [52,54]. Subsets of “regulatory DCs”, which directly suppress antitumor immune responses and promote Treg expansion, have also been described [55,56]. Tumor cells and other TME components (TAMs, MDSCs and Tregs) produce several cytokines (IL-6, IL-10 and transforming growth factor, TGFβ1) chemokines (C-C motif chemokine ligand 2 (CCL-2), C-X-C motif chemokine ligand 1 (CXCL1) and CXCL5) growth factors (vascular endothelial growth factor, VEGF and granulocyte-macrophage colony-stimulating factor, GM-CSF) and soluble factors (prostaglandin, PGE_2) that affect the differentiation, maturation, and functionality of DCs into a skewing towards regulatory DCs, which, in turn, produce immunosuppressive factors [53,57-59].

Besides secreting immune suppressive cytokines and chemokines, tumor cells produce high amounts of lactic acid, which cause an acidification of the TME with profound consequences for the immune populations. However, there are few studies and conflicting evidence on the effects of lactic acid and/or extracellular acidosis on the activation and the functions of DCs. Two studies on murine bone marrow (BM)-derived DCs (BMDCs) showed that acidification of the culture medium to a pH of 6.5 enhanced endocytosis by DCs and increased the surface expression of CD11c, MHC-II and costimulatory molecules (CD40, CD80, and CD86). Furthermore, low extracellular pH augmented MHC-I, but not MHC-II, restricted antigen presentation, as well as the ability of DCs to induce the proliferation of allogeneic T cells during mixed lymphocyte reaction (MLR) and specific CTL responses [60,61]. This effect appears to be mediated by a family of Na^+ channels, the ASICs. Indeed, BMDCs express functional ASIC1, ASIC2, and ASIC3. Interestingly, ASIC2 is expressed on the plasma membrane, while ASIC1 and ASIC3 are localized in the endoplasmic reticulum and mitochondria, respectively [61].

Opposite effects are instead described about the impact of lactic acid on the phenotype and functionality of DCs. Lactic acid produced by tumor cells or by DCs cultured at high density, or added to culture medium during DC differentiation, reduced both basal CD1 expression and toll-like receptor (TLR)-induced expression of CD1a, CD83, and HLA-DR. These DCs displayed a tolerogenic phenotype, characterized by reduced IL-12 and increased IL-10 secretion in response to TLR stimulation, and impaired migratory response to lymph node-derived chemokine CCL-19. Moreover, lactic acid inhibited the proliferation of allogeneic T cells during MLR and the proliferation of antigen-specific CTLs

stimulated with autologous peptide-pulsed DCs. The restoration of physiological pH or the inhibition of LDH with oxamic acid (*in vitro*) or diclofenac (*in vivo*) reversed this suppressive effect [62-64]. Similarly, LDHA inhibition by diclofenac, which is associated with a reduction of lactate production by glioma cells, promotes a regained ability of tumor-infiltrating DCs to produce IL-12 upon TLR stimulation [64].If DC activity does not seem to be interfered by low medium pH, it should be underlined that instead the detrimental effects mediated by lactic acid are pH dependent, as they were reverted by buffering pH to neutral values [62,63]. This indicates that acidity may impact on DC activity indirectly, *i.e*. by facilitating or synergizing with the tolerogenic effect of lactic acid. This hypothesis is in line with the emerging evidence about the crucial role of metabolic changes in the maturation and activation of DCs. Indeed, while resting immature DCs exploit β-oxidation of lipids and oxidative phosphorylation (OXOPHOS) to maintain their energetic balance, maturation and activation (for instance upon TLR stimulation) induce a switch in DC metabolism to aerobic glycolysis, and in turn, to generation of lactic acid [65,66]. Thus, the extrusion of lactate is necessary to maintain the high glycolytic flux required for DC activity. As MCTs cotransport lactate and protons following the concentration gradient [24], the high concentration of lactic acid in the TME can block endogenous lactate export and increase extracellular lactate import, hence impairing DC metabolism.

5. Shaping of Natural Killer cells by microenvironmental acidic pH

NK cells are effector lymphocytes belonging to the innate immunity and constitute the first-line defense of the immune system in the control of tumor growth and metastasis diffusion. NK cells are also important immunoregulatory cells, interacting with T cells, DCs, macrophages and endothelial cells, *via* the secretion of a broad array of cytokines including IFNγ [67]. NK cells are widespread throughout lymphoid and nonlymphoid tissues and represent a minor fraction of total lymphocytes, *i.e.* from 2% to 18% in human peripheral blood [68]. Circulating NK cells are mostly in their resting phase but activation by cytokines leads to their tissue infiltration [69]. Once NK cells recognize the target, their cytotoxic ability is mainly mediated *via* two predominant pathways, shared with activated T cells and leading to caspase-dependent tumor cell apoptosis: i) the secretion by exocytosis of granules containing perforin and granzymes, ii) the expression of pro-apoptotic molecules such as Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL), interacting with cognate receptors (e.g. Fas and TRAIL-receptor, respectively) expressed on tumor cells [70]. NK cells in humans are present in peripheral blood and lymphoid organs in different activation status, differing in homing properties, cytokine secretion and cytolytic potential [71-73]. NK cell activity in the peripheral blood is associated with increased risk to develop cancer [74], and different NK cell-based immunotherapies have recently shown significant clinical potential particularly in hematological malignancies [75,76].

Similarly to the other immune cell populations, NK cells can be affected by tumor metabolism and its products. Indeed, as demonstrated for T cells, also NK cells appear to be sensitive to increased lactate, which inhibits NFAT upregulation, and consequently impairs NK cell function and survival [40]. Similarly, reduced cytolytic activity together with lower expression of granzyme, perforin and Nkp46 is detected in NK cells upon exposure to tumor-derived lactate [77]. In contrast, during the killing of yeast cells or cryptococcoma,

NK cells appeared to profit from acidic pH of the microenvironment by displaying enhanced perforin degranulation and killing capacity [78]. These results suggest that NK cells might amplify their killing activity at acute low pH conditions, thus maintaining microbicidal host defenses against quickly replicating organisms despite an acidic microenvironment. This is clearly different from cancer, whose acidity mediates a chronic long-term exposure of immune cells to the hostile milieu of the TME.

Indeed, tumor acidity is acknowledged to exert a key role in facilitating tumor escape from NK cell immune surveillance, as already reported in the early 90s, with studies showing how acidic pH can significantly blunt antitumor activity of both NK cells and lymphokine-activated killer (LAK) cells. Physical characteristics of the TME, such as low oxygen tension, low glucose concentration and acidic pH, are responsible for alterations of the cytolytic activity of tumor-infiltrating NK cells [79]. Combinations of either moderate (1% O_2, 26 mg/dl glucose, pH 6.7) or extreme (0% O_2, 6 mg/dl glucose, pH 6.4) alterations of physical conditions reduced NK activity. In another study on LAK cells, the *in vitro* cytotoxic activity was considerably reduced under acidic conditions (pH 6.8; 6.3; 5.8). Interestingly, this effect was independent of NK donors, time of exposure, effector:target ratio [76], and it could be extensively reproduced using both human NK and LAK cells and different tumor cell line targets [80-82], indicating a quite efficient and readily inhibition of NK-mediated lysis by local acidity. Furthermore, an extracellular pH below 7.2 influenced NK activation and LAK generation by IL-2 [83]. Of note, alterations of the cytotoxic activity appeared to be of permanent nature. At difference with what observed in T cells [41], NK cells first cultured at low pH did not recover upon culture medium buffering, indicating an irreversible damage.

Importantly, tumor products linked to acidic pH can subtly tune NK activity, as reported by Crane *et al.,* who showed that LDH isoform 5 (LDH5) secreted by glioblastoma cells and detectable in sera from glioblastoma patients caused down-regulation of natural killer group 2, member D (NKG2D) on NK cells *via* induction of NKG2D ligands on myeloid cells [84]. The importance of NKG2D in tumor immune surveillance is underscored by the observation that NKG2D-deficient mice are more susceptible to the development of oncogene-induced tumors [85]. The inhibition of NK-mediated tumor surveillance due to the accumulation of LDHA-associated lactic acid has been recently demonstrated in immunocompetent C57BL/6 mice, in which tumors with reduced lactic acid production have been found to develop in a significantly slower manner and to show increased infiltration with IFNγ-producing T and NK cells, with respect to controls [44]. Similar results were obtained with a pancreatic cancer model, corroborating the evidence that lactate can inhibit NK cell activity *in vivo* [77].

Impaired NK cell activity has been commonly reported in cancer patients. Leukemic patients displayed defects both in the interaction of NK cells with tumor cells and in their cytotoxic activity [86]. Also in colorectal cancer patients, NK cells exhibited profound inability to degranulate and produce cytokines [87]. Although not directly demonstrated, it could be speculated that many of the reported defects of NK cell activity in cancer patients could be at least in part attributed to the acidity characterizing the TME.

Based on a broad panel of tumor models, we extensively demonstrated that a class of antiacid drugs, the PPIs, has a potent antitumor effect both *in vitro* and *in vivo*. This effect is due to the inhibition of proton pumps expressed in tumor cells [88-93], but it could also

involve the boost of immune function as clearly demonstrated in a melanoma model [41]. Indeed, since PPI administration results in a stable buffering of acidic pH in the TME, as shown by *in vivo* tumor pH measurements through magnetic resonance spectroscopy [94], it is reasonable to hypothesize that a recovery of physiological pH levels would be beneficial to antitumor immune responses including those mediated by NK cells.
NK-based immunotherapy is gaining a great relevance particularly in the field of hematological cancers [95]. A paradigmatic example is provided by the NK-activating antibodies directed against SLAM-F7 and CD38 (elotuzumab and daratumumab, respectively), which have been recently approved for the treatment of patient with multiple myeloma [96]. In this scenario, combination therapies with drugs reducing tumor acidity could be envisaged as a promising strategy for improving clinical efficacy by overcoming low pH-associated NK functional defects.

6. Myeloid cells as potent allies of cancer progression recruited by tumor acidity

Multiple subsets of myeloid cells are key components of protumor immune responses. Patients with different cancer histologies commonly display pathological accumulation of aberrant myeloid cells in the blood. This phenomenon stems from the conditioning of BM myelopoiesis by systemic factors produced by tumor cells and causes a mobilization of immature cells. Collectively defined as MDSCs, this heterogeneous population includes both granulocytic and monocytic subsets [97]. MDSCs are among the most potent negative regulators of adaptive and innate immunity, and their accumulation in blood and immune organs is associated with immunosuppression and cancer aggressiveness. When attracted to the tumor site by selective chemokines (e.g. CCL-2 and CCL-5) and homing factors, MDSCs mediate immune tolerance and promote tumor growth by favoring angiogenesis, matrix remodeling and epithelial-to-mesenchymal transition (EMT), through a program resembling wound-healing process. Once in the TME, MDSCs can trans-differentiate into TAMs or TANs, however retaining and even exacerbating their protumor inflammatory properties, generally defined as type 2 [98].
Low pH conditions and accumulation of associated metabolites including lactic acid, expression of molecules involved in ions transport/pH regulation and hypoxia, can profoundly impact on the multistep process of myeloid cell conditioning in cancer. Indeed, thanks to their intrinsic functional plasticity, myeloid cells can sensitively detect changes in local pH and promptly respond by molding their differentiation status and activity. Notably, the BM undergoes significant decrease in pH levels under certain pathological conditions, even in distant organs. An interesting example, although not cancer-related, is provided by acute myocardial infarction, where intracoronary BM transplant is applied as therapeutic strategy to exploit the anti-inflammatory and wound-healing properties of BM mononuclear cells. Indeed, the curative activity of BM cells appears to be strongly associated with the degree of acidity of the originating BM, suggesting a boost of immunosuppressive myeloid cells by a low-pH environment [99].
It is well established that acidity, characterizing the inflammatory sites including cancer, is a major “attractor“ of myeloid cells, although the molecular mechanisms underlying this process are presently poorly defined. A possible explanation could come from the

evidence that molecules involved in immune cell extravasation, such as selectins, are influenced in their binding dynamics by acidity. Selectins are proteins regulating the multistep process leading to the recruitment of circulating leukocytes to the sites of injury and inflammation. Under acidic conditions, binding of P and L-selectins to P-selectin glycoprotein ligand 1 (PSGL-1) is significantly enhanced, causing increased adhesion of neutrophils and monocytes to the vascular endothelium [100]. In contrast, E-selectin activity is unaffected by acidic pH, indicating that activated antigen-specific T cells, mostly relying on E-selectin for extravasation, may not be favored by low pH values featuring tumor site [101].

Low environmental pH is also responsible for the direct induction of a series of proinflammatory mediators in various tumor-associated myeloid cells, although dedicated studies to address this effect in literature are scantly. The inducible isoform of nitric oxide synthase (iNOS) is significantly enhanced both at transcriptional and protein level in macrophages exposed to culture pH values below 7.0, through a process that involves increased nuclear factor k-light-chain-enhancer of activated B cells (NF-κB) translocation and TNFα secretion [102]. Since nitric oxide (NO) impairs intracellular pH recovery in macrophages following acid loading, this amplification loop tends to auto-maintain the proinflammatory effect of TME acidity. Stimulation of human neutrophils by extracellular acidosis triggers activation via PI3K/Akt and ERK pathways [103], a feature resembling the functional profile of MDSCs. The evidence that low pH is also associated with enhanced phagocytosis and HLA-mediated antigen presentation to T cells, prompts to hypothesize that neutrophils activated in low pH might acquire an antigen-specific immunosuppressive activity, a feature that is well established for murine MDSCs [104].

The effect of acidity on myeloid cells is mostly based on experiments involving lactate, a key player in cell signaling and intercommunication between myeloid stroma and tumors that is released by TME cells during aerobic and anaerobic glycolysis. Lactate broadly influences immune responses, not only by blunting T and NK cell activation [44] but also by promoting cancer survival and progression through the induction of protumor immunosuppressive immunity, including MDSCs. In turn, MDSCs are able to inhibit NK cell activity, suggesting that lactate can act directly and indirectly through other immune cells [77, 105]. Of note, in tumor-infiltrating monocytes this metabolite plays a fundamental role in the transcriptional regulation of MHC I which is crucial in antitumor immune responses [106]. Lactic acid induces M2-like polarization of TAMs through HIF-1α and the expression of arginase I and VEGF in macrophages [107,108]. The knock down of LDHA in pancreatic cancer cells is associated with a remarkable decrease in MDSC frequency, which is promptly lost in the presence of IL-6 and GM-CSF [77].

HIF-1α was found to be a crucial inducer of myeloid differentiation and function at tumor site, through rapid and pronounced upregulation of arginase I and iNOS and concomitant down-regulation of NADPH oxidase and reactive oxygen species (ROS) [109]. More recently, Noman and collaborators showed that HIF-1α triggers the expression in MDSCs of immune checkpoint receptors and ligands (including molecules of the PD-1/PDL-1 and CTLA-4/CD80 axis), which are involved in the production of the immunosuppressive cytokines IL-6 and IL-10 by these cells [110]. Activation of HIF-1α is essential for the regulation of glycolytic capacity, aggregation, motility, invasiveness and functions of myeloid cells, thus proving how hypoxia and the consequent local acidity are direct

regulators of MDSC survival and activity in the TME [111]. Importantly, the differentiation of TAMs in the TME from MDSC precursors appears to be controlled by down-regulation of the activity of the transcription factor STAT3, which is caused by hypoxia through the activation of the CD45 tyrosine phosphatase [112]. This evidence strongly suggests that local biochemical changes involving hypoxia and acidity drive MDSC conversion into TAMs at tumor site.

If the contribution of TME acidity to the accrual and activation of protumor myeloid cells has been clearly illustrated, the involved molecular pathways still need to be fully dissected, particularly in view of potential druggable targets. Nevertheless, some data are emerging about the role of pH regulators in myeloid cells. A relevant example is provided by V-ATPase, which has been reported to be involved in M2 polarization of TAMs in murine tumor models, with a clear involvement of the a2 isoform (a2V) in the upregulation of mannose receptor-1, arginase I, IL-10 and TGFβ [113]. Of note, studies in influenza A (IVA)-infected cells demonstrated that ERK/PI3K activation is linked to increased activity and acidification of the intracellular compartments, with kinases colocalizing with V1 domain of V-ATPase in IVA-infected cells [114]. This pH regulator could thus represent a therapeutic target, particularly in view of the inhibition of PI3K activity, which has been recently found to mediate resistance to ICIs when upregulated by the TME myeloid components [115]. V-ATPase can be also targeted by PPIs, including drugs like omeprazole, lately exploited for their potential antineoplastic therapeutic properties linked to the reversion of altered H^+ homeostasis in the TME. *In vivo* administration of the PPI pantoprazole in lymphoma-bearing animals led to enhanced recruitment of macrophages with an antitumor M1 phenotype and potent antitumor activity, contributing to *in vivo* tumor control [116]. Interestingly, the effect of pantoprazole is detectable also in the BM, where tumor-bearing mice developed enhanced myelopoiesis involving antitumor $TLR2^+$ macrophages, thus indicating that V-ATPase targeting can alleviate acidity-induced myelosuppression even at systemic level [117].

Another therapeutic tool is potentially represented by dichloroacetate (DCA), an inhibitor of pyruvate dehydrogenase kinases that antagonizes arginase I expression induced by lactic acid. In a tumor murine model, $CD8^+$ T cell proliferation and tumor infiltration is restored though a mechanism relying on the inhibition of macrophage activity. DCA acts not only on tumor cells to suppress glycolysis but also on immune cells to improve the immune status modulated by lactic acid and to increase the effectiveness of antitumor immunotherapy [118].

The GPCRs, including OGR1, GPR4, and TDAG8, have been identified as important regulators of pH homeostasis in physiological conditions and during inflammation. Myeloid cells, including monocytes and macrophages, express OGR1 after NF-κB activation *via* TNF, and data in knocked-out mice depict a pathological role for pH-sensing receptor OGR1 in precancerous mucosal inflammation. Multiple studies show that acidic environment, through the local increase of proton concentration and lactate production, triggers the production of the proinflammatory cytokine cascade (including TNF, IL-6, IFNγ, and IL-1β) in activated macrophages. In this context, OGR1 senses extracellular protons and induces a broad functional program including phospholipase C activation, inositol trisphosphate formation, and Ca^{2+}. Interestingly, OGR1 expressed in myeloid cells has been recently shown to play a key role in chronic colitis, while OGR1 deficiency protects

from spontaneous inflammation at preclinical level [119]. These data favor the hypothesis that pH sensors expressed by immune cells may be early involved in sustaining precancerous inflammation, thus representing a potential target for tumor prevention in inflammation-based cancer.

Polarized macrophages adapt their glucose metabolism based on their differentiation status and tissue environment [120]. Since TAMs tend to accumulate in tumor hypoxic areas expressing HIF-1α and associated genes, such as *GLUT1*, *HK2*, *PFKFB3*, and *PGK1*, they preferentially use the glycolytic metabolism to survive the hostile TME. The shift to glycolysis, associated with the activation of the IL-1β/TNF program, is mediated *via* the AKT-mTOR-HIF-1α pathway, corroborating the role of HIF-1α in myeloid cell-mediated inflammation [111]. Furthermore, it could be speculated that infiltrating myeloid cells may contribute to TME acidification and to the self-maintenance of a protumor phenotype through their own lactate production. In addition to drugs specifically interfering with these pathways, dietary intervention could also be considered a strategy for the metabolic reprogramming of inflammatory myeloid cells, particularly to prevent cancer onset or recurrence in high-risk patients. Of note, the ketogenic diet, based on very low carbohydrate intake and thus low blood glucose levels in tumor-bearing mice, is associated with decreased MDSC frequency and activity, restored T cell function and significant slowdown of tumor growth [77].

7. Regulatory T cells at the tumor site: the orchestrating role of hypoxia

The metabolic environment of cancer is characterized by acidity, hypoxia and nutrient starvation, detrimental factors for the activation and expansion of T effector antitumor responses while it is permissive for the accumulation of immune suppressive cells such as Tregs. Accumulated in the TME, they play a crucial role in dampening antitumor response. The frequency and activation status of Tregs often correlates with poor prognosis in several human cancers [121,122]. Recent studies clearly demonstrate that tumor infiltrating Tregs display unique transcriptional signatures, which, besides testifying an enhanced state of activation, also indicate that Tregs express a large variety of chemokine receptors. “Chemokine signaling” and “cell migration” are categories of genes overexpressed in tumor-associated Tregs [123]. Hypoxia and acidity enhance the intra-tumor expression of chemokines such as SDF1/CXCL12, CCL-17 or CCL-28 and thus facilitate the active recruitment of Tregs expressing the corresponding receptors [124-126]. Although the direct role of hypoxia in favoring the differentiation of Tregs is still controversial [127,128], several studies agree in documenting that HIF-1α activation strongly increases the expression of transcription factor forkhead box P3 (Foxp3), a crucial transcription factor for Treg cell lineage commitment and functions, and fosters their immune suppressive functions [129-131]. *Ex vivo* studies showed that in human tumors hypoxia is associated with a tolerogenic environment mediated by the selective accumulation of Tregs [125,126]. This is also true for human hepatocellular carcinoma (HCC), a tumor displaying acidic and hypoxic features. Here, Tregs not only suppress antitumor responses but also play an active role in promoting neo-angiogenesis [122]. Treg induction at tumor site relies either on the direct binding of HIF-1α to Foxp3 regulatory regions or on the hypoxia-induced expression of TGFβ by tumor cells [132]. Hypoxia modulates Treg pathways in the tumor setting but also in autoimmune related

diseases, such as encephalomyelitis (EAE) and colitis [133,134]. Moreover, hypoxia supports the differentiation of type 1 regulatory cells (Tr1), characterized by a high production of IL-10 and by an elevated expression of LAG-3 and CD49b [135,136].
The metabolic/acidic TME can affect Treg homeostasis and potentiate their suppressive functions, which are mediated by multiple still not fully elucidated mechanisms. A new vision of cell-to-cell communication is now emerging, and in this functional network a prominent role is played by exosomes. Also in T cell physiology, these nanovesicular structures are assuming a strong relevance as physiological mediators in T cell receptor driven synapses [137]. Similarly, a recent study indicates that Treg exosomes and their specific miRNA content contribute to the Treg mediated suppression of conventional T cell proliferation and activity [138]. In several cell types, especially in tumor cells, the release of exosomes is strongly augmented by hypoxia and acidic environment [139,140]. Thus, it is reasonable to speculate that tumor induced acidosis and hypoxia boost Treg exosome release, thus providing an additional possible mechanism of improving immunosuppression.
How other TME factors influence and actively sustain the suppressive functions, differentiation and local homeostasis of Tregs still requires deep exploratory studies. However, accumulating evidence indicates that Tregs possess metabolic requirements in compliance with the tumor-conditioned low glucose availability, ensuring Treg differentiation and survival. Tregs face these extreme conditions by obtaining energy through oxidative phosphorylation (OXPHOS). Metabolic waste of glycolytic pathways, such as lactate and kynurenin, which suppress conventional T cell activation and cytotoxic activity, indeed promote Treg activities [141-143].
As opposed to $CD8^+$ and Th1, Th2, Th17 polarized $CD4^+$ T cells, Treg metabolism is dynamic and finely tuned by external factors which include, in addition to TCR triggering and cytokine milieu, also nutrient availability [144,145]. For their energy supply, Tregs can exploit fuel sources alternative to glucose, and thus are not likely affected by glucose competition occurring at the tumor site [146]. In addition to glucose consumption, to respond to increasing biosynthetic demands imposed by the accelerated growth, tumor cells display continuous *de novo* synthesis of fatty acids, which accumulate in the tumor interstitial spaces [147]. Treg metabolism is based on fatty acid oxidation more than glycolysis, and their expansion relies on the activation of AMP-activated protein kinase (AMPK), a sensor of nutrient stress. Since for their self-maintenance Tregs primarily utilize FAO and exhibit low mTOR activity, this fatty acid microenvironment likely provides a perfect soil for Treg homeostasis [148]. Most importantly, it has been shown that the addition of fatty acids to the culture medium, together with *in vitro* inhibition of glucose uptake and glucose oxidation, prompted Treg differentiation [149]. Moreover, lipid uptake and oxidation are mandatory for the expression of Foxp3, as demonstrated in murine models [149].
The immunometabolism is an emerging field that may provide new opportunities to develop novel Treg-based immunological interventions. The use of rapamycin, an mTOR-inhibitor exploited to prevent acute graft rejection in humans, has been demonstrated to selectively expand Tregs. To date, no metabolic modulator is specifically used to inhibit Tregs in cancer patients, but studies *in vivo* highlighted new possible metabolic targets. One interesting example is represented by the pharmacological inhibition of the

mevalonate pathway through the administration of statins. These cholesterol-lowering drugs appear to impair Treg suppressive activity [148]. Interestingly, treatment with etomoxir, an inhibitor of carnitine palmitoyl-transferase 1 (CPT1), a FAO mitochondrial enzyme, abrogates Treg development, probably due Treg FAO dependence [149].
In conclusion, low glucose, high fatty acid, hypoxia and local acidosis are all factors that synergistically operate towards an advantageous Treg environment in cancer patients. Indeed, a recent proteomic *ex vivo* analysis demonstrates that Tregs isolated from tumor sites have a different profile from peripheral blood Tregs, which are highly glycolytic, and anergic [150]. Thus, the metabolic conditions of the TME actively induce a selective reprogramming of Tregs and further stress the plasticity of this T cell compartment and their superior ability in adapting to tumor-driven conditions.

8. The possible effect of pH on antibody activity

Most of the complex network of cells belonging to innate and adaptive immunity can interact with tumor cells and impact their expansion either by impairing or promoting cancer progression. Nevertheless, the role of tumor-specific B cells and Abs spontaneously arising in cancer bearing host is still controversial and no consensus on a unique effect of these immune effectors in cancer immunosurveillance has been reached [151]. On the other hand, monoclonal antibodies (mAbs) represent an emerging class of therapeutic drugs widely used for the treatment of various solid tumors, for their reliable and specific ability to impair tumor growth either by direct targeting or, more recently, *via* immune modulation [152-154]. For this reason, understanding whether tumor acidity might impact on the activity and function of therapeutic Abs could provide novel insights into the potential resistance mechanisms and the possible strategies to improve clinical efficacy of this universal approach of cancer treatment.
Albeit no direct study addressing this topic is to our knowledge available in literature, some interesting speculations could be made on the basis of the molecular/structural features of Abs, and their biodistribution properties in the TME. Low extracellular pH and the resulting pH gradient across the plasma membrane of tumor cells, high interstitial fluid pressure and low oxygen tension constitute the TME hallmarks of solid tumors and represent a fundamental barrier to drug delivery and efficacy for many chemotherapeutic agents [155]. Indeed, in an acidic environment, weak bases such as for instance doxorubicin, acquire a charged state that inhibits their transport across biological membranes.
The therapeutic activity of mAbs depends on their direct interaction or binding to the target antigen expressed either by cancer or immune cells. Cells linked by Abs act as a marker for the immune system that recognizes the Fc sequence of Abs through the Fcγ-receptor (FcγR) and kills them through phagocytosis, complement-dependent cytotoxicity (CDC) or antibody-dependent cellular cytotoxicity (ADCC) [156]. Different subclasses of immunoglobulin G (IgG), the class mostly used as therapeutic proteins, exhibit differential ADCC and CDC ability due to the diversity in their constant regions. IgG1 mAbs interact efficiently with most FcγRs, proficiently engaging the immune system, while IgG2 and IgG4 ones show a reduced affinity to a number of FcγRs and cause low immune-mediated cell killing. Recently, mAbs functioning to boost the immune response by regulation of T cell function have been introduced in the clinics (e.g. anti-CTLA-4 and anti-PD-1). Their therapeutic effect is based on the antagonist activity on the binding of negative-regulatory

immune receptors (mostly immune checkpoints) to cognate ligands. These mAbs are specifically modified to avoid interaction with Fc-receptors and the potential elimination of immune effectors by ADCC. The majority of studies concerning Abs and pH are related to the optimization of therapeutic Ab production and formulation to minimize their deterioration. Indeed, mAbs are subjected to chemical (fragmentation, oxidation, isomerization, and polymerization) and physical (aggregation, denaturation) deterioration, the extent of which depends on the biochemical and biophysical properties of the mAb itself, as well as the physico-chemical environment to which the mAb is exposed during processing and storage, including pH [157]. Considering that weakly acidic conditions (pH 6-7) seem to represent the optimum for most Abs [157], it is plausible that acidic extracellular pH in solid tumors may influence only weakly the deterioration of therapeutic mAbs. However, since some tumors display a lower pH in the TME, the possibility that mAb degradation might reduce their therapeutic activity cannot be excluded. Indeed, the rate of oxidation and Fc aggregation has been described to increase with decreasing pH [158,159]. Moreover, acidic pH-induced chemical degradation of aspartate amino acid in the complementarity-determining regions (CDR), induced the loss of binding activity of the antibody to its antigen, as described *in vitro* for a mAb directed against epidermal growth factor receptor (EGFR) [160].

Interactions between biomolecules, such as receptor-ligand, antibody-antigen or Fc-FcγR, are generally characterized by their affinity, specificity, and affected by environmental features, such as pH [161]. Therefore, since high affinity to their target is a prerequisite of mAbs to achieve therapeutic benefits, the activity of these biomolecules is predicted to be influenced by pH. Acidic pH is indeed used to elute mAbs from immunoaffinity columns during the purification process and directly reduces the equilibrium dissociation constant between the antibody and its antigen decreasing their association. Moreover, histidine residues in the interacting sites could augment pH-mediated dissociation, because these residues are protonated in acidic conditions favoring electrostatic repulsion between rigid domains [162]. These chemical characteristics are exploited for mAb engineering. Introducing histidine residues in the complementary CDR generates pH dependent Abs, which are able to link the antigen at neutral pH and release it in the endosomes (pH 5.5-6) [163]. This allows the recycling of free Abs and antigen elimination [164]. A potential involvement of pH in the therapeutic activity of mAbs is also supported by enhanced antitumor responses to ICIs against CTLA-4 or PD-1 upon neutralization of tumor acidity with bicarbonate, as shown in B16 melanoma and Panc02 pancreatic cancer models [43]. Even though higher activation of T cells *in vitro* under alkaline compared to acidic pH conditions and the inefficacy of bicarbonate to further increase antitumor activity of double blockade with anti-CTLA-4 and anti-PD-1, an increment in receptor-antigen affinity, derived from the increased pH, may participate in enhancing the antitumor activity of ICIs. Future investigations, especially in solid tumors characterized by a highly acidic environment like melanoma [165], are warranted to understand the impact of pH on the therapeutic activity of mAbs and possible interventions.

9. Concluding remarks and clinical implications

Many of the known mechanisms of tumor immune escape appear exquisitely and selectively tailored for defined molecular immune pathways, as if tumor cells, through a

Darwinian pressure, were forced to lose specific features in order to survive immune attack. A classical example of this process is the loss of tumor or HLA-class I antigens, rendering cancer cells completely invisible to T lymphocytes but at the same time more susceptible to NK cell killing [166]. In a totally opposite trend, tumor acidity could instead be envisaged as a sort of “global protection shield”, by which cancer cells, through a single and relatively simple biochemical pathway, simultaneously wipe out the activity of all antitumor immune effectors and convert regulatory immune cells to protumor allies (**Figure 3 should be placed here**). If so, correcting tumor pH should specularly lead to a rebalance of physiological immune responses and the concomitant recovery of multiple antitumor functions.

As a matter of fact, despite research investigating the immunological effects of cancer acidity is still in its infancy, data emerging from preclinical investigations depict a rich scenario of promising candidates for potential immunomodulation in clinical setting. Findings available up to date predict that buffering tumor pH should contribute to a recovery of antitumor T and NK cells and a relief of the detrimental effects exerted by immunosuppressive stroma components. Such an approach might be applied to improve spontaneous cancer immune control, or most likely to potentiate the efficacy of tumor immunotherapy. Contrasting the protumor activity of stromal myeloid cells, by specific immune depletion or blockade of selective signaling pathways, has been convincingly reported to overcome resistance to ICIs at preclinical level [115]. In this view, the hypothesis of introducing an alternative strategy to interrupt cancer/myeloid interaction by antagonizing tumor acidity sounds quite appealing.

Reversion of acidity in the TME might be obtained by systemic buffering with bicarbonate fostering an improved efficacy of PD-1 blockade [43]. The antagonism of cancer acidity is also obtainable by administration of PPIs including omeprazole and analogues. These drugs, recently receiving much attention for their unexpected therapeutic potential in oncology, have shown to reproducibly increase tumor pH in a selective manner, thanks to their nature of prodrugs specifically activated by low-pH of tumor milieu (in addition to gastric environment). PPIs potentiate DC-based cancer vaccines and adoptive T cell transfer in tumor murine models, and synergize with chemotherapy in breast cancer and sarcoma patients [93,167]. Their safety and accessibility promote omeprazole-related PPIs as promising therapeutic strategy to revert the detrimental effects on antitumor immune responses, simultaneously interfering with autocrine signaling pathways that sustain tumor growth and progression [168]. Indeed, it is established that administration of esomeprazole is associated with increased extracellular tumor pH and this is paralleled by a beneficial effect on antitumor immunity in murine models [41]. Although PPIs have been developed to bind the gastric H,K-ATPase, and definitive data on their actual cross-reactivity with V-ATPase are still scantly [169- 171].

The possibility to selectively buffer the acidic TME, independently of the underlying molecular mechanisms, underscores the potential role of PPIs as innovative strategy of immunomodulation in cancer patients. Studies focused on the binding and inhibitory activity of PPIs on V-ATPase expressed in tumor or immune cells and the related functional outcomes are encouraged to improve our knowledge about the clinical potential of these promising drugs in cancer.

This could be the case of myeloid cells, which are known to upregulate V-ATPase and CAs under different conditions in response to hypoxic and dysmetabolic stress [172,173]. Thus, the administration of drugs interfering with the activity of these pumps could help reconditioning the whole immunosuppressive context of the TME, favoring an optimized scenario for conventional T cell activity and antitumor responses. We have collected evidence that esomeprazole can reduce the frequency of MDSCs in melanoma-bearing mice and patients, and this effect is associated with concomitant activation of T cells [Umansky and Rivoltini, unpublished observation]. Interestingly, novel therapeutic tools for blocking V-ATPase function in different pathological conditions are under development, including small molecules, for more selective activity and possibly a better tolerability in clinical setting [174].

Old and novel drugs interfering with TME acidity could thus be envisaged as potential innovative tools of immunomodulation in cancer patients, particularly in synergy with immunotherapeutic strategies. In fact, pH-modulating drugs might represent one possible choice to overcome tumor resistance and potentiate clinical benefit of ICIs. A potential prediction of this synergism may come from the established evidence that cancer patients with high LDH plasma level usually fail to respond to both CTLA-4 and PD-1 blockade [175,176].

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Figure Legends

Figure 1. Pathways inducing spontaneous T cell immunity in cancer-bearing host (*tumor immunity cycle*). Tumor cells developing in an organ are initially recognized and lysed by NK cells, leading to the release of tumor cell debris containing antigenic material, which is removed by local phagocytes including dendritic cells (DCs). DCs migrate to regional nodes where they prime T cells expressing T cell receptors able to recognize the tumor antigens. Upon activation T cells acquire the ability to leave the lymph nodes, enter the blood stream, undergo clonal expansion and home to the tumor site, to mediate antigen-specific recognition and killing of tumor cells. This cycle may lead to cancer cell elimination (tumor immunosurveillance). However, in the likely case of tumor cell persistence, the chronic stimulation of the tumor immunity cycle induces the onset of negative feedback mechanisms, including the accrual of immunosuppressive cell subsets such as regulatory T cells and myeloid-derived suppressor cells.

Figure 2. Mechanisms leading to low pH in the TME. Low oxygen supply and activation of oncogenes upregulate glycolysis, in turn eliciting HIF-1α expression. These pathways lead to the accumulation of protons and acidification of the TME. Simultaneously, HIF-1α promotes the activation of multiple genes including those encoding pH regulators contributing to a further acidification of the TME. HK, Hexokinase; MMP, Matrix metalloproteinase; IGF, Insulin growth factor; GAPDH, glyceraldehydes-3-P-dehydrogenase; LDHA, lactate dehydrogenase A; NOS, nitric oxide synthase.

Figure 3. Low pH in the tumor microenvironment works as a "global protection shield" for tumor cells. As illustrated in the review's text, tumor acidity acts as a broad immune escape mechanism by which cancer cells, simultaneously wipe out the activity of all antitumor immune effectors (including T cells, NK cells and crucial antigen-presenting cells such as dendritic cells), at the same time favoring the accrual and conversion of regulatory T cells and myeloid cells into immunosuppressive and protumor cells. Protons and lactate, potentiated by common driving factors represented by hypoxia and dysmetabolic pathways, create a hostile milieu for T cells, while myeloid cells and Tregs can survive thanks to specific metabolic reprogramming and expression of pH regulators.

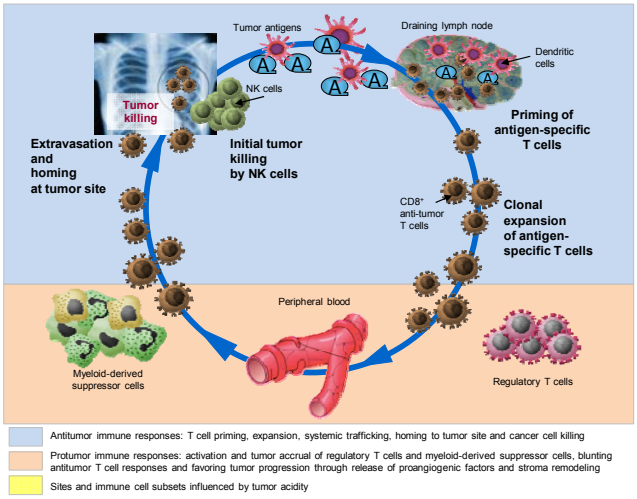

Figure 1

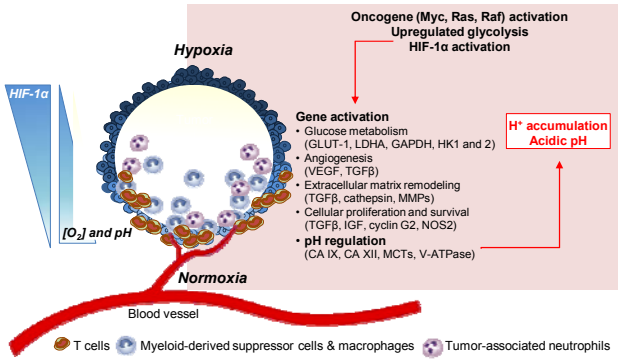

Figure 2

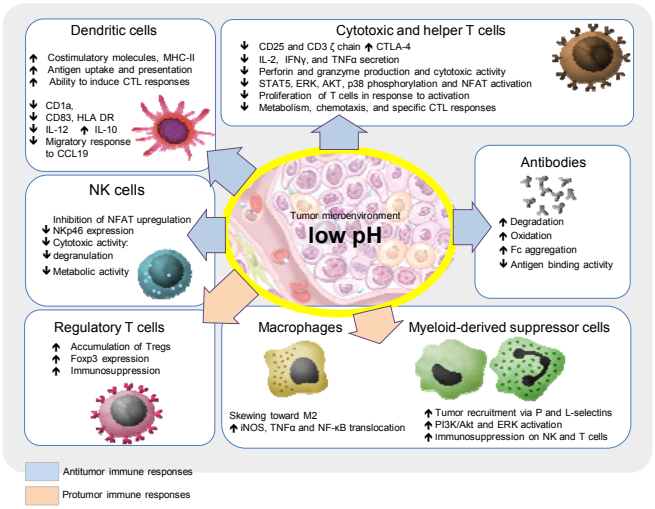

Figure 2

Figure 3

Table 1

Effects of microenvironment acidity on T lymphocytes

Species	Tumor/cell line/ pathology	Cell Type	Effects of acidic pH compared to physiological pH and key mechanism proposed	Ref.
Human	MEL 15 primary melanoma cells	T lymphocytes	pH_e 6.6 (obtained by adding HCl to the culture medium): -Impairs proliferation, CD25 and CD71 expression, and IL-2 and IFNγ production upon PHA and anti-CD3 stimulation -Upregulates IFN-γ2R and CTLA-4 upon PHA stimulation -Blocks the ability of T cells stimulated with allogeneic MEL15 cells to differentiate into specific CTLs Anti CD28 mAb partly reverses low pH_e effects	[40]
Human	Multicellular tumor spheroids (MCTSs) of MelIm melanoma cells	Melan-A-specific $CD8^+$ CTLs Tumor spheroid-infiltrating CTLs	High extracellular concentration of lactic acid (added to cell culture medium or produced by MTCS), but not sodium lactate: -Inhibits CTL proliferation but does not modulate activation and maturation markers (CD25, CD69, CD71, TCRβ-chain, ICAM-1, HLA-DR, CD45RO, and CD95) -Increases CTL apoptosis (upon 24-hour incubation) -Reduces IL-2 and IFNγ production by CTLs and MCTS-Infiltrating CTLs -Decreases perforin and granzyme production and the cytotoxic activity of CTLs and MCTS-infiltrating CTLs -Impairs CTL metabolism and decreases intracellular pH by blocking endogenous lactic acid export and by inducing fast lactic acid uptake Acidification of culture medium with HCl exerts these effects only in part These suppressive effects are reversed by culturing CTLs at physiologic pH for 24 h or by blocking lactic acid production with oxamic acid (lactate dehydrogenase inhibitor)	[35]
Human	RCC 26 (HLA-A2+) and KT195 (HLA-A2-) Renal cell Carcinoma	Cytotoxic T effector clones TCR transduced effector $CD8^+$ T cells	High extracellular concentration of lactic acid (added to cell culture medium): -Inhibits CTL degranulation and TCR-induced cytokine production (IL-2, IFNγ, and TNFα) -Strongly decreases p38 and JNK phosphorylation, but does not affect MEK1, ERK, or AKT phosphorylation These effects are rapidly reversed by incubation in medium at physiologic pH	[49]

Mouse	EL4 thymoma and P815 mastocytoma cell lines	Specific CTL lines (from splenocytes of previously immunized mice)	High extracellular concentration of lactic acid (added to cell culture medium): -Does not induce apoptosis and does not modulate CD25, CD69, and PD-1 expression -Strongly reduces IFNγ and granzyme B secretion and specific cytolytic activity against target cells -Impairs the induction of functional antigen-specific CTLs from memory T cells -Abrogates the increase in the induction of CTLs mediated by hypoxia These effects are reversed by neutralization of medium culture with NaOH	[42]
Human and mouse	Reumathoid Arthritis Mouse model of peritonitis	$CD8^+$ and $CD4^+$ T cells (both human and murine)	High extracellular concentration of lactic acid (added to cell culture medium): -Inhibits CXCL10 and RANTES-induced chemotaxis of activate $CD8^+$ T cells independently of glycolysis inhibition -Reduces cytolytic activity of CD8+ T cells against allogeneic endothelial cells -Blocks CD8 T cells in inflammatory sites Sodium lactate or acidification by HCl do not affect the motility and cytolytic activity of $CD8^+$ T cells, but sodium lactate inhibits basal or chemokine induced chemotaxis of $CD4^+$ T cells through interference with glycolytic pathway	[50]
Human and mouse	Stage IV melanoma (human) and B16 melanoma (mouse)	Human and murine TILs $CD3^+$ T cells from HD and Ag -specific $CD8^+$ T cells from melanoma patients	Acid pH_e (6.6): -Does not affect cell viability, but reduces proliferation upon anti CD3/anti CD28 stimulation -Strongly decreases the expression of CD25 and CD3 ζ-chain, perforin degranulation, and the release of IL-2, IFNγ, and TNFα upon stimulation with mitogens, CD3/anti CD28, or autologous tumor cells -Impairs STAT5 and ERK phosphorylation in response to activation T cell functions are restored by a 24-hour recovery period at physiologic pH Esomeprazole improves TIL recruitment and restore TIL effector functions in melanoma bearing mice by buffering of tumor pH	[41]
Mouse	B16 melanoma, Yumm 1.1 melanoma, and Panc02 pancreatic cancer	$CD8^+$ T cells (from Pmel and OT-I mice) $CD4^+$ T cells (from OT-II mice)	Acid pH_e (6.6): -Does not alter cell viability and pH_i -Reduces IFNγ production by specific $CD8^+$ and $CD4^+$ T cells -Decreases glycolysis and increases oxygen consumption upon T cell stimulation These effects are reversible by culturing T cells at physiologic pH for 24h Bicarbonate treatment increases TIL recruitment and improves the effects of adoptive cell transfer and checkpoint inhibition (PD-1 and CTLA-4) by increasing tumor pH in murine models	[43]

Mouse	LDHA^{low} and control B16.SIY mouse melanoma LDHA^{null} and control Panc02-H7 mouse adenocarcinoma	Mouse CD8^{+} T lymphocytes and NK cells	High extracellular concentration of lactic acid (added to cell culture medium), but not sodium lactate: -Diminishes cytokine production (IL-2 and IFNγ) but does not modulate activation or exhaustion markers by activate CD8^{+} T lymphocytes -Causes intracellular acidification through increase of lactic acid uptake and blockage of lactic acid export -Impairs energy metabolism, decreases AKT and p38 phosphorylation, and inhibits the up-regulation of NFAT upon activation Acidification with HCl reduces cytokine secretion, although to a lesser extent than lactic acid CD8 T cells co-cultured with LDHA^{low} tumor cells produce more cytokine than CD8 T cells co-cultured with control tumor cells LDH-A ^{low} tumors contain higher percentages of IFNγ and granzyme B producing CD8^{+} T lymphocytes	[44]

Abbreviations: Ag antigen; pH_e extracellular pH; pH_i intracellular pH; ICAM intercellular adhesion molecule