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Review Article

Key indexes and the emerging tool for tumor microenvironment editing

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Abstract: Many cancer management approaches including immunotherapies can not achieve desirable therapeutic efficacies if targeting tumors alone or could not effectively reach tumor cells. The concept of tumor microenvironment and its induced gene reprogramming have largely extended our current understandings on the determinants of tumor initiation/progression and fostered our hope in establishing first-line therapies targeting cancer microenvironment or adjuvant therapies enhancing the efficacies of existing oncotherapeutic modalities such as immunotherapies for efficient cancer management. This review identifies key indexes of tumor microenvironment, i.e., hypoxia, acidosis, hypo-nutrition and inflammation, which collectively determine the feature and the fate of adjacent tumor cells, and proposes cold atmospheric plasma, the fourth state of matter that is largely composed of various reactive oxygen and nitrogen species, as a promising tool for tumor microenvironment editing. We propose that cold atmospheric plasma represents an emerging onco-therapeutic strategy alone or complementing existing treatment approaches given its multi-modal nature through tumor microenvironment modulation.

Keywords: Tumor microenvironment, cold atmospheric plasma, reactive oxygen and nitrogen species, microenvironment editing

Introduction

Tumor microenvironment (TME) refers to the environment where a tumor originates. It dynamically alters during carcinogenesis and constantly interchanges signals and biomasses with tumors. Tumors can edit TME by, e.g., promoting tumor angiogenesis, creating metabolic symbiosis with stromal cells, and inducing peripheral immune tolerance, while immune cells in the microenvironment can affect the proliferation and evolution of cancerous cells [1]. The critical roles of TME and its interplay with tumors during cancer initiation and progression such as vascularization and immunosuppression [2, 3] have been increasingly recognized. Understanding the unique features of TME not only helps us create desired efficacies from conventional anticancer therapies (e.g., immunotherapies [4]), but also leads us to the identification of novel onco-therapeutic targets and treatment possibilities. We are thus motivated to comprehensively review key indexes for TME measurement, based on which we propose cold atmospheric plasma (CAP), incompletely ionized plasma and the fourth state of matter besides solid, liquid and gas, as an emerging first line or adjuvant approach for cancer control given its efficacies in TME editing.

Factors influencing tumor microenvironment

Hypoxia

Hypoxia, being one of the most characterized properties of TME, arises in tumors through excess tumor mass resulting from uncontrolled tumor cell proliferation and insufficient oxygen supply. Hypoxia, in turn, up-regulates the production of angiogenic factors and triggers the vascularization of the tumor mass, resulting in tumor angiogenesis [5]. Unlike normal vascularization, vessels formed from tumor angiogenesis have chaotic architecture that often lead to vascular leakiness and non-laminar blood flow [6]. Therefore, the functionalities of these abnormally generated vessels are not guaranteed,

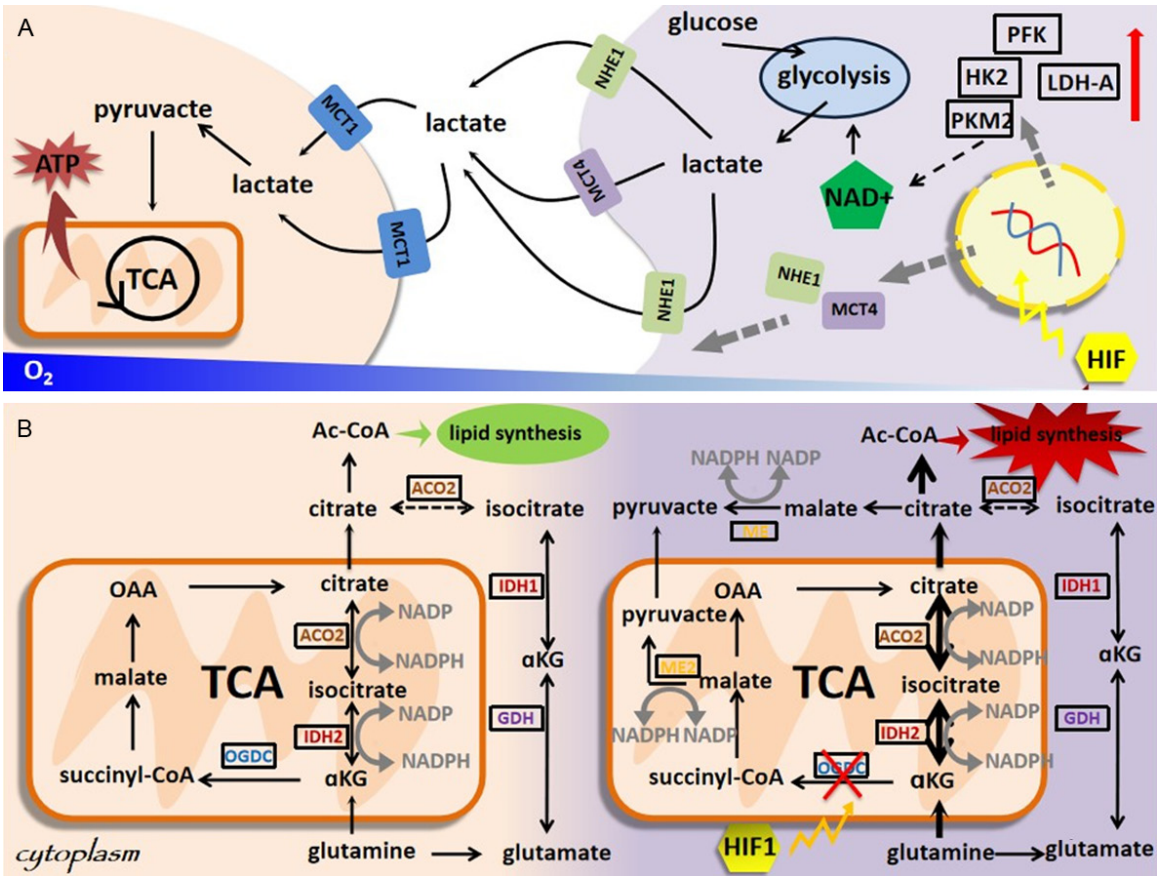

Figure 1. Hypoxia is a characterized feature of tumor microenvironment. Hypoxia can (A) rewire the glucose metabolic fate of cancer cells, and (B) alter glutamine flux. In (A), under hypoxic conditions, glycolytic rate is enhanced by up-regulating and/or activating a series of glycolysis-stimulating enzymes such as PFK, HK2, PKM2 and LDH-A, several acid and/or lactate-extruding transporters such as MCT4 and NHE1 are up-regulated by hypoxia through HIF-1α signaling, leading to the secretion of lots of lactates and other acid equivalents by tumor cells. Metabolic coupling occurs between cancer cells in hypoxic and well-oxygenated tumor regions, and between cancer and stromal cells, i.e., lactates produced by hypoxic tumor cells are taken up via MCT1 in normoxic cancer cells, followed by conversion to pyruvate, sparing the limited supply of glucose for the hypoxic tumor regions. In (B), despite decreased mitochondrial respiration and increased activity of reductive carboxylation, hypoxic cells can maintain and in some cases even up-regulate oxidative glutamine metabolism, accounting for the majority of ATP synthesis through oxidative phosphorylation under hypoxic conditions.

and typically subjected to alterations in the direction and velocity of the flow that likely to lead to blood clotting and local tissue oedema [7, 8]. Adaptation to hypoxia is primarily mediated through transcription factor hypoxia-induced factors (HIFs). There are two forms of HIFs, HIF1α and HIF2α, each being composed of a β subunit and an oxygen-labile α subunits that distinguishes the two HIFs. The α subunit is rapidly degraded through PHD-mediated hydroxylation following pVHL-dependent ubiquitylation under normoxic conditions [9-11]. HIFs regulate the expression of target genes playing critical roles in, e.g., tumor angiogenesis and metabolic adaptations through recognizing hypoxia-responsive elements located in either proximal or distal to their promoters [12-16].

Hypoxia can rewire the glucose metabolic fate of cancer cells (**Figure 1A**). Under hypoxic conditions, glycolytic rate is enhanced by up-regulating and/or activating a series of enzymes boosting glycolysis including, e.g., lactate dehydrogenase A (LDH-A), pyruvate kinase M2 (PKM2), hexokinase II (HK2), and phosphofructokinase (PFK) [17, 18]; several transporters extruding acids and/or lactates such as monocarboxylate transporter isoform 4 (MCT4) and the Na^+/H^+ exchanger NHE1 are up-regulated by HIF-1α [19, 20], leading to the secretion of vari-

ous acid equivalents including lactates by tumor cells; these lactates produced by hypoxic tumor cells are taken up by normoxic cancer cells via MCT1 and spared for tumor energy and biomass supply [21], resulting in metabolic coupling between cancer cells located in differentially oxygenated regions, and between cancer and normal cells.

Hypoxia can alter glutamine flux (**Figure 1B**). Being essential to the anabolism of most cells [22], glutamine is oxidized through both the tricarboxylic acid cycle (TCA) and anabolic building blocks to support cell proliferation under normoxic conditions. Once exposed to hypoxia, glutamine flux is decreased due to reduced pyruvate oxidation and mitochondrial respiration, glutamine oxidation dropped which can be mimicked in cells with dysfunctional mitochondria [23-26], reductive carboxylation of glutamine-derived alpha-ketoglutarate (αKG) occurs in response to increases in the αKG/citrate ratio to produce sufficient citrate for lipid synthesis [27-31]. It is worth mentioning that hypoxic cells can maintain or even up-regulate glutamine oxidation in some cases to make cells generate ATP mostly via oxidative phosphorylation under hypoxic conditions, regardless of reduced mitochondrial respiration and enhanced reductive carboxylation [32-34].

Acidosis

Tumor acidosis has been gaining increased recognition as another major TME index. Tumor acidosis mainly results from lactic acid excretion and CO_2 hydration. H^+ ions may alter the functionalities of various proteins by influencing the ionization of some of their amino acid residues [35], underlying a delicate balance between the intracellular pH (pH_i) and the extracellular pH (pH_e) that represents the metabolic features of a given cohort of cancer cells.

Acidosis alters the glucose metabolism of cancer cells. A lactate gradient is generated to adapt to TME acidosis, where the most hypoxic tumor areas has the lowest pH. While lactates are released through MCT4 from cancer cells as the end-products of glycolysis, other cancer cells can capture lactates via MCT1 and convert them into pyruvates [21, 36-40], resulting in metabolic symbiosis [23, 41-49]. The fact that H^+ ions need to be co-transported with lactates for inward flux boosts the requirement on neutralizing intracellular H^+ for sustainable lactate shuttle, where carbonic anhydrase helps solving this problem by neutralizing H^+ ions and hydrating CO_2 (**Figure 2A**). Using lactates secreted from cancer cells to support the TCA cycle and fatty acid synthesis of other cancer cells is one important survival strategy for cancer cells under low glucose availability. First, local acidification plays promotive roles on tumor invasion [50], partly through increasing extracellular levels of VEGF-A [51, 52] and proteases [53, 54]. Second, lactates can be used as energy substrate by adjacent stromal cells to support growth or produce pyruvates that are extruded to TME and taken up by cancer cells [55, 56].

Lipid metabolism is also metabolically reprogrammed under acidosis (**Figure 2B**). Acidosis can rewire the fates of citrates and/or acetates toward acetyl-CoA production and ultimately fatty acid synthesis, accompanied with fatty acid oxidation [41, 42]. It is worth noting that though these two fatty acids synthesis supporting pathways are also stimulated in response to hypoxic stress [25, 26, 43, 44], fatty acid oxidation is only concomitantly activated under acidosis. In many cancers such as colon, oropharyngeal and cervical tumors, fatty acid synthesis and oxidation are simultaneously promoted under acidosis by down-regulating ACC2 [45]. Fatty acid oxidation derived acetyl-CoA can fuel the TCA cycle and lead to a sharp increase in the non-enzymatic acetylation of many proteins, including electron transport chain (ETC) complex members [45]. However, restraining the activity of acetylated ETC complex I could limit the production of reactive oxygen species (ROS) that enhances the fitness of cancer cells, and reduced activities of acetylated ETC complex I has been measured in various types of cancer cells adapted to acidosis [45].

Hypoxia is advantageous for tumor cells to survive under acidosis exposure [47]. Alteration in the key pH_i regulating proteins under hypoxia provides tumor cells with survival advantages over normal cells [48]. The hypoxia-induced transcription factor HIF2α plays critical roles in the regulation of metabolic adaptation to acidosis [41, 46, 57, 58], and the activity of the *bona fide* HIF1α is generally down-regulated under such conditions [41, 59, 60]. Acidosis leads to increased activities of NAD-dependent protein

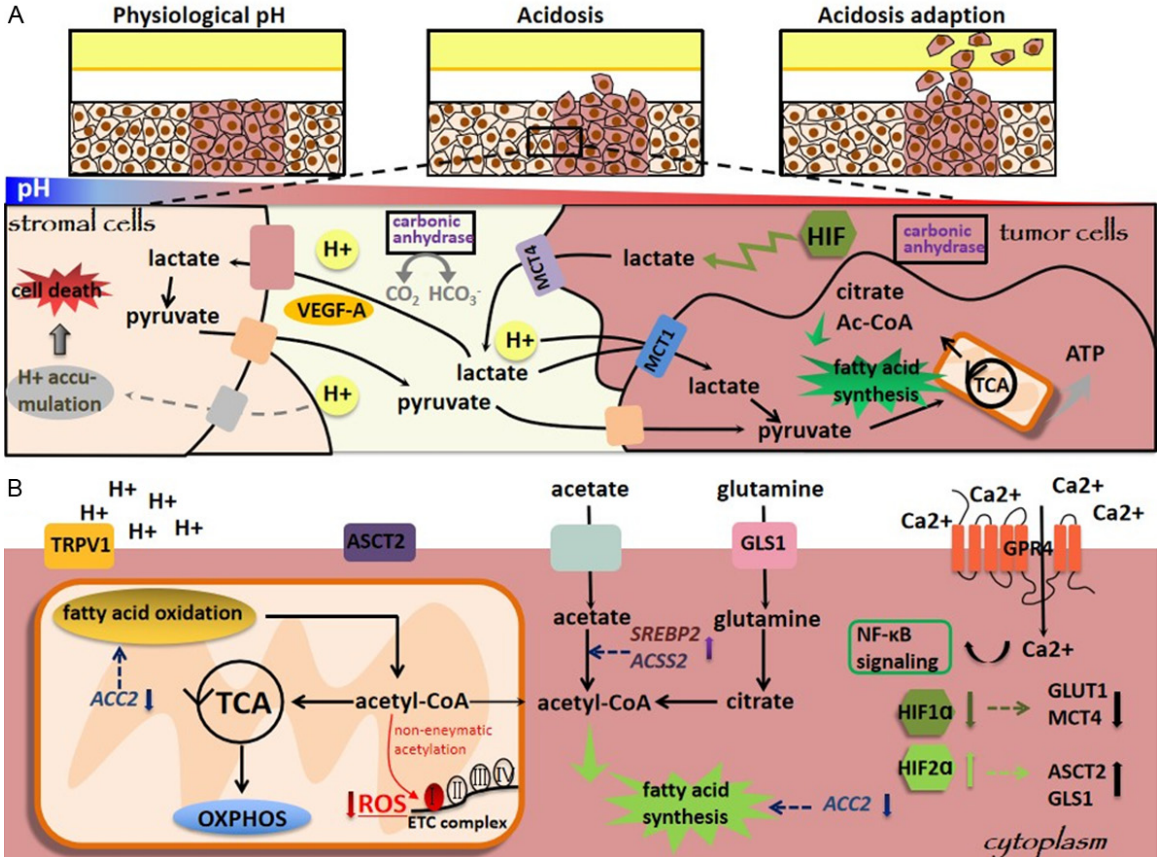

Figure 2. Tumor acidosis is a major tumor microenvironment index. Acidosis alters (A) the glucose and (B) lipid metabolism of cancer cells. In (A), acidosis adaption occurs, i.e., generating a lactate gradient, with the highest concentration being in the most hypoxic tumor areas, following metabolic symbiosis, i.e., while lactates are released through MCT4 from cancer cells as the end-products of glycolysis, other cancer cells can capture lactates via MCT1 and convert them into pyruvates. In (B), both fatty acid synthesis and fatty oxidation are enhanced under acidosis, where fatty acid synthesis is mediated via the production of acetyl-CoA from citrates and acetates.

deacetylase sirtuin-1 (SIRT1) and -6 (SIRT6) which differentially affect the activities of both HIFs [52, 61, 62]. While deacetylation of lysine residues in the amino-terminal transactivation domain of HIF2α is associated with its increased regulatory activities [62], similar deacetylation in HIF1α represses its transcriptional functionalities [63]. In cancer cells of the cervix, colon and pharynx, Up-regulated HIF2α and down-regulated HIF1α could drive the switch from glucose to glutamine metabolism on acidosis exposure via elevating the expression of the glutamine transporters ASCT2 and GLS1 (regulated by HIF2α) [41] and reducing the expression of the glucose transporters GLUT1 and MCT4 (modulated by HIF1α).

Various types of pH sensors function in sensing extracellular acidosis and transducing it into intracellular reprogramming [64]. G protein-coupled receptors (GPCRs) such as GPR4, GPR65 and GPR68 could be activated if the histidine residues of their extracellular domains were protonated [65], and transduce signals via activating various pathways such as phospholipase C and adenylyl cyclase through the use of different G proteins [66] (**Figure 2B**). Non-GPCRs such as transient receptor potential cation channel subfamily V member 1 (TRPV1) and acid-sensing ion channel 1 (ASIC1) can also sense extracellular acidosis [66]. Calcium influx, under acidosis exposure, directly or indirectly opens these channels that activates NF-κB signaling in, e.g., breast [67] and prostate [68] cancers. Alterations in pH can also be detected through the protonation of various signalling proteins [35, 69]. For instance, the R337H substitution in the tetramerization domain of TP53, a famous tumor suppressor, under increased pH_i can result in DNA

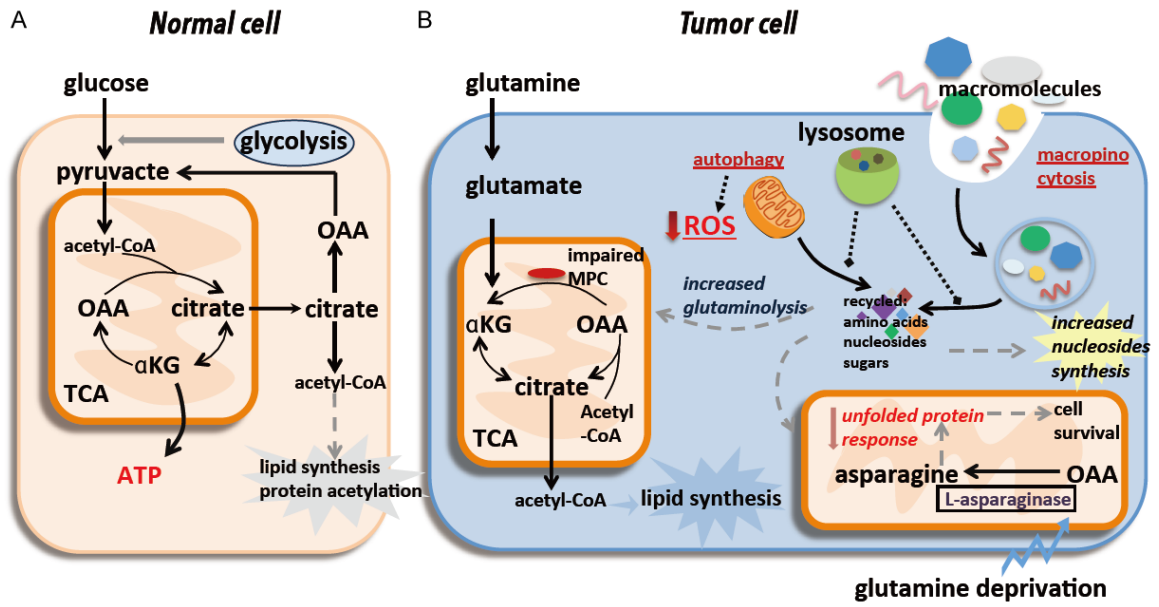

Figure 3. Hypo-nutrition is characterized in tumor microenvironment. Tumors (A) use glutamine to fuel alternative forms of metabolism and (B) activate autophagic degradation of macromolecules under hypo-nutrition. In (A), glucose deprivation in cancer cells stimulates a pathway whereby glutamine carbon is re-routed to acetyl-CoA. In (B), autophagy functions in degrading damaged organelles and their macromolecular components to provide recycled small molecule nutrients to feed intermediary metabolism under hypo-nutrition, and functions to eliminate defective mitochondria, thereby reducing ROS accumulation and improving cellular fitness.

binding inhibition [70]. Unveiling various types of pH sensors and altered signaling pathways in response to their activation can significantly advance our understandings towards tumor acidosis and avail in any oncotherapeutic design targeting tumor acidosis and the associated metabolic reprogramming.

Hypo-nutrition

As nutrient supply cannot typically support the growth of tumor cells due to the rapid expansion of tumors and relatively poor angiogenesis, cells residing in the inner part of tumors typically suffer from hypo-nutrition. Warburg effect, the observation that cancer cells favor anaerobic glycolysis rather than the more energy-efficient aerobic glycolysis suggests that tumor cells require enhanced nutrient supply to support their increased metabolism and biosynthesis than normal cells. To survive under hypo-nutrition, cancer cells show extreme metabolic flexibilities. It was recently reported both *in vitro* and *in vivo* that, glucose deprivation imposes a selective pressure for *KRAS* mutations in colon cancer cells [71]. Similarly, cancer cells are capable of rewiring their metabolism towards the use of alternative nutrients to compensate for the loss of, e.g., glucose [32, 72-75].

Glutamine can complement the synthesis of acetyl-CoA as an alternative of glucose [76], which is crucial for cell survival on lack of glucose (**Figure 3**). For instance, converting glutamine to lactate can produce sufficient NADPH that is needed for the synthesis of fatty acids [39]. Lymphoma cells with Myc over-expression can reroute glutamine carbon to produce acetyl-CoA once deprived of glucose [32], and such metabolic reprogramming can also be produced by silencing the mitochondrial pyruvate carrier (MPC) [77, 78]. Therefore, metabolic vulnerability as such is required and glutamine oxidation is indispensable for tumor cells to survive if MPC was impaired [78].

Maintaining an asparagine pool provides survival advantages to tumor cells when deprived of glutamine. For example, citrate synthase maintains the functionalities of the TCA cycle by condensing glutamine-derived OAA with acetyl-CoA under normal conditions [76], which was found to be lost once deprived of glutamine [79]; further, shunting OAA towards asparagine rather than citrate was shown to be favorable

for cell survival [79]. On the other hand, asparagine synthetase expression is positively associated with poor prognosis in cancers such as glioma and neuroblastoma [79], providing an *in vivo* evidence of our notion that an asparagine pool favors cancer cell survival.

Macropinocytosis adds further metabolic flexibilities to cancer cells under glutamine deprivation. Macropinocytosis enables cells to scavenge fluid and macromolecules, where extracellular proteins were important cargoes captured and internalized in macropinosomes, and these proteins provide starved cells with materials to generate pools of glutamine and other amino acids required for survival [80]. It was reported that glutamine deprivation could stimulate macropinocytosis in cancer cells expressing *Ras* [80]. Therefore, macropinocytosis provides another mode of metabolic flexibility for cancer cells to overcome hypo-nutrition.

Cancer cells can recycle molecule nutrients by degrading macromolecules through autophagy under hypo-nutrition [81-84] (**Figure 3**). During autophagy, damaged organelles are degraded and recycled to feed intermediary metabolism [83, 85, 86]. Autophagy can also reduce cellular redox level by eliminating defective mitochondria, leading to improved cellular fitness. Autophagy was shown to be required in *Kras*-driven pancreatic tumors [87] and *BRaf*V600E lung tumors [88] for maximal growth, and both autophagy and normal mitochondrial function were needed in forming aggressive *Kras*-driven carcinomas [89]. Therefore, autophagy provides tumor survival advantages through extracellular nutrient supply and intracellular nutrient recycle.

Inflammation

Inflammation is a defensive response against foreign invasion or in response to physical and chemical hazards [90, 91]. A clear evidence between inflammation and tumorigenesis was established in the last decade [92], and tumor-associated inflammation was implicated as a enabling cancer hallmark in 2011 [93].

Tumor-associated inflammation is characterized by the presence of a large amount of leukocytes in tumor tissues and high expression of inflammatory mediators in TME (**Figure 4**). Macrophages in the TME are composed of tumor-associated macrophages (TAMs) and monocytes recruited from blood vessels, where TAMs have two phenotypes, i.e., M1 and M2 macrophages which are pro-inflammatory and anti-inflammatory, respectively [94]. M2 macrophages are polarized by factors derived from tumors to sustain tumor proliferation and enable immunosuppression [95-97]. Cancer-associated fibroblasts (CAFs) are originated from normal fibroblasts with acquired characteristics similar to myofibroblasts. Both TAMs and CAFs are abundant in a TME and play crucial roles in tumor initiation, progression, evasion, and chemotherapeutic resistance [98].

Tumor-associated inflammation is a chronic process that fosters tumor progression [99] (**Figure 4**). During tumorigenesis, cancer cells, TAMs, CAFs or other innate immune or activated resident cells produce a variety of chemokines and cytokines such as interleukins and interferons in response to signals originated from tumor cells. Cytokines are major players in chronic inflammation, and are indispensible throughout the whole process of cancer initiation and progression mediated by inflammation [100]. For example, cytokines can activate and constitute the so-called cytokine storm (a type of systemic inflammatory response which can be caused by infection or adverse effect of some immunotherapies) by recruiting massive amounts of additional bone marrow-derived innate immune cells [101]; and this prolonged reaction favors immunosuppression via accumulating myeloid suppressive cells and inhibiting effector immune cells, and promotes tumor cell proliferation as well as angiogenesis [102]. With our increased knowledge on signalings involved in tumor inflammation and its crosstalk with TME, we will be able to create more effective immunotherapies with little side effects by concomitantly modulating TME [103, 104].

Cold atmospheric plasma targets tumor microenvironment indexes

CAP is a near room temperature ionized gas comprised of various reactive species, such as charged particles, neutral gas molecules, UV radiation, localized electric, reactive species and so on [105-107]. Dominant radical sources are reactive oxygen and nitrogen species (RONS) formed from oxygen and nitrogen molecules [105]. These complicated substances

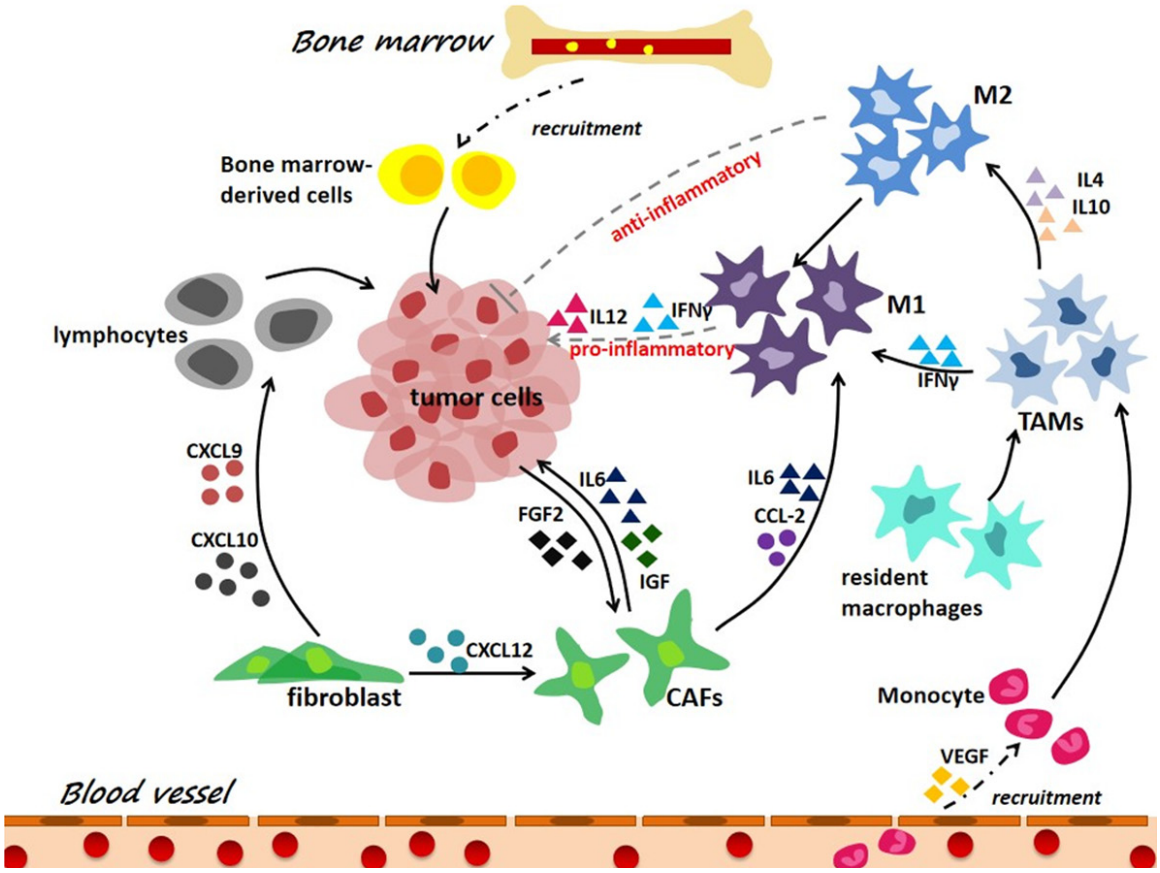

Figure 4. Tumor associated inflammation is an essential mark of tumor microenvironment. Tumor-associated inflammation is characterized by a high number of leukocytes in tumor tissues, and high expression of inflammatory mediators in TME, where tumor-associated macrophages (TAMs) have two phenotypes, i.e., M1 macrophages (pro-inflammatory) and M2 macrophages (anti-inflammatory). During tumor-associated inflammation, innate immune cells such as TAMs and activated resident cells such as CAFs produce a variety of chemokines and cytokines in response to the danger signals originated from tumor cells.

lead to numerous interactions between CAP and cells or tissues [107, 108], and triggers complex chemical kinetics. RONS have been implicated in tumor microenvironment modulation [109]. CAP has been proposed as a promising oncotherapeutic approach [106, 110, 111], which largely relies on its ability in tumor microenvironment modulation apart from its other functionalities controlling cancer progression (**Figure 5**).

Cold atmospheric plasma reduces hypoxia

Hypoxia is a major cause of cancer cell resistance to some treatment modalities such as radiotherapy. CAP can reduce hypoxia, where a rapid fourfold increase in tissue oxygen partial pressure (pO2) was observed in mouse skin upon plasma treatment [112].

As oxygen is the terminal electron acceptor, electrons leak out from the mitochondrial electron transport chain under hypoxia [9, 10], which creates a redox stress in tumor mitochondria, CAP can create temporal openings of the cell membrane, usually over a microsecond time scale, to allow for the transportation of RONS into cells [113, 114]. Once entering cells, RONS increases cellular redox level that leads to selective death of cancer cells as the baseline redox level of malignant cells is higher than that of normal cells and cells undergo apoptosis when cellular redox level exceeds a certain threshold [115-118]. Redox change is quite often accompanied with signalings tilting apoptosis. CAP can induce ATM expression that phosphorylates p53 [119] and p73 [120], where phosphorylated p53 and p73 can induce mitochondria mediated apoptosis through acti-

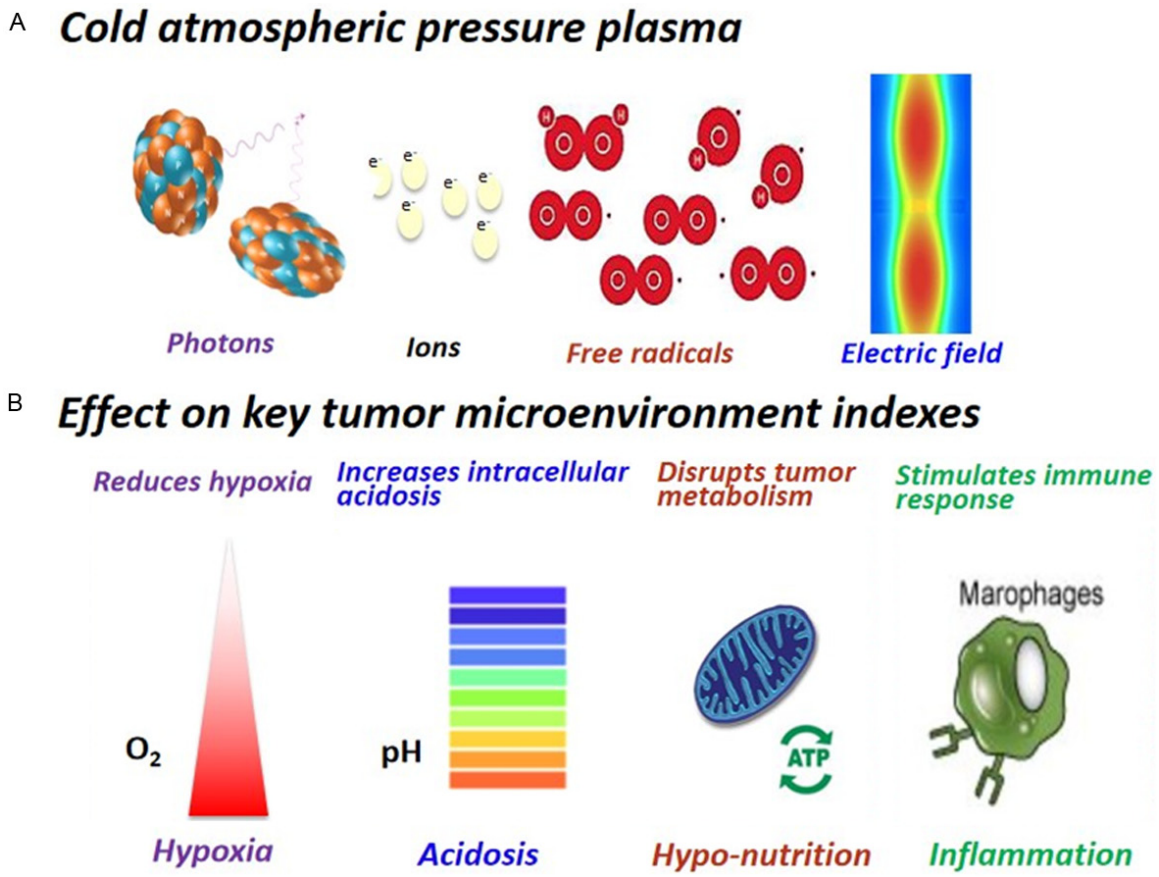

Figure 5. Cold atmospheric plasma composition and its effects on modulating key tumor microenvironment indexes. A. CAP is a cocktail therapy with multi-modality nature. It is composed of, e.g., free radicals, photons, ions and elective fields. B. CAP reduces hypoxia, increases intracellular acidosis, disrupts tumor metabolism and stimulates immune response.

vating the expression of pro-apoptotic factors such as Bax, PUMA, and NOXA [120, 121].

Cold atmospheric plasma increases intracellular acidosis

Reactive species generated by CAP at the gas-water interface triggers intricate reactions within the bio-system that often lead to acidification of the culture medium and ultimately increased intracellular acidosis. For example, with the increased dose of CAP, the pH of the cell culture medium might drop from 8.5 to 5.5 [122]. An alkaline pH_i was shown necessary for mechanisms involved in driving or facilitating cellular transformation and proliferation [123-125], as many intracellular metabolic enzymes, such as phosphofructokinase (the rate-limiting step of glycolysis), have alkaline pH optima [123]. As elevated pH_i is associated with both cell transformation and cell proliferation [126], increased intracellular acidosis, though does not necessarily lead to cell apoptosis [122, 127, 128], may arrest cell growth.

Cold atmospheric plasma disrupts tumor cell metabolism

Cancer cells adopt aerobic glycolysis to support their excess needs on biomass supply required for rapid growth [129]. Through whole metabolism profiling, CAP was found to suppress beta-alanine metabolism in myeloma cells [130]. Beta-alanine is crucial for acetyl-CoA synthesis that plays critical roles in TCA cycle and biomass production such as the synthesis of fatty acids, cholesterols and acetylcholines [112, 131]. Therefore, CAP can disrupt cellular metabolism favorable for tumor cell growth through, e.g., suppressing biomass production.

Cold atmospheric plasma stimulates immune response

Immunogenic cell death (ICD) can induce an effective antitumor immune response through activation of dendritic cells and consequent activation of specific T cell response [132]. ICD is accompanied by changes in the influx of many signal molecules on the surface of cell membrane, and the synthesis and release of immune-effector factors. These substances are called damage-associated molecular patterns (DAMPs), including early cell death calreticulin (CRT), release of heat-shock proteins, late cell death ATP and HMGB1 [133]. CRT is a main cause of ICD, which is capable of emitting 'eat me' signaling to mediate the engulfment of tumor cells by macrophages and dendritic cells [134] and recruiting immune cells to the tumor immune site to promote inflammation. ATP and HMGB1 are important signaling molecules of DAMPs, where extracellular ATP promotes secretion of key pro-inflammatory cytokines, activates macrophages that play an important role in anti-tumor effects. CAP can significantly enhance the secretion of CRT, ATP and HMGB1 in tumor tissues, inducing ICD [135, 136].

Macrophages are crucial mediators of cellular inflammation [135]. M1 macrophages are pro-inflammatory, including secrete pro-inflammatory cytokines and cytotoxic substances, and M2 macrophages are immunosuppressive and release anti-inflammatory cytokines [134]. Tumor cells can polarize macrophages into M2 to support tumor growth, and CAP can modulate this switch in reverse [136]. It was demonstrated that the expression of CD206 (marker of M2) decreased remarkably while iNOs (Marker of M1) significantly increased on CAP treatment [135, 136]. CAP has also been shown to upregulate the influx of INF-γ in cell culture supernatants derived from splenocytes [133], which play a key role in activating M1 macrophages. Altogether, M1 macrophages were enhanced to induce antitumor responses including secretion of cytokines such as TNFα, IL-1 and IL-6, kill tumor cells, and provoke inflammation in the tumor microenvironment upon CAP treatment. Other immune cells including monocytes and neutrophils have also been detected increased in CAP activated medium that lead to enhanced immune response against tumor cells [129].

Endoplasmic reticulum (ER) stress is a protective stress response of eukaryotic cells that reduces abnormal aggregation of intracellular proteins by activating unfolded protein response (UPR) [137]. UPR is coupled with inflammatory signaling pathways through various mechanisms including RONS, NFkB, and release of calcium ions in ER. ATF4 and STC2 are typical markers of ER stress, and CAP was shown capable of increasing ATF4 and STC2 generation that triggers inflammatory signaling [111].

Conclusion

Given the multi-modality nature, CAP has demonstrated its unique properties in TME modulation including, e.g., reducing hypoxia, increasing intracellular acidosis, disrupting tumor cell metabolism and stimulating immune response. Importantly, CAP can selectively target cancer cells [130, 138-140] with its safety being systematically validated by several studies [110, 140-143]. Thus, CAP represents a promising onco-therapeutic approach, alone or in combination with existing treatment modalities to achieve improved efficacies with little side effects.

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Disclosure of conflict of interest

None.

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