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Hypoxia Reduction Sensitizes Refractory Cancers to Immunotherapy

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Abstract

In order to fuel their relentless expansion, cancers must expand their vasculature to augment delivery of oxygen and essential nutrients. The disordered web of irregular vessels that results, however, leaves gaps in oxygen delivery that foster tumor hypoxia. At the same time, tumor cells increase their oxidative metabolism to cope with the energetic demands of proliferation, which further worsens hypoxia due to heightened oxygen consumption. In these hypoxic, nutrient-deprived environments, tumors and suppressive stroma evolve to flourish while antitumor immunity collapses due to a combination of energetic deprivation, toxic metabolites, acidification, and other suppressive signals. Reversal of cancer hypoxia thus has the potential to increase the survival and effector function of tumor-infiltrating T cells, as well as to resensitize tumors to immunotherapy. Early clinical trials combining hypoxia reduction with immune checkpoint blockade have shown promising results in treating patients with advanced, metastatic, and therapeutically refractory cancers.

VASCULAR DISRUPTION AND ELEVATED OXIDATIVE METABOLISM ESTABLISH AND MAINTAIN TUMOR HYPOXIA

Hypoxia is a predominant feature of rapidly proliferating, aggressive solid tumors and leads to poor outcomes across a wide range of cancers by mediating resistance to chemotherapy, radiotherapy (RT), and immunotherapy (1, 2). Tumor hypoxia results from a combination of disrupted oxygen supply from disordered vasculature and excessive oxygen consumption by tumor cells. In contrast to healthy blood vessels, tumor vasculature is distorted, resulting in perfusion-limited as well as diffusion-limited hypoxia. While perfusion-related hypoxia occurs due to structurally and functionally abnormal vasculature that is ineffective in oxygen delivery, diffusion-related hypoxia is a result of increasing diffusion distances (>70 μm) of oxygen to continuously growing tumor cells (3). Indeed, solid tumors are not homogeneously hypoxic but instead are mosaics of high and low oxygen tension representing diverse regions of metabolic stress and immune function (**Figure 1**) (4). To adapt to hypoxic stress, tumor cells rely on the expression of hypoxia-inducible

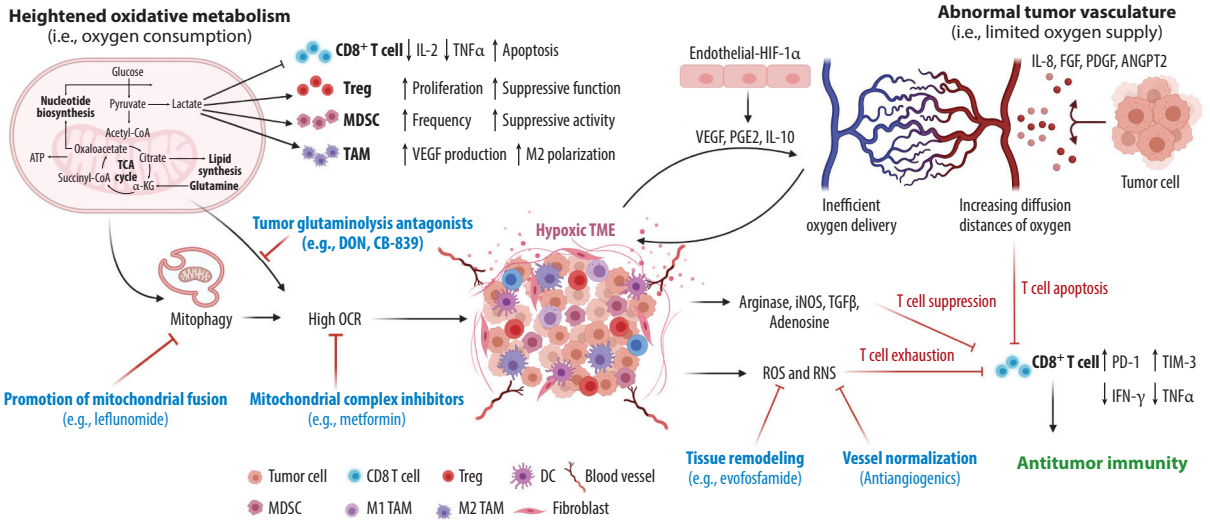

Figure 1

Disordered vasculature and excessive tumor oxidative metabolism perpetuate tumor hypoxia. Tumor hypoxia creates a cytokine milieu to sustain abnormal angiogenesis, resulting in a structurally and functionally dysfunctional vasculature that is ineffective in oxygen delivery. Rapid growth of tumor cells also increases diffusion distances of oxygen, further enhancing hypoxia. In addition to poor oxygen supply, excessive oxidative metabolism of tumor cells effectively outcompetes T cells for oxygen and nutrients, while generating toxic metabolites, such as lactate. In the metabolically hostile TME, $CD8^+$ T cells fail to thrive, exhibiting decreased proinflammatory cytokine production and undergoing apoptosis. In contrast, lactate supports the generation and suppressive polarization of Treg cells, MDSCs, and TAMs, resulting in both direct and indirect suppression of antitumor $CD8^+$ T cells. Inhibition of hypoxia through tissue remodeling (e.g., evofosfamide), vessel normalization (antiangiogenics), mitochondrial respiration inhibitors (e.g., metformin), or glutaminolysis inhibitors (e.g., DON) can reverse immune suppression in the TME. Specifically, levels of numerous mediators of immune suppression including arginase, iNOS, TGF-β, and adenosine diminish with hypoxia reduction, fostering a T cell permissive TME supportive of tumor regression. Abbreviations: ANGPT2, angiopoeitin-2; DC, dendritic cell; DON, 6-diazo-5-oxo-L-norleucine; FGF, fibroblast growth factor; HIF-1α, hypoxia-inducible factor 1α; IFN-γ, interferon γ; IL, interleukin; iNOS, inducible nitric oxide synthase; MDSC, myeloid-derived suppressor cell; OCR, oxygen consumption rate; PD-1, programmed cell death protein 1; PDGF, platelet-derived growth factor; PGE2, prostaglandin E_2; RNS, reactive nitrogen species; ROS, reactive oxygen species; TAM, tumor-associated macrophage; TCA, tricarboxylic acid; TGF, transforming growth factor; TIM-3, T cell immunoglobulin and mucin domain containing protein 3; TME, tumor microenvironment; TNFα, tumor necrosis factor α; Treg, regulatory T cell; VEGF, vascular endothelial growth factor. Figure adapted from image created with BioRender.com.

factors (HIFs), HIF-1α and HIF-2α, to drive an angiogenic switch, which is characterized by enhanced secretion of proangiogenic factors—vascular endothelial growth factor (VEGF), fibroblast growth factor, interleukin (IL)-8, and angiopoeitin-2 (5–7). The resulting blood vessels are tortuous and leaky, and they lack the adhesion receptors required for T cell extravasation. Despite their abnormal features, tumor neovascularization has long been associated with promoting tumor progression and metastasis (8, 9). Consistent with the role of hypoxia in vessel growth and cancer progression, tumor-bearing mice with HIF-1α-deficient endothelial cells (ECs) experience significantly reduced vascularization in the tumor bed with a corresponding reduction in tumor burden. This effect is mediated by deficits in VEGF secretion by ECs with concomitant disruption of VEGFR1 and VEGFR2 expression (10). Similarly, while EC-specific HIF-2α deletion increased formation of small vessels (<30 μm), these vessels failed to fully mature, resulting in poor tumor perfusion (11, 12). Despite differing roles of HIF-1α and HIF-2α in vascular remodeling, absence of either isoform in ECs led to a reduction in tumor burden, indicating that the tumor vasculature generated in the absence of HIF-1α or HIF-2α is inadequate and unable to support tumor progression.

Excessive fibrosis or desmoplasia in the tumor microenvironment (TME) also contributes to hypoxia both through secretion of angiogenic factors by cancer-associated fibroblasts (CAFs) and through excessive deposition of extracellular matrix that compresses blood vessels and exacerbates diffusion of oxygen (13). HIF-1 promotes secretion of transforming growth factor β (TGF-β), platelet-derived growth factor receptor β (PDGFR-β), and basic fibroblast growth factor, thereby driving differentiation of CAFs, which contribute to disorganized neovascularization in tumors. Coinjection of CAFs with tumor cells enhances tumor growth relative to coinjection with normal fibroblasts, supporting their role for vessel formation in tumor progression (14). Hypoxia induces HIF-1α- and HIF-2α-dependent transcription of integrin subunits α5 and β1 and procollagens, while regulating lipoxygenase enzymes that are critical for extracellular matrix remodeling by CAFs (15). Interestingly, a positive feedback loop exists between desmoplasia and hypoxia whereby pancreatic stellate cells, activated by hypoxia, differentiate into myofibroblasts, resulting in accelerated collagen deposition (16). Collagen deposition further exacerbates poor oxygen diffusion, driving hypoxia-exposed pancreatic cancer cells to secrete high levels of Sonic Hedgehog ligand, which then promotes further fibroblast-derived desmoplasia (17). This progressive cycle of desmoplasia and further worsening of hypoxia has been observed in numerous cancer types and plays key roles in metastatic spread and exclusion of immune cells from the tumor bed (18, 19).

Beyond disordered vasculature, there is a distinct role for tumor oxidative metabolism in maintenance of a hypoxic microenvironment and in resistance to immunotherapy. Oxygen consumption occurs primarily in the mitochondria, where the terminal stage of the electron transport chain (ETC) donates protons (H^+) and free electrons to oxygen atoms to generate H_2O and ATP. While aerobic glycolysis, or the Warburg Effect, was initially hypothesized to result from deficits in tumor mitochondrial function, we now appreciate that functional mitochondria are essential to tumor cell viability. Indeed, though aerobic glycolysis is thought to generate metabolic byproducts necessary to support rapid proliferation, disruption of glucose uptake fails to slow tumor progression or sensitize tumors to checkpoint blockade. Conversely, disruption of tumor oxidative metabolism through destabilization of mitochondrial complex I of the ETC improves oxygen tensions and infiltrating immune cell function and sensitizes aggressive tumor models to single-agent programmed cell death protein 1 (PD-1) blockade (20). Indeed, the rate of oxygen consumption by biopsied tumor cells corresponds with hypoxia burden and can predict clinical outcomes in patients receiving checkpoint blockade therapy (20–22).

HYPOXIA NUCLEATES AN IMMUNOSUPPRESSIVE TUMOR MICROENVIRONMENT AND DRIVES CELLULAR DYSFUNCTION

While hypoxia promotes immunosuppressive alterations in tumor and stromal cells, it also enforces significant changes in the tumor-infiltrating immune milieu with relevance to clinical outcomes. Tumor hypoxia increases the recruitment and/or polarization of immunosuppressive cell populations, including myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), and regulatory T (Treg) cells. Further, hypoxia accelerates the differentiation of tumor antigen–specific $CD8^+$ T cells to a dysfunctional fate known as terminal exhaustion (**Figure 2**) (22). Enrichment of these populations correlates with poor patient outcomes and a failure to respond to immunotherapy. We detail in this section the impact of hypoxia exposure on immune cells, and in the next section how therapeutically replenishing oxygen in the TME can augment antitumor modalities.

Adaptive Immune System

Hypoxia represents a formidable barrier to T cell infiltration and function within solid tumors. While HIF-1α stabilization has distinct positive roles in T cell activation (23), trafficking (24), and cytolytic functions (25, 26), excessive hypoxia enforces immune suppression both directly (21) and indirectly (27). Culturing activated $CD8^+$ T cells in vitro under hypoxic conditions enhances HIF-1α-dependent, antigen-specific tumor killing with the consequence of diminished secretion of proinflammatory cytokines, IL-2, and interferon γ (IFN-γ) (28, 29). Hypoxia also directly limits IL-2 mRNA production in naïve T cells (30) and induces their apoptosis through CCR7 inhibition (31). In vivo hypoxia-induced secretion of VEGF, IL-10, and prostaglandin E2 drives apoptosis of extravasating $CD8^+$ T cells through the Fas:FasL pathway (32), suggesting a role of hypoxia in immune exclusion from tumor. Conversely, hypoxia supports the peripheral generation of Treg cells through induction of FOXP3 expression (33) and increases their recruitment toward ovarian cancer cells in a CCL28-dependent manner (34).

Hypoxia-driven metabolic alterations play a prominent role in suppression of T cell responses. For example, through enhanced lactic acid secretion and carbonic anhydrase–mediated carbonic acid production, hypoxia drives down the extracellular pH into the acidic 5.8–6.5 range. While tumor cells continue to thrive under low pH, exposure of $CD8^+$ T cells to lactic acid blunts their viability, proliferation, cytolytic potential, and proinflammatory cytokine production (35–39). Importantly, increasing intratumoral pH rescues T cell suppression and increases IFN-γ expression in $CD8^+$ T cells (40). In contrast to $CD8^+$ and $CD4^+$ effector T cells, Treg cells are metabolically supported by the lactate-rich TME. Lactate supplementation can enhance Treg cell generation and suppressive activity (41, 42).

In addition to lactate, hypoxia also promotes the accumulation of the suppressive metabolite adenosine by mediating HIF-1α-dependent transcription of the extracellular ATP hydrolyzing ectonucleotidases CD39 and CD73 on Treg cells. Restriction of extracellular ATP limits T cell receptor (TCR) signaling and activation, while increasing free adenosine inhibits cytotoxicity via A2AR and A2BR receptors (43–46). Consistently, hypoxia inhibition by respiratory hyperoxia decreases adenosine accumulation and improves T cell infiltration (47). Apart from adenosine, nitration of TCR subunits and associated CD8 by reactive nitrogen species generated under hypoxia disrupts MHC:antigen complexing (48). In addition, nitration of chemokines such as CCL2 inhibits recruitment of effector T cells while retaining myeloid cell recruitment (49).

Persistence of $CD8^+$ T cells in the TME enforces progressive loss of mitochondria and oxidative metabolism over time (50). Chronic exposure to cognate antigen drives constitutive TCR signaling, which blunts PGC1α-mediated mitochondrial biogenesis and results in inefficient

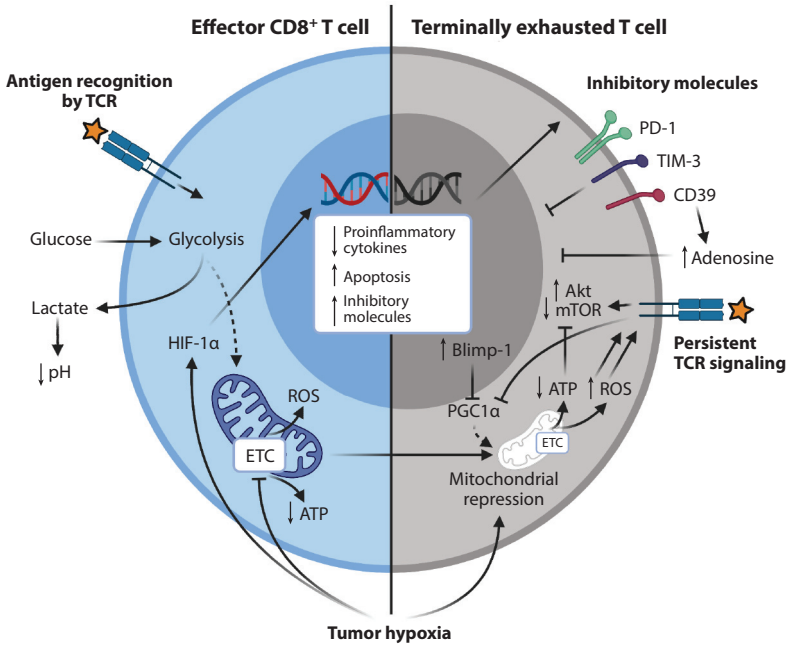

Figure 2

Hypoxia suppresses effector CD8$^+$ T cell function and drives differentiation to terminal exhaustion. Tumor cells produce a metabolically stressful microenvironment that prevents effective antitumor immune responses in infiltrating CD8$^+$ T cells. Rapidly proliferating tumor cells with high oxidative metabolism restrict available oxygen and prevent effective propagation of the ETC in CD8$^+$ T cells, requiring a glycolysis-predominant program and enriching the tumor microenvironment with acidic metabolic byproducts. Constitutive HIF-1α activity, stabilized by low oxygen tensions and persistent TCR signaling, limits effective proinflammatory immune responses, predisposes CD8$^+$ T cells to apoptosis, and reinforces expression of inhibitory molecules (PD-1, TIM-3, CD39). CD8$^+$ T cells in the presence of tumor hypoxia and continuous TCR stimulation differentiate to terminal exhaustion, a state marked by low proliferative potential, poor tumor control, loss of polyfunctional cytokine production, and metabolic and epigenetic derangements. Terminally exhausted T cells maintain elevated expression of coinhibitory receptors PD-1 and TIM-3 and the ectoenzyme CD39. CD39 breaks down extracellular ATP and ADP to AMP, which can be further catabolized to the immunosuppressive molecule adenosine. Persistent TCR signaling via Akt and upregulation of Blimp-1 in terminally exhausted CD8$^+$ T cells represses the function and expression of PGC1α, a critical transcriptional coactivator for mitochondrial biogenesis. Compounding hypoxia and loss of PGC1α drives progressive loss of mitochondrial respiration, further depleting intracellular ATP stores, restricting T cell activation, and inhibiting mTOR activity. Metabolically blunted, ineffectual, terminally exhausted T cells are incapable of controlling tumor progression but persist in the hypoxic, nutrient-deprived tumor microenvironment. Abbreviations: ATP, adenosine triphosphate; ETC, electron transport chain; HIF-1α, hypoxia-inducible factor 1α; mTOR, mechanistic target of rapamycin; PD-1, programmed cell death protein 1; PGC1α, peroxisome proliferator-activated receptor-γ coactivator 1α; ROS, reactive oxygen species; TCR, T cell receptor; TIM-3, T cell immunoglobulin and mucin domain containing protein 3. Figure adapted from image created with BioRender.com.

control of mitochondrial reactive oxygen species (ROS). An excess of ROS inhibits phosphatase activity, preventing appropriate control of kinase activity, and further propagates signaling downstream of the TCR. Continuous TCR signaling in the context of tumor hypoxia accelerates the differentiation of effector CD8$^+$ T cells to a terminally exhausted state, marred by epigenetic and transcriptional dysfunction, absence of polyfunctional cytokine production, and expression of immunosuppressive effector molecules—specifically CD39, IL-10, Fas, and cytolytic molecules

(**Figure 2**) (22). Indeed, in vitro exposure to continuous TCR stimulation in tumor-hypoxia conditions, induction of intracellular ROS, or inhibition of phosphatase activity can each recapitulate features of cellular exhaustion. Restoration of effector functions in terminally exhausted $CD8^+$ T cells represents a major barrier in immunotherapy. Significant efforts to restore functionality by checkpoint blockade have revealed that these modalities instead promote function in active effector $CD8^+$ T cells while failing to recover cells that have reached terminal exhaustion (51, 52). Rescue of tumor oxygenation, by targeted deletion of tumor mitochondrial complex I (20) or its inhibition with the antidiabetes drug metformin (21), can restore functionality in infiltrating $CD8^+$ T cells and resist their differentiation to terminal exhaustion. These effects can be recapitulated by normalization of tumor vasculature with the small-molecule tyrosine kinase inhibitor axitinib, which targets VEGF receptors 1, 2, and 3. Low-dose axitinib monotherapy has shown efficacy in multiple murine models of cancer through increased infiltration of T cells and a reduction of suppressive capacity in MDSCs (53, 54). These responses can be further improved with combination checkpoint blockade, oncolytic virotherapy (55), or RT (56).

Innate Immune System

MDSCs are a heterogeneous population of immature myeloid cells that are recruited to the TME and have potent immunosuppressive activity. Hypoxia in the TME plays a crucial role in both the recruitment and suppressive polarization of MDSCs. Hypoxia fosters a milieu rich in chemokines such as CCL2, CXCL8, and VEGF that enhance the recruitment of monocytic MDSCs, polymorphonuclear MDSCs (PMN MDSCs), and TAMs (57–59). Hypoxia in the primary tumor has been shown to drive PMN MDSC recruitment into the lung and drive the formation of a premetastatic niche (60). Hypoxia-induced CAIX expression is involved in this process through stimulating nuclear factor κB transcriptional activity and regulating granulocyte colony-stimulating factor production (61). In addition to driving recruitment, hypoxia also facilitates TAM retention. Tumor necrosis factor α produced in the hypoxic TME reduces CCR2 expression on TAM, preventing tumor egress (62) Hypoxia-driven inhibition of mitogen-activated protein kinase signaling (63) and downregulation of Semaphorin 3A (64) also facilitate TAM entrapment within the TME. Besides enhancing MDSC accumulation in tumors, hypoxia facilitates their suppressive reprogramming wherein immature myeloid cells infiltrating tumors differentiate toward MDSCs and TAMs, in contrast to their propensity to become proinflammatory macrophages and dendritic cells (DCs) in peripheral lymphoid organs. In this case, hypoxia-induced increases in CD45 tyrosine phosphatase activity and a resultant reduction in STAT3 activation contribute to this altered differentiation (65). In addition, lactic acid, generated as a metabolic consequence of hypoxia within the TME, plays an important role in polarizing TAM toward a protumorigenic, immunosuppressive M2 phenotype (66). Consistently, M2 TAMs are preferentially localized within hypoxic regions (67, 68).

Hypoxia influences multiple suppressive mechanisms of the tumor myeloid stroma. Tumor-derived MDSCs were found to be more suppressive than their splenic counterparts and to upregulate programmed death ligand 1 (PD-L1), arginase, and CD80 specifically in the TME (69). PD-L1 is a direct transcriptional target of HIF-1α, and blockade of PD-L1 was able to reverse hypoxia-driven T cell suppression by MDSCs in an IL-6- and IL-10-dependent manner (70). L-arginine is a crucial nutrient for T cell metabolic fitness and survival (71). MDSC-expressed arginase depletes L-arginine, diminishing T cell effector functions and antitumor immunity. HIF-1α-dependent arginase upregulation in MDSCs increases their suppressive function within the TME. Myeloid-specific HIF-1α knockout mice were able to revert MDSC suppressive functions (68, 72). The microRNA miR-210 is upregulated in hypoxia and is responsible for enhanced

arginase expression. Consistently, downregulation of miR-210 decreased arginase expression in MDSC and enhanced T cell effector functions (73).

In summary, hypoxia regulates both antitumor immune functions and the counterbalanced immunosuppressive populations in the TME. Hypoxia-driven alterations in cellular metabolism and transcriptional programs culminate in a hostile microenvironment wherein tumor-reactive T cells are forced to disadvantageously compete for nutrients while being inundated with suppressive metabolic byproducts, cytokines, and effector molecules.

RESTORATION OF INTRATUMORAL OXYGEN TENSION AUGMENTS ANTITUMOR IMMUNITY

Considering the detrimental impact of hypoxia on patient outcomes, multiple approaches to restore oxygen tension in tumor and to augment antitumor immune functions have been pursued. Since tumor hypoxia results from a combination of ineffective oxygen delivery and excessive oxygen consumption, strategies to improve oxygen supply while inhibiting heightened tumor oxygen metabolism are being investigated to overcome this adverse metabolic state.

Hypoxia-Activated Prodrugs

Hypoxia-activated prodrugs (HAPs) are prodrugs of antineoplastic agents that become activated under hypoxia by oxygen-inhibited enzymatic reduction. HAP efficacy depends on the presence of tumor hypoxia, the expression of 1e- and 2e- oxidoreductases capable of bioreductive activation of the prodrug, and the intrinsic sensitivity of the tumor to respond to the cytotoxic effector moiety released after prodrug activation (74). Evofosfamide (TH-302) is a HAP that undergoes bioreductive activation by cytochrome nicotinamide adenine dinucleotide phosphate P450 (POR) enzymes to release the cytotoxic effector molecule bromo-isophosphoramide mustard (Br-IPM), which can alkylate DNA of proliferating cells, leading to their apoptosis. Consistently, the presence of a proliferative gene signature and hypoxia correlate with better sensitivity to evofosfamide (75, 76).

We found that transplantable and spontaneous murine prostate tumors harbor regions of hypoxia that act as islands of immune privilege, excluding T cells while recruiting immunosuppressive MDSCs (77). Evofosfamide alone can diminish hypoxia in these tumors and reduce tumor growth. Importantly, addition of evofosfamide sensitizes these resistant tumors to immune checkpoint blockade therapy combining anti-CTLA-4 (cytotoxic T lymphocyte–associated protein 4) and anti-PD-1. In the spontaneous TRAMP (transgenic adenocarcinoma of the prostate) model, which mimics human prostate cancer in its resistance to immunotherapy, combining evofosfamide and checkpoint blockade drastically reduces tumor burden. Efficacy coincides with improvements in $CD8^+$ and $CD4^+$ T cell proliferation and effector function along with decreases in MDSC infiltration and suppressive polarization. Interestingly, combinatorial treatment leads to persistent defects in the ability of the tumors to replenish their myeloid stroma. In addition, $CD31^+$ vessel density increases in response to the combination therapy. These healthier, more complete vessels are likely responsible for reoxygenation of the tissue and resultant reduction of hypoxia and may improve T cell extravasation capacity (77). Consistent with these findings, evofosfamide has also been shown to reduce hypoxia in lymph nodes disseminated from an orthotopic head and neck squamous cell carcinoma (HNSCC) model and to augment CTLA-4 blockade in a syngeneic model (76).

While the efficacy of evofosfamide has been thought to be dependent on hypoxia and POR expression, CRISPR (clustered regularly interspaced short palindromic repeats) knockout and reductase-focused short hairpin RNA screens determined the presence of a functional ETC to

be important for evofosfamide activation. Interestingly, evofosfamide inhibits mitochondrial respiration in a dose-dependent manner, hinting at the possibility that the effect of evofosfamide on hypoxia reduction might stem from a combination of direct ablation of hypoxia regions and inhibition of excessive tumor oxygen consumption (78).

Systemic Oxygenation

Lack of oxygen in the TME confers resistance to RT, so combining oxygen delivery with RT has the potential to improve therapeutic responses. Accelerated RT combined with hyperoxic breathing (ARCON) is one such approach, where fractionated RT is combined with carbogen (95% O_2 + 5% CO_2) and nicotinamide to reduce diffusion-limited and perfusion-limited hypoxia, respectively. ARCON reduces hypoxia and improves tumor control. However, 95% O_2 is reported to cause oxygen toxicity and excessive nonspecific inflammation (79), highlighting a need for alternative approaches.

To circumvent the toxicity arising from carbogen, respiratory hyperoxia with 60% O_2 has been preferred to normalize oxygen tensions in the tumor bed. Tumor-bearing mice exposed to 60% O_2 achieve a lower tumor burden than those breathing atmospheric O_2 (21%). Hyperoxia reduces intratumoral hypoxia (80), improves effector $CD4^+$ and $CD8^+$ T cell infiltration, reduces Treg cell infiltration, and enhances response to CTLA-4/PD-1 blockade (47). It is key to note that this approach has been tested in pulmonary tumors with direct access to oxygen; it is not a universal approach to hypoxia ablation. In the case of peripheral tissues, strategies will need to rely on the use of heme-based protein carriers to deliver oxygen.

Heme-Based Oxygen Carriers

OMX-4.80P is an oxygen carrier protein that is derived from the heme-based nitric oxide/oxygen (H-NOX) family of bacterial proteins and has a tunable oxygen binding affinity. In contrast to hemoglobin-based oxygen carriers, OMX-4.80P releases oxygen specifically into highly hypoxic tissues such as tumors. Consistent with its function, OMX-4.80P has been shown to reduce hypoxia and improve $CD8^+$ T cell infiltration and function in a murine brain tumor model. Similar to the efficacy of antitumor immunity, OMX-4.80P monotherapy is reported to cure up to 55% of mice with early-stage GL261 brain tumors, comparable to PD-1 blockade therapy. In late-stage tumors, OMX-4.80P cooperates with PD-1 blockade in curing up to 40% of mice, compared to 5% with PD-1 blockade alone (81).

Mitochondrial Respiration Inhibitors

A complementary approach to hypoxia ablation is inhibition of excessive tumor oxidative metabolism. Murine tumors with elevated oxidative metabolism have exacerbated hypoxia, enrichment of a terminally exhausted signature in tumor-infiltrating lymphocytes, and resistance to checkpoint blockade. Perturbation of tumor oxidative metabolism in an aggressive murine melanoma, B16-F10, through disruption of mitochondrial complex I of the ETC significantly reduces hypoxic burden in the TME and improves T cell function in response to immunotherapy. Importantly, blockade of glycolytic metabolism via deletion of glucose transporter Glut1 failed to improve tumor hypoxia and response to immunotherapy (20). Consistently, inhibition of mitochondrial complex I with the antidiabetic drug metformin reduces tumor hypoxia and cooperates with PD-1 blockade to cure up to 70% of mice with B16-F10 melanoma. Importantly, metformin is not detrimental to T cell function and instead drives T cell metabolic fitness and effector functions (21). Metformin also reverts MDSC-mediated T cell suppression by downregulating the

expression of ectonucleotidases CD39 and CD73 on MDSCs. This improves survival and $CD8^+$ T cell–mediated immunity in ovarian cancer patients (82).

IACS 010759, another mitochondrial complex I inhibitor developed by MD Anderson Cancer Center's Therapeutics Discovery Team, shows therapeutic efficacy in acute myeloid leukemia and glioblastoma multiforme xenograft models by impairing tumor viability and reducing tumor hypoxia (83). In addition, the combination of IACS 010759 with RT can reverse resistance to PD-1 blockade in a non–small cell lung cancer xenograft model (84). A phase I clinical trial recently concluded at the MD Anderson Cancer Center (NCT03291938) encompassing patients with relapsed or refractory lymphoma, colorectal cancers, and castration-resistant and other prostate cancers reported evidence of antitumor activity, with 7/18 patients achieving RECIST (response evaluation criteria in solid tumors)-stable disease. The study has been expanded to identify the maximum tolerated dose (MTD) (85). Further validating tumor oxidative metabolism as a viable therapeutic target, IM-156, a mitochondrial complex I inhibitor developed by Immunomet, shows efficacy in preclinical models, is well tolerated, and was developed for a phase I clinical trial in patients with lymphoma and solid tumors (NCT03272256). This trial, which concluded in 2020, demonstrated modest clinical activity and no dose-limiting toxicities (86).

Hence, multiple approaches targeting hypoxia have seen success in sensitizing resistant tumors to therapy. It is noteworthy that a single approach might not work for all cancer types and, therefore, stratifying tumors according to their hypoxic and metabolic signatures is critical for evaluating therapeutic responses. In addition, tumors might evolve multiple overlapping strategies to generate hypoxia, necessitating combinations of hypoxia reduction approaches to achieve superior therapeutic efficacy.

CLINICAL OUTCOMES IN CONCURRENT IMMUNOTHERAPY AND HYPOXIA REDUCTION

Evofosfamide has seen success as monotherapy and in combination therapeutic regimens. Evofosfamide achieved single-agent efficacy at 480 mg/m^2 in HNSCC patients, with 2/5 patients achieving partial responses and 3/5 achieving stable disease, resulting in a disease control rate of 100% (76). In patients with refractory glioblastoma, evofosfamide plus bevacizumab, a monoclonal antibody that binds circulating VEGF, resulted in a disease control of 60.9% (87).

Based on our promising preclinical findings indicating the ability of evofosfamide to sensitize immune-restricted prostate tumors to immune checkpoint blockade, we launched a clinical trial of the combination of evofosfamide and ipilimumab (anti-CTLA-4) in 2017 (NCT03098160) to assess the safety and efficacy of the combination as well as to identify the MTD of evofosfamide. Patients with metastatic or locally advanced pancreatic cancer, human papillomavirus–negative HNSCC, immune checkpoint blockade–refractory melanoma, or castration-resistant prostate cancer were treated with escalating doses of evofosfamide (400 mg/m^2, 480 mg/m^2, 560 mg/m^2, and 640 mg/m^2) in combination with 3 mg/kg of ipilimumab in a standard 3+3 design. Of the 21 enrolled patients, 18 had measurable disease at baseline as per irRECIST (immune-related RECIST) and were evaluated for overall response rate. The proportion of patients with partial response was 16.7%, while 66.7% had stable disease. The overall disease control rate (complete response + partial response + stable disease) was 83.3% (15/18). The MTD of evofosfamide was identified as 640 mg/m^2 in combination with 3 mg/kg ipilimumab. The recommended phase II dose was 560 mg/m^2. No dose-limiting toxicities were observed.

Analysis of peripheral blood mononuclear cells from patients obtained prior to, during, and after treatment revealed statistically significant increases in effector $CD4^+$ and $CD8^+$ T cell proliferation in responders relative to nonresponders. Responders also accumulated fewer $PD\text{-}1^+LAG\text{-}3^+$

exhausted $CD8^+$ T cells. Arginase levels in PMN MDSC and monocytic MDSCs trended toward downregulation in responders at every time point examined, while both arginase and PD-L1 expression in DCs were significantly reduced in responders. These data suggest the potential of a suppressive phenotype in the circulating myeloid repertoire as a potential pretreatment biomarker, and of peripheral effector T cell proliferation as a pharmacodynamic biomarker of response to cotreatment with evofosfamide and ipilimumab.

In addition to assessment of peripheral immune responses, comprehensive flow cytometric analyses of paired biopsies obtained prior to and on week 7 of treatment indicated greater percentages of proliferating effector $CD4^+$ and $CD8^+$ T cells in responders. In addition, greater hypoxic exposure of T cells and DCs correlated with better response. Increased PD-L1 expression—characteristic of an inflammatory TME—was observed on T cells, DCs, and PMN MDSCs in responders.

Differential gene expression profiles were observed in response to therapy in all patients. Interestingly, responders exhibited distinct gene expression profiles from nonresponders both pretreatment and on-treatment. Gene set enrichment analyses against the hallmark gene signature indicated that responders showed evidence of pre-existing innate and adaptive immunity. This implies that T cell recruitment and survival in formerly hypoxic areas improves their function and sensitivity to CTLA-4 blockade. On the other hand, cases that progressed on therapy exhibited a hypermetabolic phenotype, indicating that in these patients, T cells continue to be at a metabolic disadvantage, and hypoxia ablation alone is not sufficient to restore antitumor immunity.

To further validate the reduction in hypoxia driven by therapy, gene set enrichment analysis against transcription factor targets was performed. Targets of HIF-1α, the master regulator of hypoxic response, accumulated in nonresponders while they decreased in responders (88).

Metformin is thought to have multiple distinct effects on cancer cell metabolism, acting to reduce mitogenic insulin/mTOR signaling, oxidative respiration through inhibition of mitochondrial complex I (89, 90), and gluconeogenesis through inhibition of mitochondrial glycerophosphate dehydrogenase 1 (91). The combination of these effects restricts tumor protein synthesis and cell cycle progression. Indeed, numerous studies have determined that patients taking oral metformin for diabetes control have significantly reduced risk of occurrence of numerous cancers (92, 93). Conversely, metformin alone has little therapeutic benefit to an established tumor. However, when metformin was utilized as an adjuvant to conventional therapy, metformin-naïve, nondiabetic patients with primary-stage colorectal, prostate, and breast cancer displayed improved progression-free survival (94, 95). While studies in murine models of cancer have revealed a positive combinatorial effect of metformin and immune checkpoint blockade, the data in cancer patients remain confined to case reports and retrospective analyses, which have obvious caveats. Several smaller studies have revealed favorable clinical outcomes in patients with late-stage disease who receive metformin with immunotherapy regimens, but, perhaps due to the limited scale of the studies, these data do not reach significance (96, 97). Interestingly, an association between adjuvant metformin and improved clinical outcomes was shown in one study that stratified patients by body mass index (BMI). Patients with BMI > 25 kg/m^2 displayed significant improvements in overall survival and disease-specific survival with the addition of metformin, with the greatest effect observed in patients with BMI > 30 kg/m^2. Infiltrating T cells from these patients had significantly reduced checkpoint molecule expression. These data follow other reports that disease outcomes following immunotherapy are better in obese patients than in those with lower BMI (98–100). Essentially all of the retrospective analyses in metformin are, expectedly, in the context of type 2 diabetes. It remains unclear whether metformin may act differently in nondiabetic cancer patients, when assessed prospectively. Several clinical trials have been proposed using biguanides such as metformin or phenformin with targeted or immune-based therapies.

CONCLUDING REMARKS

Immunotherapeutic success relies critically on the ability of reinvigorated T cells to infiltrate the TME, mediate effector functions, and persist after engagement with tumor cells. These biologic processes are energetically demanding and are driven in part by mitochondrial processes dependent on oxygen availability. Further, many tolerogenic populations thrive in hypoxic tissue and can survive on metabolites enriched under low oxygen tension. In this way, targeting hypoxia represents a means to broadly improve the immunometabolic status of the TME. Through specifically targeting hypoxic tumor cells, improving angiogenesis, or inhibiting the metabolism of the tumor itself, hypoxia reduction may tip the energetic balance in favor of antitumor immunity, ultimately supporting robust and durable responses.

DISCLOSURE STATEMENT

M.A.C. is the founder of, has an ownership interest in, and receives consulting fees from ImmunoGenesis, Inc., which owns evofosfamide (although this acquisition occurred after collection of all of the evofosfamide data presented herein).

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