Hypoxia as a Potential Inducer of Immune Tolerance and a Driver of Tumor Mutational Burden: Impact on Cancer Immunotherapy

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ABSTRACT: In cancer patients, immune cells are often functionally compromised due to the immunosuppressive features of the tumor microenvironment (TME) which contributes to the failures in cancer therapies, including immunotherapy. Targeting immune systems can benefit patients across a wide range of tumors and immune checkpoint blockers drive current revolution in immunotherapy across multiple tumor types. Nevertheless, most cancer patients do not respond, and the determinants of response and resistance are not well understood. To survive, developing tumors adapt to the immunological environment and create a local microenvironment that inhibits immune function by inducing immune tolerance and invasion. The TME is an integral part of tumor physiology that nurtures the malignant process. In this context, microenvironmental hypoxia, which is a hallmark of solid tumors, may result in pleiotropic effects contributing significantly to tumor aggressiveness and therapy resistance. It controls tumor resistance and plasticity and promotes the differentiation and expansion of immune-suppressive stromal cells. More importantly, hypoxia also drives genomic instability in cancer cells, and it may hinder the DNA damage response and DNA repair. Recent advances place hypoxia as a potential driver of tumor mutational burden. Here, we review the current knowledge on how hypoxic stress in the TME impacts on the tumor heterogeneity, plasticity and immune resistance, with a special interest in tumor immunogenicity. A detailed and integrated understanding of the dual effect of hypoxia might lead to innovative approaches for accurately exploiting hypoxia-associated pathways in the clinical setting.

KEY WORDS: hypoxic stress, antitumor immunity, immune evasion, tumor immunogenicity

ABBREVIATIONS: CTL, cytotoxic T lymphocyte; **EMT**, epithelial-to-mesenchymal transition; **HIF**, hypoxia inducible factor; **MDSC**, myeloid derived suppressor cell; **NK**, natural killer; **TAM**, tumor-associated macrophage; **TMB**, tumor mutational burden; **Treg**, T regulatory cell

I. INTRODUCTION

The role of the tumor microenvironment (TME) during the initiation and progression of carcinogenesis is presently considered to be of critical importance, both for better understanding of fundamental cancer biology and for exploiting this source of relatively new knowledge to improve molecular diagnostics and therapeutics. Carcinomas are no longer considered a singular mass of tumor cells, but rather a complex and dynamic pseudo-organ, comprising transformed epithelial cells residing within a complex microenvironment with unique physiology, rich in different non-malignant cell types that interact physically and via paracrine signaling molecules. The TME represents a complex macrocellular complex network which consists of host-derived stromal, immune and endothelial cells with potential dual and paradoxical role in tumor development and dissemination. In fact, to ensure tumor growth and immune evasion, the stromal component of the tumor mass undergoes numerous metabolic adaptations and changes favoring tumor development and dissemination.

Several mechanisms contribute to the hypoxic milieu of solid tumors. The major mechanism underlying reduced tumor oxygenation is the insufficient and aberrant vasculature that cannot deliver the necessary blood supply to all sites of the tumor tissue. There is ample evidence that angiogenesis induced by the growing tumor is leaky and ineffective.[1]

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Hypoxia, or gradients of hypoxia, occurs in most growing solid tumors and may result in pleiotropic effects contributing significantly to tumor aggressiveness and therapy resistance.[2] Indeed, the generated hypoxic stress has a strong impact on tumor cell biology. For example, it may contribute to increasing tumor heterogeneity, helping cells to gain new functional properties and/or select certain cell subpopulations, facilitating the emergence of therapeutic resistant cancer clones, including cancer stem cells coincident with tumor relapse and progression. When oxygen availability is reduced, tumor cells respond to restore energy production, minimize the potential damage of aberrant oxidative phosphorylation and secrete angiogenic factors to increase oxygen delivery. This extensive re-calibration of the metabolic and signaling pathways is under the control by hypoxia-inducible factor-1α (HIF-1α), a central transcription factor that mediates hypoxia responsive genes.[3] This transcription factor has been widely accepted to play a critical role in tumor invasion and metastasis due to its increased cell survival, angiogenesis and cell migration and invasion. Moreover, extensive research has shown that hypoxia increases the generation of reactive oxygen species in both the tumor cells as well as the stromal cells. Hypoxia has been found to correlate with a state of oxidative stress, and the induction of reactive oxygen species is one of the most common regulatory mechanisms under hypoxic conditions.[4] It should be noted that hypoxia is not only induced by a lack of oxygen and several biochemical pathways or gene mutations may lead to a pseudohypoxic state despite high tumor oxygen levels. For instance, in renal cell carcinoma, a mutated or lost the *VHL* gene prevents pVHL from participating in E3 ubiquitin ligase complex formation and interaction with its HIF-1α substrate, allowing stabilization of HIF-1α even in physiological levels of oxygen.[5] This phenomenon is called "pseudo-hypoxia" and promotes cellular mechanisms following VHL mutation that resemble those induced during exposure to non-physiological low pO_2 levels. VHL loss causes pseudohypoxia that profoundly alters the cellular secretome and interactions, impacting cancer cells and the TME. Furthermore, hypoxic stress can activate a variety of oncogenic signaling pathways that can lead to the development of tumor clones resistant to anti-tumor immune attacks by cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells.[6,7] It should be reminded that immune resistance may arise from both genetic instability and tumor heterogeneity driven by microenvironmental hypoxia that promotes carcinoma cell plasticity as well as extrinsic or intrinsic mechanisms of immune resistance. In this regard, hypoxia remains a threat to genome integrity and has been shown to down-regulate DNA damage response and repair mechanisms.[8]

The possible existence of a broad influence of the hypoxia-associated mutator phenotype[9] that may direct the tumor subclonal architecture and tumor immunogenicity should be considered. Clearly, a detailed and integrated understanding of the dual effect of hypoxia might lead to innovative approaches for accurately exploiting hypoxia-associated pathways in order to better target tumor stroma and design innovative integrative and instructive immunotherapy approaches.

II. NK CELLS: KEY ACTORS OF THE TUMOR STROMA

CTLs and NKs are known to infiltrate tumors and are at the forefront of efforts to develop immuno-oncologic treatments. These cells are attractive targets for immunotherapies because of their efficient mechanisms for killing cancer cells. While it is admitted that both innate and adaptive immunities are involved in immune surveillance in cancer patients, the cytotoxic response mediated by adaptive and innate killer cells is often altered. Most of the immunotherapy approaches are devoted to manipulating the T lymphocyte system. Because, in contrast to other treatment agents, T cells display a big diversity and specificity and more importantly have a memory that is capable of lasting for decades. In addition to the cytokines and monoclonal antibodies, the manipulation of T cell response has attracted a lot of attention in the field of immunotherapy of cancer as the adaptive immune system is considered as an ideal anti-cancer agent as these cells have a strong diversity, specificity and are effective for decades thanks to their memory. With this respect, the ultimate goal of most cancer immunotherapies is to

induce a strong cytotoxic T lymphocyte (CTL) response, with the prevailing view being that induced CTLs will eradicate tumor cells. However, this view has been challenged by clinical observations showing that tumor rejection does not follow a successful induction of CTL response. Tumor-specific $CD8^+$ T cells found in human solid tumors are often dysfunctional and the microenvironmental immunosuppression is a rate limiting step to effective anti-tumor immunity.[10] Strong evidence indicates that neoplastic cell responses to immunotherapy are not solely dependent on the qualitative and/or quantitative features of the T cells or the complexity of the genomic aberrations they harbor, but is also regulated by numerous dynamic properties of the TME.[11] With this respect, the hypoxic TME limits the anti-tumor activity of killer cells by promoting immune checkpoint activation, limiting access to key nutrients and recruiting a variety of immunosuppressive cell types to the tumor site, thereby reducing the effectiveness of the anti-tumor immune response.

III. DYNAMIC CROSS-TALK BETWEEN MALIGNANT CELLS AND THE TUMOR STROMA: ROLE OF THE TME

Many reports indicate that a fundamental albeit deranged relationship between the tumor and the stromal cells is essential for tumor cell growth, progression, and development of life-threatening metastasis. Therefore, insight into this interaction and the underlying signaling, transcriptional, and metabolic pathways, can reveal valuable opportunities for therapeutic intervention during cancer progression. The TME and associated multiple factors are found to contribute to the failures in cancer therapies, including immunotherapy. It has become clear that it is critical as a source of improved molecular diagnostics and therapeutics. It is composed of proliferating tumor cells, blood vessels, infiltrating inflammatory cells, and a variety of other associated stromal cells. The dynamic crosstalk between malignant cells and the tumor stroma in the TME determines the trajectory of tumor progression, its aggressiveness, heterogeneity, and response to cancer treatment. In fact, tumor stroma components are engaged in an active and complex molecular cross-talk that compromises immunological recognition of tumors by killer immune cells. Obviously, the immune suppressive shaping of the TME may be considered an initial immune checkpoint. Strong evidence indicates that neoplastic cell responses to immunotherapy are not solely dependent on the qualitative and/or quantitative features of the T cells or the complexity of the genomic aberrations they harbor but is also regulated by numerous dynamic properties of the TME.[11]

Among the microenvironment factors that play a dominant role in determining therapeutic responses to immunotherapy, hypoxia is central. It is a hallmark of most solid tumors with the potential for mediating metabolic and phenotypic changes (cell plasticity) as well as direct immune suppression. As a pervasive feature of the TME, hypoxia plays also a significant role in cancer progression and ultimately clinical outcome.[12] It is now known that microenvironmental stress during tumor development is frequently accompanied by cellular plasticity such as the epithelial-mesenchymal transition (EMT) that facilitates adaption and selection of lethal cancer clones.[13] Targeting carcinoma cell plasticity is in this regard an important strategy to better control the emergence of resistant variants and ensure a more effective cancer therapy.

IV. HYPOXIA: A MAJOR COMPONENT OF METABOLIC TUMOR MICROENVIRONMENT IN MOST ADVANCED SOLID TUMORS

The TME involves several soluble factors and metabolic changes. Among these metabolic changes, hypoxia plays a pivotal role in shaping the TME.[14] Hypoxic stress is a feature of most solid tumors and is associated with poor clinical outcomes in various cancer types.[15–17] Hypoxia arises due to a combination of excessive oxygen consumption by growing tumor cells and the disorganized tumor-associated vasculature.[18] Considerable evidence now suggests that hypoxia plays an important role in tumor progression, affecting both metastatic spread and selection of cells with more aggressive phenotypes.[19–21] Hypoxia in the TME has been shown to be a strong indicator of tumor aggressiveness and a potential

predictor for resistance to chemotherapies, radiotherapy, and immunotherapy.[22] Tumor hypoxia can also lead to immune suppression.[23] The master regulator of the hypoxic response is HIF-1 involved in the adaptive responses to hypoxic stress. In mammalian cells, the response to hypoxia depends in large part on the activation of HIF-1, a heterodimeric transcription factor consisting of a hypoxia-inducible HIF-1α subunit and a constitutively expressed HIF-1β subunit. HIF-1 transactivates target genes containing *cis* acting hypoxia response elements that contain the HIF-1-binding site sequence. HIF-1α protein levels are tightly regulated by the cellular pO_2. Under hypoxic stress, hypoxia-dependent stabilization of HIF dimers allows for the induction of numerous genes regulating various biological processes and functions in cells, including angiogenesis, cell survival, proliferation, pH regulation, and metabolism. It should also be noted that one key cellular consequence of hypoxic stress is the dysregulation of DNA repair pathways, which contributes to the genomic instability and mutator phenotype observed in human cancers. Recently, cell plasticity has emerged as a potential contributor to therapy evasion through regulation of cancer cell phenotypic and functional heterogeneities.

A. Hypoxia-Induced Immune Suppression

When T cells infiltrate the TME they encounter a myriad of metabolic stressors including hypoxia. *In vitro* and *in vivo* studies indicate that tumor cells can avoid the immune response by promoting an immunosuppressive microenvironment. We and others have demonstrated that hypoxia shapes the immunosuppressive microenvironment. It is well established that hypoxic stress results in the recruitment of tumor-associated macrophages (TAMs), T-regulatory cells (Tregs), tumor-associated neutrophils, myeloid-derived suppressor cells (MDSCs) or the expression of negative immune checkpoints including CTLA4 (cytotoxic T lymphocytes associated protein 4) and PDL1 (programmed death-ligand 1). IDO (indoleamine 2,3-dioxygenase 1) is also a potential immunosuppressive enzyme within the hypoxic TME. We have previously shown that hypoxia-induced Nanog[24] favors the intratumoral infiltration of regulatory T cells and macrophages via direct regulation of TGF-β1. Inhibition of hypoxia resulted in an increase in $CD8^+$ T effector cells in the tumor bed in part by modulating TGF-β1 production. All these factors play a key suppressive mechanism that hypoxic cells exploit to escape immune destruction. Currently many efforts are oriented towards finding mechanisms by which the suppressive impact of hypoxia can be minimized that will likely be critical to sensitizing these tumors to immunotherapeutic interventions such as checkpoint blockade, cancer vaccines and adoptive cell therapy. In recent years, it has become increasingly clear that an immunosuppressive TME is one of the major obstacles to an efficient therapy response, not only limiting the IO therapy response but also limiting the clinical efficacy of other conventional therapies, thus major research efforts are currently underway to elucidate the cellular and molecular mechanisms involved in search for novel targets.

V. INDUCTION OF IMMUNE RESISTANCE BY HYPOXIA

While immune suppression is considered an important mode of immune escape, another important mechanism of immune escape in the TME is related to the hypoxic stress-regulated resistance of tumor cells toward the cytotoxic machinery of immune effector cells. Tumors use hypoxic stress to their own advantage by activating key biochemical and cellular pathways that are important in their progression and survival. Clearly, hypoxic stress induces tumor target adaptation that compromises the effectiveness of CTL and NK cell activities.[25]

Several reports from our laboratory and others have clearly demonstrated that hypoxia is efficient in inducing target resistance to cell-mediated cytotoxicity including CTLs and NK cells by several mechanisms. In this regard, hypoxia protects target cells from specific lysis by stabilizing hypoxia-inducible factor-1α and inducing STAT3 phosphorylation.[26] In addition, we showed that NANOG, a transcription factor associated with stem cell self-renewal, is a new mediator of hypoxia-induced resistance to specific lysis.[27] More importantly, we have provided evidence indicating that hypoxia induced

autophagy[28] and this results in granzyme B degradation in hypoxic tumor target cells.[29] It has also been reported that tumor hypoxia is known to promote the accumulation of extracellular adenosine, which can be measured at high concentrations in multiple malignancies.[30] Free adenosine can inhibit both effector T-cell and NK cell proliferations and cytotoxicities.[31] Hypoxia further contributes to adenosine accumulation through HIF-1α induction of the ATP hydrolyzing ectonucleotidases CD39 and CD73 on $FoxP3^+$ regulatory T cells (Tregs).[32] Hypoxia also promotes the generation of reactive nitrogen species[33] and it is well documented that nitrative stress directly affects T-cell signaling molecules leading to T-lymphocyte dysfunction.[34]

VI. INDUCTION OF TUMOR CELL PLASTICITY BY HYPOXIA

Tumor plasticity is an adaptor that contributes to the generation of diverse cancer invasion programs, enhanced tumor heterogeneity, and ultimately resistance to cytotoxic treatments.[35] In this regard, EMT facilitates the escape of tumor cancer cells from the primary tumor site, which is a key early event in metastasis. It can be reactivated during cancer progression, providing certain cancer cells with increased capacity to migrate, invade, and resist cell death. EMT can be partial, with cells conserving many salient features of epithelial cells, and transient, with cells transitioning from an epithelial to a mesenchymal state and then reverting to an epithelial state, either partially or fully mesenchymal-epithelial transition. The EMT phenomenon endows epithelial cells with enhanced migratory and invasive potential and, as such, have been implicated in many physiological and pathological processes requiring cell migration.[36,37]

HIFs, angiogenesis and inflammatory factors might exert important regulatory functions and can stimulate EMT and/or support a mesenchymal state.[38–40] When lung adenocarcinomas included in the TCGA-LUAD[41] were analyzed, hypoxia signatures, as well as HIF1A mRNA expression, were significantly and positively correlated with EMT-TF expression levels. Taken together, this underscores the importance of hypoxic stress in mediating tumor plasticity and heterogeneity. Furthermore, HIF-1α activates Twist, Snail and SIP1 expressions, thereby leading to E-cadherin repression.[42,43] In addition, it has been reported that hypoxia increased the expression of ZEB1 in cervical squamous cell carcinoma (CSCC) cells, which resulted in increased infiltration of TAMs. Hypoxia-induced ZEB1 was found to be closely correlated with the CD47-SIRPα,[44] which enables cancer cells to evade phagocytosis by macrophages and promotes tumor progression.[44] Analysis of datasets derived from hundreds of patients with CSCC revealed that ZEB1 expression was correlated with CD47 and SIRPα expression and patient mortality.[45]

VII. HYPOXIA PROMOTES GENOMIC INSTABILITY AND MAY REGULATE TUMOR IMMUNOGENICITY

It is widely admitted that one key cellular consequence of the hypoxic stress is the dysregulation of DNA repair pathways, which contributes to the genomic instability and mutator phenotype observed in human cancers. In this regard, tumor hypoxic environments can affect DNA repair mechanisms in a variety of ways.[46–48] Hypoxia increases the DNA damage response through pan-nuclear activation of gamma-H2AX in both acute and chronic hypoxia. While chronic hypoxia can cause a considerable decrease in DNA repair proteins and mechanisms, excessive hypoxia/anoxia can cause replication stress. Transcriptional and translational downregulation of DNA repair pathways under chronic and intermittent hypoxia are the major contributor of chromosomal and genomic instability.[49,50] Using *in vitro* breast cancer models, we have shown that chronic and intermittent hypoxic stress contributes to downregulation of several DNA repair pathways, induction of replication stress that contributes to an increase in mutational load and concomitant potential neoantigens.[51] Data analysis of TCGA datasets from different cancer types has shown that defects in DNA damage signaling like ATR/Chk1, deficiency of DNA repair mechanisms like mismatch repair, double strand break repair (DSB) and base excision repair (BER) are the major contributors for TMB and neoantigens.[52] Chronic hypoxia can also

repress mismatch repair genes and can inactivate MMR pathway genes through epigenetic mechanisms thus participating in MSI.[52] A recent study demonstrated that cyclic hypoxia induced replication stress provides single strand DNA substrates for enhanced APOBEC3B activity. In addition, they demonstrated an association for high hypoxia with increased APOBEC-mediated mutagenesis in breast and lung cancer cohorts from TCGA.[53]

VIII. CONCLUSION

The introduction of immune checkpoint inhibitors has marked a prominent paradigm shift in cancer treatment. However, despite the significant progress in the field of cancer immunotherapy, we are currently facing a wide range of challenges and there is still a need for more effective treatments to maximize cancer patient survival rates. The high degree of non-responders and relapsing patients, notably in highly prevalent malignancies such as in lung and breast cancers, is a major concern and a good reminder that we possess only partial understanding of the events controlling anti-tumor immunity and tumor immune resistance. In this regard, novel strategies for tumor target selection and immunostimulatory intervention are being developed in the context of the TME. At present, the design of innovative integrative immunotherapy approaches is the subject of intense investigation. Currently, the important clinical challenge lies within the design of optimal combination regimens with the potential to enhance the fraction of responders and/or improve patient responses. Accordingly, the combination of immunotherapies with small molecules targeting the TME to reduce its hostility might be an interesting option in the future.

It is widely known that tissue oxygenation is an important component of the microenvironment and can alter cell behavior through the direct regulation of genes involved in cell survival, apoptosis, glucose metabolism, and angiogenesis. Hypoxia, a hallmark of most tumors, appears to be a key parameter of the TME, with the potential for mediating metabolic and cell plasticity, phenotypic changes such as EMT as well as immune suppression. It plays an important role in the development of tumors, modulation of neoangiogenesis, immune suppression, tumor resistance and plasticity. Accumulating evidence indicates that tumor hypoxic signatures are correlated with poor prognosis in several cancer types. Hypoxic zones in solid tumors are infiltrated by a large number of immunosuppressive cells, such as TAMs, MDSCs, and Tregs. More importantly, several studies have demonstrated that the hypoxic microenvironment drives the selection of more aggressive cancer cell populations through cellular adaptations referred as epithelial-mesenchymal plasticity (Fig. 1). Therefore, exploiting hypoxia-associated tumor escape capacities holds great promise for attenuating tumor heterogeneity, overcoming alteration of antitumor cytotoxic response and may improve its effectiveness in cancer patients and hypoxic stress has become an attractive target for cancer immunotherapies.

Very recently, convincing evidence has been provided indicating that hypoxia interferes with genomic instability, DNA repair and the subsequent tumor immunogenicity. In this respect, a detailed and integrated understanding of the dual effect of hypoxia might lead to innovative approaches for accurately exploiting hypoxia-associated pathways in the clinical setting. Recent data from cohorts of cancer tissue clearly show a connection between hypoxia and genomic instability, as well as tumor mutational burden. While studies have shown that hypoxia is an early event during tumor development, and often associated it with clonal genomic

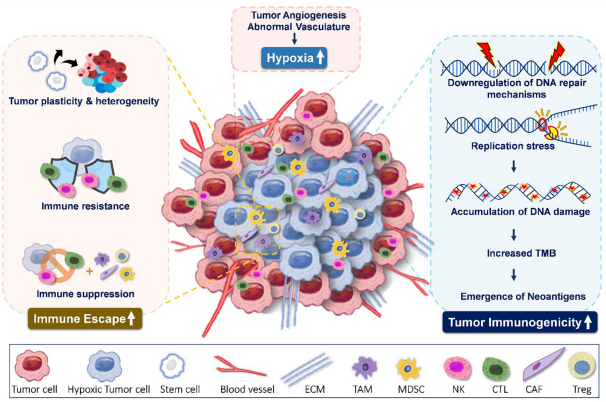

FIG. 1: The tumor microenvironment: A dual effect in regulating tumor escape and tumor immunogenicity

variations; it remains to be determined whether hypoxia-linked TMB actually results in the production of clonal or sub-clonal neoantigens that are distinct from normoxic tumors. Another consideration is that even when hypoxia-specific neoantigens are expressed, an immune-privileged microenvironment is necessary for successful ICB. Future studies with independent trials assessing the impact of hypoxia on TMB, PD-L1 and neoantigen quality will be beneficial in guiding patient selection and treatment strategies concerning ICB. Indeed, whether a hypoxic tumor microenvironment in the context of immune checkpoint blockade is a boon or a bane remains to be further investigated.

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