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Tumor hypoxia: an important regulator of tumor progression or a potential modulator of tumor immunogenicity?

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Introduction

Hypoxia in the tumor microenvironment has been shown to be a strong indicator of tumor aggressiveness, metastases, and promotes cancer progression.[1] Several studies have indicated that tumor hypoxia is a predictor for resistance to chemotherapies, radiotherapy, and immunotherapy.[2] Tumor hypoxia can also lead to immune suppression and can induce resistance to immune checkpoint blockade (ICB).[2] However, the mechanisms through which hypoxia contributes to immunotherapy resistance remain unclear. Current predictive biomarkers of response to ICB efficacy include tumor mutational burden (TMB), neoantigen load, PD-L1 expression, Microsatellite Instability (MSI) status, and diversity of immune cells in the TME.[3] High TMB with neoantigen load has been reported as an independent predictor of favorable outcome across various cancer types and there are well-defined factors (both cell intrinsic and extrinsic) contributing to TMB generation.[3] MSI-High tumors arise due to germline variations, as well as epigenetic repression of mismatch repair genes, and MSI is currently tested as a marker for several immunotherapy trials.[3] The most well-established biomarker, PD-L1, has been beneficial in guiding patient selection for immune checkpoint blockade. The regulation of PD-L1 in tumors is quite complex and other immune checkpoints contribute to negative immune regulation.[3] Tumor immunome (the TME's immune cell repertoire) contributes to ICB effectiveness and is used as a biomarker for long-term response in many cancers, but it is extremely complex and varies spatiotemporally.[3] According to our current understanding, TMB development, neoantigen load, PD-L1 expression, and immune cell infiltration at the tumor site are all thought to be influenced by tumor genomic instability.[4] However, the effect of hypoxia on these biomarkers remains unclear.

Hypoxia is associated with immune evasion

While hypoxia may play a role in TMB induction, it can also lead to intratumor heterogeneity and immune evasive phenotypes[5] (Figure 1). Hypoxia in the TME 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-cells (CTLs), Natural Killer (NK) cells as well as macrophages.[4] It has the potential to modulate non-genetic heterogeneity and phenotypic switches of tumor cells (also referred to as tumor plasticity) representing another means through which tumors can evade anti-tumor immunity. Converging evidence exist that tumors containing clones or contingents with a more mesenchymal phenotype frequently exhibit elevated expression of PD-L1 and immune suppressive factors.[6–9]

Moreover, hypoxic cells often downregulate Type-I interferon signaling and MHC (major histocompatibility complex) presentation mechanisms and activate autophagy to escape CTL- and NK cell-mediated killing.[10,11] There have been contradictory reports on the effect of hypoxia on MHC-class I antigen presentation pathway necessary for activating CD8 + T-cells.[12,13] On one hand, exposure of murine cancer cells to 24 hours of 1% oxygen enhanced MHC I antigen presentation through the HIF-1α-inducible ER-resident oxidase ERO1-α.[12] On the other hand, in 3D-cultured tumor cells exposed to acute hypoxic conditions and in a tumor bearing mouse model, hypoxia was shown to downregulate MHC molecules and antigen presenting proteins in a HIF-1α-dependent manner.[13] Hypoxic suppression of IFN-γ was further found to inhibit IFN-γ-induced MHC I expression in a HIF-1 independent manner in mouse melanoma and colon cancer cells exposed to up to 48 hours of 0.5% oxygen.[14] Hypoxia was shown to downregulate the NK-activating ligands MHC class I polypeptide-related sequence A/B (MICA/B)[15] and to induce their shedding.[16–18] HIF-1α-induced downregulation of MICA/B was shown to diminish NK cell-mediated cytotoxicity in a lung cancer cell line exposed to 24 hours of 0.6% oxygen.[15] In prostate cancer cells maintained for 24 hours at 0.5% oxygen, hypoxia-associated impairment of nitric oxide (NO) signaling led to MIC shedding and inhibition of NK cell-mediated cytotoxicity.[16] Alternate mechanisms for hypoxia-induced MIC shedding have also been reported.[17,18] Importantly, a milieu of immunosuppressive cytokines is
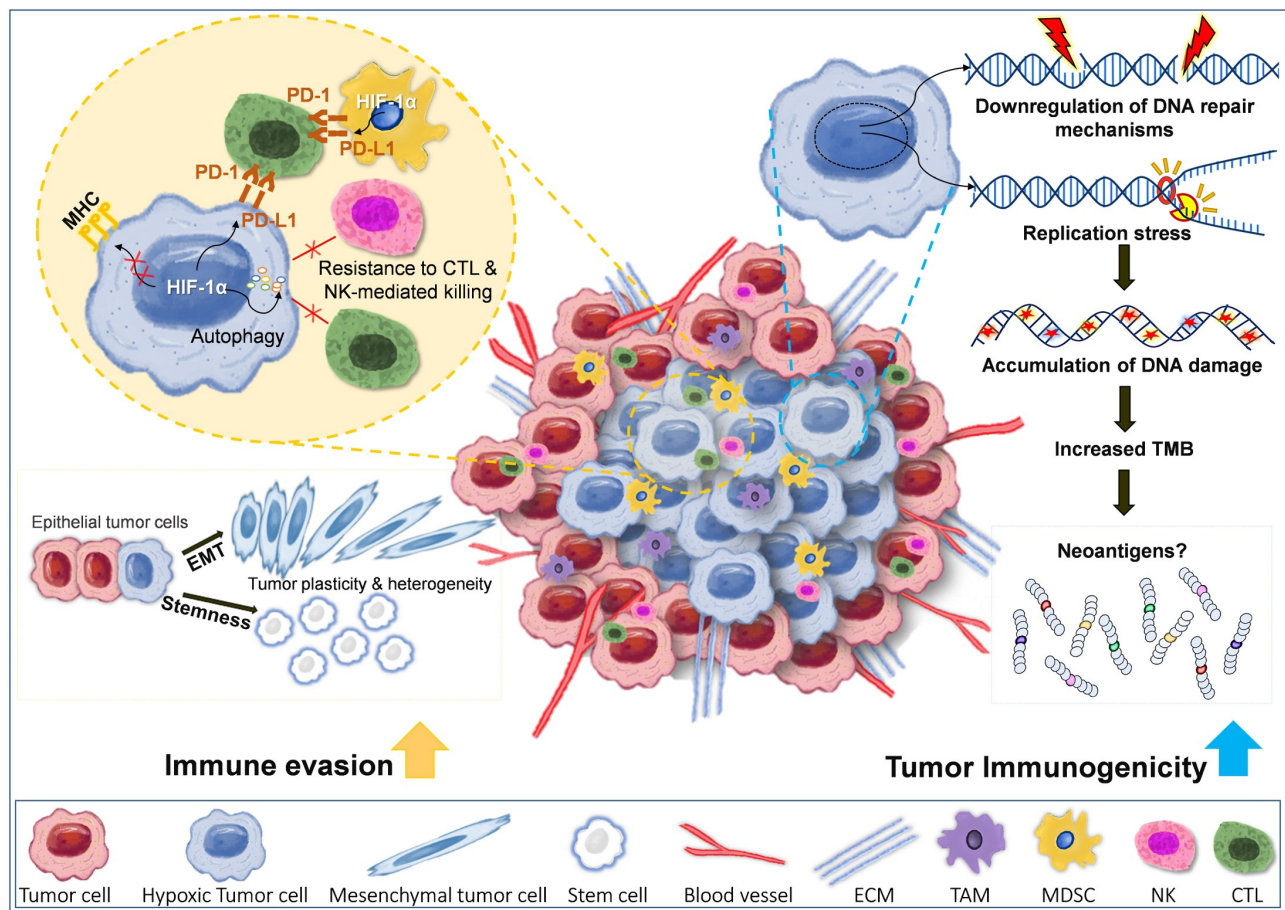
Figure 1. The dual effect of hypoxia in regulating the antitumor immune response: the left panel represents hypoxia's role as a key driver of immune evasion and tumor plasticity. Hypoxia promotes resistance to cytotoxic T-lymphocyte (CTL) and NK-cell mediated killing by inducing Hypoxia inducible factor-1α (HIF-1α)- mediated autophagy, loss of MHC class I molecules, and increased PD-L1 expression in tumor cells as well as myeloid-derived suppressor cells (MDSCs). With respect to tumor plasticity, hypoxia promotes epithelial to mesenchymal transition (EMT) and cancer stemness. On the other hand, the right panel highlights the tumor immunogenicity promoting role of hypoxia; wherein the hypoxia-associated downregulation of DNA repair mechanisms and replication stress result in increased tumor mutational burden (TMB) that could give rise to potential neoantigens. ECM: extracellular matrix; TAM: tumor associated macrophage.

induced by the hypoxic TME, impairing T-cell and NK-cell function.[4] In addition, under hypoxic conditions, tumor cells, and myeloid-derived suppressor cells (MDSCs) express PD-L1 to induce immune suppression.[19] Preclinical evidence in the prostate cancer model TRAMP-C2 suggests the presence of a 'cold' tumor microenvironment with poor T-cell infiltration in hypoxic zones in comparison to normoxic zones of the same tumor.[20] In the context of immunotherapy, hypoxia can pose significant challenges for ICB and can lead to resistance by establishment of an immune excluded phenotype in the TME and decreased immunogenic cell death.[21–23] Various hypoxia alleviating strategies have been applied in preclinical models and were found to enhance response to immune checkpoint inhibitors and revitalize the anti-tumor immune response. Such strategies have been reviewed elsewhere[22–24] and include directly targeting hypoxia with hypoxia-activated prodrugs,[20] as well as indirectly attenuating hypoxia using inhibitors of oxidative phosphorylation to decrease oxygen consumption and improve tumor oxygenation.[24] Recently, it was also shown that blocking HIF-1α transcriptional activity in B16-F10 melanoma promoted immune cell infiltration into the TME and increased levels of the chemokine CCL5.[25] Co-

targeting melanoma-bearing mice using an inhibitor of HIF-1 α/β dimerization, coupled with anti-PD-1 and a peptide vaccine completely stalled tumor growth.[25] Such studies underline the significance of targeting hypoxia-associated pathways to ameliorate immunotherapy efficacy.

Hypoxia promotes genomic instability

Hypoxic environment can affect DNA repair mechanisms in a variety of ways, as has been thoroughly studied.[16,17] 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 contributors of chromosomal and genomic instability.[26,27] Using *in-vitro* breast cancer models, we have recently 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.[28] Enigmatically, while downregulation of DNA repair pathways makes the cells susceptible to enhanced DNA damage accumulation and subsequent activation of immune response, it can also cause immune suppression through upregulation of PD-L1.[29] 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.[30] In addition, depletion of DNA repair factors related to DSB and BER leads to direct upregulation of PD-L1 expression on tumors.[30] Chronic hypoxia can also repress mismatch repair genes and can inactivate MMR pathway genes through epigenetic mechanisms thus participating in MSI.[27] 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.[31]

DNA repair processes are intricately dependent on several metabolic pathways for maintenance of genome stability. DNA damage signaling kinases regulate the metabolic state of the cell through production of nucleotides required for repair, lactate mediated chromatin remodeling to enhance DNA repair gene transcription.[32] Chronic hypoxia promotes the buildup of various enzymes related to glycolysis and tricarboxylic acid cycle oncometabolites such as 2-hydroxyglutarate, fumarate, and succinate, that could impede DNA repair.[4] For example, 2-hydroxyglutarate inhibits the lysine demethylase KDM4B which acts on histone H3 proteins and contribute to impediment of homology-directed rejoining.[4] Furthermore, reoxygenation after acute hypoxia can result in an increase in reactive oxygen species (ROS) and a decrease in mitochondrial ATP generation, which in turn may induce alterations in DNA damage checkpoints and contribute to genomic instability.[4]

Alongside DDR, chronic hypoxia can activate Unfolded Protein Response (UPR) independent of HIF signaling, but the interaction between these two key processes under hypoxic conditions remains unclear.[33] Upregulation of DDR and/or activation of UPR process can regulate common downstream process like autophagy, apoptosis, metabolic reprogramming, redox homeostasis, and immune regulation.[33] As levels of hypoxia and chronicity can vary among tumor types, understanding the coordination between DDR and UPR will be important, specifically in the context of a tumor's immunogenicity.

Another important contributing factor may be *TP53*. The interplay between p53 and hypoxia plays a key role in the DNA repair process.[34] In a recent study of lung tumors, Sun and collaborators reported that while *TP53* missense and nonsense mutations are equally associated with elevated TMB, neoantigen levels, and DNA damage repair deficiency, *TP53* missense and nonsense mutations were significantly different in terms of associations with PD-L1 and response to anti-PD-L1.[35]

Given the enormous complexity of DNA repair mechanisms and TMB in human tumors, analyzing the exclusive impact of hypoxic tumor microenvironment on these disruptive events has been challenging to study in *in-vivo* models.

Computational analysis of publicly available datasets links hypoxia with TMB

In this regard, pan-cancer and TCGA studies have paved the way for understanding the association of hypoxia with genomic instability and TMB. The application of hypoxia gene signatures as a surrogate biomarker for this condition enabled the concurrent evaluation of other genomic elements and tumor environmental features. Clear indications have been established between higher levels of hypoxia and increased percentage of copy number aberrations,[36,37] a marker of genomic instability, as well as a higher number of single nucleotide variants (SNVs) per Mbp;[37] therefore, hypoxia could drive clonal diversification in tumors. As hypoxia is an early event in tumor progression, it also contributes to the selection of a number of clonal alterations as opposed to subclonal alterations.[37] In addition, more hypoxic tumors have been found to exhibit a higher proportion of mutations attributed to single base substitution (SBS) signatures derived from defective homologous recombination-based repair and DNA mismatch repair.[37] In one study on primary soft tissue sarcoma, the concurrent application of a hypoxia signature and a signature measuring the degree of genomic instability revealed a significant enrichment of hypoxic tumors in the group classified as having high genomic instability.[38] The positive correlation between hypoxia and TMB has been further validated in independent studies for hepatocellular carcinoma,[39] gastric cancer[40] and pancreatic cancer.[41] In gastric cancer, TMB positivity was further correlated with higher positive rate of ERO1A protein, a marker of hypoxia, in 73 tumors tested by immunohistochemistry.[40] In addition, lung adenocarcinoma patients classified as high-risk based on a signature of hypoxia-related alternative splicing events have been shown to harbor significantly higher TMB compared to the low-risk group.[42] While hypoxia could be driving TMB through its downregulation of DNA repair genes and pathways, a recent study suggests otherwise.[43] This study applied a hypoxia biomarker gene signature (named as NB-hop) to classify neuroblastoma patients into two groups with favorable (hypoxia-low) and unfavorable (hypoxia-high) prognosis. The authors showed that several DNA repair pathways (MMR, BER, and DSB repair) were upregulated in samples with unfavorable prognosis. Similarly, key DNA repair genes like *FEN1, PCNA, TERT, BRCA1, BRCA2, CHEK1, CHEK2, TPX2*, and *TP53* were also upregulated.[43] A prior work from our group found that chronic and intermittent hypoxia exposed cells had varied gene expression patterns, with a few DNA repair genes being upregulated.[28] DNA repair appears to be downregulated *in vitro*, however *in vivo* and transcriptome data from publicly available datasets of neuroblastoma patients seems to indicate otherwise. There are a few unanswered questions in this area that require further investigation.[44] How can we assure that

in vitro and *in vivo* data is accurately reported and interpreted? Are there various patterns of DNA damage response in hypoxic tumors for different cancer types? How can we keep track of hypoxic tumors during a patient's illness and use that information to guide treatment?

The immune contexture of hypoxic tumors remains inconclusive

Studies in cancer patient cohorts have similarly correlated the presence of hypoxia with an immunosuppressed TME in breast cancer,[45,46] clear cell renal cell carcinoma,[47] colorectal cancer,[48] hepatocellular carcinoma,[49–51] melanoma,[52] osteosarcoma,[53] neuroblastoma[43] and pancreatic cancer,[41,54] among others.[55] These studies used hypoxia gene signatures to determine the hypoxic state and immune fraction or gene set enrichment analysis to highlight the immune microenvironment. In addition, the coupling of an immune score with a hypoxia score in lung adenocarcinoma,[42] pancreatic cancer,[41] and soft tissue sarcoma[38] revealed significantly lower immune activation in tumors with high hypoxia, compared to their low hypoxia counterparts. Of interest, colorectal tumors classified as immune-cold based on IHC assessment of CD3, CD4, and CD8 were linked to a hypoxic biology and poor prognosis.[56] Despite these recurring and complementary findings associating hypoxia with an immunosuppressive TME, a recent report on hepatocellular carcinoma showed diverging results.[39] By applying a hypoxia-associated risk score, the authors distinguished two different clusters of hypoxic tumors that varied in terms of their degree genomic instability and TMB, immune infiltration, and expression of immune check point inhibitors.[39] They reported increased immune infiltration and expression of immune checkpoint inhibitors in tumors of the hypoxia-high cluster. Patients in this cluster were also predicted to have enhanced response to immunotherapy.[39]

Furthermore, a study on gastric cancer reported TMB-high GC tumors as being positively correlated with NK cells and negatively correlated with CD8 + T-cells.[40] TMB-high tumors were also associated with the presence of hypoxia, however how the immune milieu varied between patients based on both the tumor's mutational load and hypoxic state was not investigated. It is intriguing to think that hypoxia could instigate an increased TMB, which could give rise to immunogenic clonal neoantigens that spur immune infiltration and enhance response to ICB. Nonetheless, studies investigating hypoxia, TMB, and immune cell infiltration are required to better elucidate the complex mechanisms at play.

Indeed, on the flipside, studies in pancreatic cancer[41] and lung adenocarcinoma[42] that have simultaneously reported on the TMB, immune TME and hypoxia, found that hypoxia-high tumors harbored higher mutations and exhibited significantly lower immune scores, compared to hypoxia-low tumors. Such discrepancies between studies suggest specific events controlled by hypoxia. The complexity of the matter is additionally exacerbated by the presence of other pro-immunogenic and anti-immunogenic factors in the TME. Such factors can skew the disparity between an immunogenic, "immune-hot" and a non-immunogenic, "immune-cold" microenvironment. In this regard, positive associations between hypoxia and the expression of PD-L1, among other immune checkpoint markers, were reported in hepatocellular carcinoma.[39,49,51] In this line, we recently observed a similar association with PD-L1 in pancreatic tumors, and further found that hypoxia-high tumors displayed significantly lower cytolytic index scores, evocative of T-cell cytolytic dysfunction.[41] Indeed, higher hypoxia scores were enriched in tumors with a low chemokine signature score, indicating inefficient T-cell activation and presumably migration.[41] Clearly a more wholistic approach that considers all these elements is warranted to better isolate the direct impact of hypoxia on the tumor immune microenvironment and its role in resistance to ICB.

Conclusion

Recent studies targeting the hypoxic tumor microenvironment through inhibition of oxidative phosphorylation,[24] inhibiting HIF-1α transcriptional activity,[25] hypoxia activated pro-drugs and anti-angiogenesis inhibitors[23] have yielded promising results. However, immunotherapeutic strategies aimed at triggering or enhancing antitumor immunity are disappointing because of diverse tumor escape mechanisms from immunosurveillance. We and others provided evidence indicating that the majority of malignancies create a hostile hypoxic microenvironment that can hamper cell-mediated immunity and dampen the efficacy of the immune response.[1] More importantly, recent data from cohorts of cancer tissue clearly show a connection between hypoxia and genomic instability, as well as TMB. Here, it would be interesting to develop models and study whether acute vs chronic hypoxia, both presumably occurring in tumors, would reveal differential effects on these parameters. Attempts to target hypoxia or hypoxia-associated pathways, to avoid hypoxia-driven immune escape, should also consider the role played by hypoxic stress and lymphocytes' exclusion from the tumor nest. Furthermore, while studies have shown that hypoxia is an early event during tumor development, and often associated it with clonal genomic 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. This could provide insight into more adapted strategies to increase immunotherapy efficiency.

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