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Abstract:

While it is widely accepted that high intratumoral heterogeneity confers serious challenges in the emerging resistance and the subsequent effective therapeutic targeting of cancer, the underlying biology of intratumoral heterogeneity remains elusive. In particular, it remains to be fully elucidated how microenvironmental factors shape genetic and non-genetic heterogeneity, which in turn determine the course of tumor evolution and clinical progression. In this context, hypoxia, a hallmark of most growing cancers, characterized by decreased O_2 partial pressure is a key player of the tumor microenvironment. Despite extensive data indicating that hypoxia promotes cellular metabolic adaptation, immune suppression and various steps of tumor progression *via* hypoxia regulated gene transcription, much less is known about the role of hypoxia in mediating therapy resistance as a driver of tumor evolution through genetic and non-genetic mechanisms. In this review, we will discuss recent evidence supporting a prominent role of hypoxia as a driver of tumor heterogeneity and highlight the multifaceted manner by which this in turn could impact cancer evolution, reprogramming and immune escape. Finally, we will discuss how detailed knowledge of the hypoxic footprint may open up new therapeutic avenues for the management of cancer.

1. Introduction

Tumor hypoxia is characterized by low-level O_2 pressure found within the malignant tumor mass. Hypoxia is well recognized for its role in mediating rapid metabolic adaptation of cells and affecting the various steps of tumor progression [1,2]. Much less is known about the role of hypoxia in mediating genetic and non-genetic driven tumor heterogeneity influencing tumor evolution and clinical progression.

Escape from immune surveillance is another hallmark of cancer. Among immune effector cells, CTLs and NK cells are known to play important roles in the immune response against cancer cells by inducing apoptosis, through different pathways, in a process commonly referred to as cell-mediated lysis. However, clinically detectable tumors have generally adapted along with the challenging immune system selecting for cancer clones able to escape immune recognition, resist to the lytic action of the cytotoxic immune effector cells NK cells and CTLs, and able to create an immunosuppressive microenvironment. By significantly boosting the host immune system to fight cancer, immunotherapy approaches targeting immune checkpoint receptors (PD-1 and CTLA-4) have significantly improved survival in a number of patients even with cancers previously considered rapidly fatal [3]. However, the high degree of non-responders in these cancers including lung, melanoma, an renal cancers, as well as the refractoriness of malignancies like prostate, colorectal 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 escape [3].

In this review, we discuss recent evidence for a role of hypoxia as a driver of tumor heterogeneity and highlight the potential consequences on tumor evolution and immune response modulation. Lastly, we discuss emerging therapeutic strategies that could benefit from these discoveries to improve treatments.

2. Tumor hypoxia

Hypoxia is characterized by a decrease in O_2 availability [2]. In a growing malignant tumor mass, hypoxic stress is typically generated because of abnormal formation of the vasculature resulting in heterogeneously distributed areas of low oxygen pressure [1,4]. Cells respond to hypoxia *via* the hypoxia inducible factors (HIFs), which are dimeric proteins composed of an O_2-sensitive α subunit (HIF-1α, HIF-2α, or HIF-3α) and a β subunit (HIF-2β). Proline hydroxylation of HIF-α in the presence of O_2 facilitates its binding to the tumor suppressor protein von Hippel Lindau (VHL), which through a degradation complex promotes ubiquitination of HIF-α and subsequent degradation by the proteasome. Hypoxic stress minimizes proline hydroxylation and

VHL binding resulting in stabilization of HIF-α thereby allowing dimerization of α and β subunits. Genetic alterations like VHL inactivation also favor HIF-α stabilization leading to pseudohypoxic states. The HIF dimers mainly act as transcriptional activators of target genes. These hypoxia-associated transcription factors are known to operate through direct binding to Hypoxia Response Elements (HREs) in target genes to induce expression of genes regulating numerous biological processes. This includes angiogenesis, cell survival and metastasis, proliferation, pH regulation, and metabolism. HIFs are now widely accepted as important regulators of tumor progression and metastasis development *via* the activation of hypoxic cascades [1,5]

3. Tumor plasticity

Certain cancer cell populations undergo molecular and phenotypic changes commonly referred to as cellular plasticity or phenotypic plasticity. Unlike genetic aberrations, epigenetics and transcriptional programs involved in these changes are reversible allowing cells to navigate and shift through different phenotypic states. Well-known examples of phenotypic changes include the epithelial–mesenchymal transition (EMT) program, and its reverse process MET. This implies epigenetic and transcriptional rewiring accompanying phenotypic switches between epithelial and mesenchymal states [6]. Another example is Neuroendocrine trans-differentiation in which epithelial-like carcinoma cells take on a neuroendocrine-like phenotype [6]. This has been studied extensively in the context of acquired therapy resistance of prostate cancer. Like EMT, this process may be reversible or partial, depending upon responses to hormonal interventions, and both processes have been associated with hypoxia.

The dynamic nature of these events still poses many challenges for basic and clinical research including the study of its dynamic in *in vivo* cancer models and in patient-derived samples. Technological advances enabled a significant gain of knowledge over the past few years. Recent evidence underlines the importance of malignant cell populations harboring intermediate transitional states, also called partial or hybrid states (e.g. partial-EMT) possessing combinations of epithelial and mesenchymal traits [6,7]. These cells can be endowed with high epithelial-mesenchymal plasticity (EMP), as well as self-renewal and drug resistance capabilities. This denotes properties shared with cancer stem cells (CSC). Thus, in most cases, cellular plasticity and stemness properties are intertwined in cancer, controlling the phenotypic equilibrium in cancer cell populations [8]. Other studies also indicate that “non-genetic” phenotypic plasticity is promoted by “genetic” variations affecting numerous genes including, but not limited to, RB loss, TP53 alterations or mutations in EGFR gene [9–11]. It should also be noted that drug exposure not only selects for dormant or drug-tolerant persister (DTP) cells with CSC features, but may also induce extensive epigenetic and transcriptional rewiring in these cells. This highlights the impact of environmental stress both on selection pressures and in the control of cellular plasticity and subsequent cellular diversity [12–15].

4. Non-genetic and genetic intratumor heterogeneity

Most tumors arise from a single mutated cell and accumulate heritable mutations as they become genetically instable and progress to advanced disease. High-throughput-sequencing technologies and the development of single-cell resolution methods have demonstrated that tumors are composed of a heterogeneous cell mixture of genetically distinct sub-clones on which strong selection pressures upon microenvironmental stress and treatments may act to drive Darwinian tumor evolution [16–18]. This evolutionary process alters intratumoral heterogeneity by changing the proportion of subclones within the tumor, and select for mutated clones that are more adept at growing and progressing towards aggressive and therapy resistant stages.

Ample evidence also indicates that a tumor can be composed of phenotypically heterogenous populations in line with epigenetic modulation, cell signaling changes and the cellular plasticity [6,19,20]. Studies analyzing phenotypic states of cancer cells within tumors now point to these non-genetic determinants as key contributors of intratumoral heterogeneity [19,20].

While we are far from understanding the mechanisms leading to such heterogeneity, high intratumoral heterogeneity is recognized as one of the main obstacles to the effective therapeutic targeting of cancer and overcoming therapy resistance [21]. Most importantly, the heterogeneity of a tumor is not only dictated by the heterogeneity of the cancer cells. In fact, growing cancers are also infiltrated by a variety of stromal cells, blood vessels, infiltrating inflammatory cells, and associated tissue cells composing the tumor immune microenvironment (TIME). The complex interplay between the cancer cells and the surrounding stromal cells, the extracellular matrix, as well as cytokines, chemokines and inflammatory factors released by these cells, in turn may influence the cancer progression and resistance to therapy by impacting the evolving heterogeneity [22–25]. Thus, the design of innovative immunotherapy approaches should consider the tumor immune contexture [23,26]. It is now increasingly clear that spatial and temporal decrease in oxygen availability throughout the tumor is also a critical determinant in shaping this continuous selection process in the TIME.

5. Hypoxia-mediated tumor plasticity and heterogeneity

The ability of hypoxia to activate EMT programs have been observed in numerous studies and in various cancer models [27–31]. Whereas we focus here on cancer cells, it is to note that hypoxia can mediate plasticity and differentiation of many, if not all, the tumor microenvironment (TME) components. This topic has been extensively covered by recent reviews to which the reader is referred [32–35]

Although our knowledge of the exact molecular mechanisms at play is still incomplete, the stabilization of HIFs under hypoxia has been shown to directly or indirectly stimulate the expression of EMT-associated transcription factors (EMT-TF) including TWIST1, ZEB1 or SNAI1 . EMT-regulatory pathways, such as TGFβ/TGFβR signaling or the NOTCH pathway also substantially contribute to promote EMT [1,2]. The discrepancies observed among the experimental models suggest context-dependent events. Environmental factors induced by hypoxic conditions such as TGF-β exert important functions given that it can act in an autocrine and/or paracrine fashion [36]. TGF-β also regulates stemness and cellular plasticity. Numerous reports have pointed out that hypoxia can sustain CSC growth [38] or promote phenotypic switches [39,40]. HIF1 and HIF2 can in some circumstances increase or stabilize expression of pluripotency genes, namely OCT4, SOX2, MYC, NANOG or NODAL [41,42]. They can activate the expression of AXL, a Receptor Tyrosine Kinase (RTK) with tumor promoting functions associated with EMT, plasticity and drug resistance [43–45]. AXL is a target of HIF through binding to the HRE in the AXL promoter, and its expression is also regulated by EMT-TFs and microenvironmental constituents [46,47].

Experimental studies using mouse and human cancer models point to the importance of hypoxia in mediating cancer cell plasticity and heterogeneity. Lehmann and colleagues showed that hypoxia induces HIF-1-dependent plasticity of cancer cells as well as their migration from a collective to a single-cell migration mode, EMT program and increased metastatic seeding [48]. In pancreatic ductal carcinoma cells, hypoxia-induced EMT occurs in varying degrees in cancer cell populations with more pronounced effects on stem-like cells [49].In a model of primary NSCLC cells, it was observed after sustained exposure to hypoxic stress that only a fraction of stressed epithelial cancer cells acquires a more mesenchymal phenotype, whereas the other fraction does not shift towards mesenchymal states, thus suggesting another mechanism by which hypoxia could drive phenotypic diversity [50].

Furthermore, recent studies applying single-cell RNA-Seq to human tumors have been informative with respect to elucidating the role of hypoxia on cancer cell heterogeneity. In glioblastoma, hypoxia signature in malignant cells was strongly associated with one particular type of mesenchymal-like cells (named MES2) [51]. In liver cancers consisting of mainly hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (iCCA), hypoxia and hypoxia-related genes including VEGFA were among the main factors contributing to cancer cell and stromal heterogeneity in tumors. Patients with higher cancer cell diversity in their tumors had reduced overall survival and disease-free survival [52]. In head and neck (HNSCC) tumors, both partial-EMT and hypoxic stress were identified in subsets of malignant cells but these clusters did not correlate as strongly [53]. A recent single cell analysis of the metabolic landscape in Melanoma and HNSCC tumors revealed that aerobic respiration via oxidative phosphorylation (OXPHOS) is highly variable in malignant cells. Moreover, OXPHOS correlated with both glycolysis and hypoxia in virtually all cell types, except in macrophages. These

observations point to the hypoxia-reoxygenation as major contributor to cellular heterogeneity [54]. If we want to decrypt hypoxia-induced tumor heterogeneity, it will be critical to develop tools to assess the extent of hypoxic stress and its dynamic in tumors.

6. Impact of tumor plasticity and heterogeneity on tumor immune escape

Previous reports covered mechanisms by which EMT and plasticity could drive therapy resistance and immune escape [55–57](cite). It was shown that EMT can reduce immunogenicity of cancer clones through altering the MHC class I antigen presentation machinery [55,56]. Cancer cells expressing the EMT factor Brachyury are protected from lymphocyte-mediated cytotoxicity likely due to pronounced resistance to apoptosis pathways(cite). Experimental evidence also exists that EMT-associated autophagic states endow cancer cells with lower susceptibility to CTL-mediated lysis [58]. In various models, EMT was found to associate with reduced recognition by immune killer cells, concurrent with defects in the establishment of immunological synapses or following loss of adhesion molecules such as ICAM1 [45,50,59]. ICAM1 on target cells binds to its cognate receptor LFA-1 (ITGAL/ITGB2) on effector lymphocytes, strengthening the interaction between the cytotoxic killer cells (CTLs and NK cells) and carcinoma target cells.

Immune co-stimulatory and co-inhibitory molecules can exhibit different expression patterns in mesenchymal-like cancer cells compared to their more epithelial counterparts. It has been demonstrated that increased mesenchymal features in cancer cells associate with increased PD-L1 expression [60,61] or reduced expression of the NKG2D ligand ULBP1 [45]. Dormant stem like cells have been demonstrated to evade immune surveillance through comparable mechanisms [62]. Furthermore, multiple lines of evidence suggest overall lower immunogenicity for CSCs (reviewed in [63]). The findings mentioned above are important with respect to resistance to various immune effector cells, including NK and CTLs. In theory, reduced expression of MHC class I molecules on cancer cells should render them more susceptible to NK-mediated killing *via* the activation of MHC class I inhibitory receptors, namely Killer cell immunoglobulin like receptors (KIRs). However, a substantial fraction of the cancer cell populations in the mesenchymal or the EMP/CSC states is defective for cell adhesion molecules and NK-cell activating ligands, which may therefore protect them from NK immunosurveillance. It is worth noting that most of these mechanisms are not only relevant to understand escape to innate immune response, they could largely contribute to immune suppression and resistance to adaptive immunity implying anti-tumor immune responses and diverse immune effector cells, such as CTLs and other tumor infiltrating lymphocytes (TILs). Supporting this notion, cells transitioning to more mesenchymal phenotypes generally upregulate TGF-β which is well-known for its immune suppressive properties [64,65].

Moreover, the impact of EMT on the expansion of immune suppressor cells like CD4+ FoxP3 immunosuppressive T-regs has been studied extensively in experimental models (reviewed in [66,56,55]. An enrichment of T-regs was reported in lung tumors harboring a mesenchymal phenotype (high EMT score) compared tumors with a more epithelial phenotype [67]. Cancer-associated fibroblast heterogeneity through TGF-β and CXCL12 pathways could greatly influence EMT while also contributing to attraction, survival, and differentiation of T-regs which in turn can affect immune responses [24,25].

Using clinical samples of NSCLC, Thompson and colleagues recently observed that patients whose tumors have a more epithelial phenotype are more likely to respond to immune checkpoint blockade (ICB). An EMT/Inflammation-based signature developed in this study may soon have clinical value to predict clinical response in this setting [68]. In another study, Hugo and colleagues discovered a gene signature associated with melanomas innately resistant to anti-PD-1 therapy. This innate PD-1 resistance (IPRES) gene signature is characterized by an upregulation of gene sets related to mesenchymal transition, cell adhesion, extracellular matrix remodeling, wound healing, monocyte/macrophage chemotaxis, and also angiogenesis.
In many of these studies, the direct link to hypoxia has not been investigated. It is tempting however to speculate that hypoxia may be one of the underlying TIME components governing tumor immune escape by promoting EMT and plasticity induced heterogeneity of the cancer cell population. More specific studies are needed to address this critical issue.

In previous work investigating cancer subclones emerging from sustained hypoxic stress, carcinoma subclones with pronounced mesenchymal phenotype were less susceptible to NK- and CTL-mediated lysis as compared to carcinoma subclones of a more epithelial phenotype [50]. This difference is at least partly explained by reduced expression of ICAM1, ULBP1, and MHC class I as well as increased TGF-β expression in mesenchymal subclones [45,50]. Moreover, in the mesenchymal subset, AXL was found differentially expressed and associated with cancer cell -resistance to NK- and CTL-mediated killing. As mentioned above, AXL can be upregulated by hypoxia, and AXL was also found to be a component of the IPRES gene signature along with other known HIF targeted genes like ROR2, WNT5A, LOXL2 VEGFA, and VEGFC [69].

We have previously demonstrated that NSCLC cells exposed to short-term hypoxic stress, accumulated of NANOG, a stem cell self-renewal factor, which protected the carcinoma from specific lysis by CTLs in a mechanism involving STAT3 phosphorylation [70]. Interestingly, NANOG expression by the carcinoma cells promoted immune suppression by directly upregulating TGF-β1 expression which resulted in Treg expansion and increased recruitment of immunosuppressive ARG1-expressing macrophages [71]. Zhang and colleagues found that HIF-1α regulated CD47 expression in breast cancer cells not only promotes maintenance of cancer stem cells, it also promotes cancer cell evasion of phagocytosis by macrophages [72]. EMT-TFs SNAI1 and ZEB-1 could also regulate CD47 expression [73]. In liver cancer, tumors showing the highest heterogeneity displayed increased marks of hypoxia, and the malignant cells, rather

stromal cells, were responsible for the high of VEGFA expression. Hypoxia signaling was linked to tumor cell diversity through VEGF expression which coincides with microenvironmental reprogramming, lower cytolytic activities, immune suppression, reduced immune responses and poorer patient overall survival [52].

Taken together, these data provide insight into how hypoxia-mediated plasticity and heterogeneity may drive tumor immune escape though multi-resistant phenotypes.

7. Potential relationships between hypoxia and genetic heterogeneity

Hypoxia, as a constantly fluctuating feature of the developing tumor core, contributes to the genetic heterogeneity through several mechanisms [74]. First, hypoxic downregulation of several DNA repair mechanisms has been documented at transcriptional and translational level, rendering the cells susceptible to genomic instability. Chronic hypoxia can lead to downregulation of homologous recombination (HR), non-homologous end joining (NHEJ), DNA mismatch-repair (MMR) capacity in hypoxic tumor cells [75]. The duration of hypoxia is an important parameter which likely contributes further towards intratumoral heterogeneity. Both chronic (prolonged) and acute (transient) hypoxia can be found within a given tumor resulting in gradients of oxygen pressure. Thus, re-oxygenation of cells following acute hypoxia can increase the levels of reactive oxygen species (ROS) and decreased mitochondrial ATP production, which in turn may induce alterations in DNA damage checkpoints and contribute to genomic instability [74,75].

Furthermore, hypoxic stress induces enormous levels of replication stress, which can possibly lead to replication errors and accompanying increase in mutation load [76]. Hypoxia boosts the accumulation of oncometabolites (2-hydroxyglutarate, fumarate and succinate) [77]. An increase in oncometabolite levels can hinder DNA repair processes [78]. Specifically, 2-hydroxyglutarate inhibits the lysine demethylase KDM4B which acts on histone H3 proteins. The inhibition of KDM4B results in hypermethylation of H3K9 perturbing the proper execution of HDR.

In a survey of prostate tumors, by using copy number alterations as a surrogate for genomic instability, Lalonde and colleagues have found that hypoxia is not consistently linked with overall genomic instability. However, in a subgroup of specimens with poor prognosis, it appeared that tumor hypoxia relates to genomic instability to promote tumor aggressiveness [79]. Others have found an increase in genomic alterations in metabolic genes in tumors displaying a high hypoxic signature that can vary between tumor types [80].

Hypoxic tumors may be characterized by an elevated mutational burden with distinct mutational signatures [81,82]. Recent work associates tumor hypoxia with increased incidence of mutations in genes like TP53 [81] and other specific molecular including alterations of PTEN, APC, or MYC, which have all been established as drivers of phenotypic plasticity in certain

tumors [9]. Interestingly, these studies also identified mutational signatures correlating with hypoxic tumors [81], some of which may reflect hypoxia-dependent mutational processes including deficiencies in DNA repair pathways like DNA double-strand break repair, HR and MMR pathways [81].

Data from this group also suggest that hypoxia acts early on during tumor evolution with an essential role on clonal, rather than subclonal, alterations [81]. Clonal (also referred to as "trunk") mutations are common to all cancer clones within a tumor while subclonal (also referred to as "branch") mutations are only present in a subpopulation of clones emerging later in the clonal evolution of a tumor. Of further interest, there might be interactions between tumor hypoxia and certain alterations (including loss of PTEN) which influence the subclonal composition of the tumor [81]. The sum of acquired alterations as a result of epigenetic, phenotypic and microenvironmental factors may eventually generate sub-clonal populations.

It is not clear to what extent these alterations occur in distinct areas of the same tumor subject to various stress conditions. One potential limitation is that DNA and/or RNAs are often obtained from large tumor specimens containing a varying degree of hypoxic and normoxic regions. How to assess heterogeneity in clinical samples is still an open question. The use of signatures to predict outcomes may be useful in some cases, but it could also lead to underestimation of heterogeneity and related genomic anomalies [83]. Multiple sampling may be required to address this issue and determine how the use of hypoxia signatures can affect the assessment of intratumor heterogeneity. Interestingly, previous reports also suggest transient hypoxia-induced site-specific copy gains (TSSGs) that could result in heterogeneity within tumors [84]. Such transient events may represent another level of complexity and additional studies are required to provide more insight into hypoxia-mediated genetic heterogeneity [83].

Multi-region sequencing or single cell technologies may help to increase resolution of the clonal diversity during cancer evolution. CNV profiling inferred by single-cell transcriptomes has been used to differentiate malignant cells from non-malignant cells and can help estimate genomic alteration [52,53,85]. Recent single-cell transcriptomic data in liver cancer indicate that transcriptomic diversity of tumor cells correlates with genomic diversity, and patient prognosis [52]. Since hypoxia/VEGFA drives transcriptomic and tumor cell diversity, hypoxia may be closely related as well to intratumor genomic diversity [52]. Additional studies are required to dissect this relationship. Functional studies validating the mutagenesis events and clonal segregation patterns exclusively under hypoxic conditions are also required to understand this ever-changing component in the tumor ecosystem. Hypoxia promotes a selective advantage for cells with loss of tumor-suppressor genes and overall defects in apoptotic pathways, thereby contributing to clonal segregation [86]. Cells in the growth-restraining environment may adapt to flawed DNA repair by enhancing the mutational rate. Such adaptive mutations affect the immunogenicity of the tumor and foster the growth of well adapted sub-clonal populations in

an otherwise hostile environment. The increased expression of cancer-specific antigens, neoantigens, can elicit an immune response through increased tumor recruitment and activity of effector T-cells [87]. In their study, Jia et al. reported that although the local mutational diversity associated with immune heterogeneity in NSCLC tumors, concurrent with T-cell clonal expansion, anti-tumor cytotoxicity did not directly correlate with neoantigen abundance [88]. Quality and the clonal status of the neoantigens may be important [89]. During tumor evolution, immunoediting of tumor cell populations by neo-epitope depletion may select for certain antigen-negative variants or subclonal neoantigens [88]. Whereas clonal neoantigens may result in better ICB response rate in many patients, accumulation of subclonal neoantigens is likely to distract efficient immune responses toward prominent clonal alterations [16]. Genomic instability as a driving force for neoantigen distribution deserves further attention [90]. The extent to which hypoxia could influence this kind of subclonal heterogeneity remains unknown. Admittedly, conflicting results exist regarding the impact of hypoxia on T cells [91]. On the other hand, it has been established that the hypoxic tumor microenvironment, through the amplification or the recruitment of immunosuppressive components, as well as the secretion of immunosuppressive growth factors and cytokines, like PD-L1 and TGF-β plays a pivotal role in establishing an immunosuppressed TIME [23,34,92,93]. Moreover, as discussed above, regional differences in subclonal characteristics due to non-genetic heterogeneity can influence the overall spatial and temporal heterogeneity of a tumor. A multi-level assessment of heterogeneity will be necessary to identify the determinants of phenotypic heterogeneity, to appreciate the intercellular communication and the contribution of the tumor immune microenvironment in tumor evolution, as well as the role of hypoxia in all of these coordinated processes [94]. Finally, we note that evidence suggests that the DNA damage response can also activate the immune response of tumors through the cGAS-STING axis [95]. The cyclic GMP-AMP synthase (cGAS) molecules recognize the damaged self-DNA particles in the cytoplasm and activate Stimulator of Interferon (STING) leading to the induction of Type I Interferons (IFNs). This point is important considering that hypoxic cells are particularly susceptible to DNA damage. Type I IFNs enhance the immune effector cell functions at the tumor site. Tumors expressing high levels of Type I IFNs signaling demonstrate better response to treatment through increased infiltration of CTLs [95]. Nevertheless, as recently argued, hypoxic cancer cells may bypass this response by repression of the cGAS-STING pathway [96]. These instances illustrate the role of hypoxia in regulating genetic events likely influencing the genetic heterogeneity. They also provide a foundation for further studies investigating the associated mechanisms underlying tumor resistance as well as evasion from the immune recognition.

8. Hypoxic influence on epigenetic heterogeneity and potential consequences on tumor immune escape

Phenotypic and behavioral heterogeneity in genetically homogeneous clones within a tumor also suggest that epigenetic mechanisms may underlie such a diversity. There is evidence for a role of hypoxia and HIFs on epigenetic changes at multiples levels resulting in histone methylation and chromatin reprogramming [97,98]. First, hypoxia regulates the expression and activity of histone modifying enzymes. The histone methyltransferase G9a (EHMT2) is stabilized under hypoxia, and the histone demethylase JMJD2C (KDM4C) as an HIF-1 target gene is induced by hypoxia. Both enzymes act on histone H3 lysine 9 (H3K9) [99,100]. Moreover, HIFs in some cells may induce global chromatin changes [101]. REST namely RE1-silencing transcription factor [also known as neuron-restrictive silencer factor (NRSF)] is regulated by HIFs [102]. REST has a pivotal role in transcriptional repression and regulation of manifold chromatin remodelers that dictate cellular phenotypic changes during development and in cancer [103,104]. Second, hypoxia has been implicated in the regulation of non-coding RNAs including miRNAs. While certain miRNAs such as miR-210 are upregulated via HIF-dependent mechanisms [105], others may be downregulated. This is explained by the fact that miRNA processing is compromised by hypoxia through epigenetic repression of DICER concomitant with inhibition of H3K27me3 demethylases KDM6A/B under hypoxia [106]. Importantly, defective expression of DICER and subsequent downregulation of the miR-200 target ZEB1 promoted the engagement of the EMT program and the acquisition of CSC-like states in various cancer systems [106].

Hypoxia has been also reported to be involved in modulation of DNA methylation. Theinpoint and colleagues demonstrated that hypoxia can cause DNA hypermethylation in cancers through suppression of Ten-Eleven Translocation (TET) enzymes that are essential for DNA demethylation [107]. In clinical tumor samples, tumor suppressor genes (TSG) promoters were markedly hypermethylated suggesting a hypoxia-dependent cellular selection of hypermethylation events. Although much remains to be learned about the landscape of TSG regulated by HIF-dependent DNA Methylation, methylation-mediated alterations of the crucial TSG genes is assumed to influence tumor heterogeneity and plasticity [108]. As discussed above, alterations of PTEN expression can lead to tumor diversity. PTEN alterations may act as clonal or subclonal event, and in certain cancers, including prostate cancer, such alterations increase lineage plasticity and phenotypic adaptability, enabling therapy resistance [108,109,9].

Epigenetic silencing of the TSGs APC or E-cadherin could potentially modulate the cellular fate of cancer cells by stimulating the Wnt/β-Catenin pathway which in turn controls the delicate balance between stemness and differentiation [108]. Epigenetic silencing of MMR factors MLH1 and MGMT, could lead to increased mutability phenotypes [108]. Of note, for a number of the mentioned deregulated epigenetic mechanisms, association with immune escape have yet to be firmly established. However, given their established relationship with EMT or tumor plasticity, we would not be surprised to see emerging among them novel immune escape mechanisms stimulated by hypoxia.

Pan-cancer analysis from the TCGA initiative data have demonstrated that global DNA demethylation was associated with increased mutational load and immune evasive signature [109]. The authors also demonstrated that global DNA demethylation was associated with poor clinical benefit from ICB therapy [109]. Rosenthal and the TRACERx consortium hypermethylation in genes that contain neoantigen mutations suggesting an epigenetic mechanism of immune evasion [90]. The dynamic interplay between tumor hypoxia, epigenetic reprogramming of the tumor and immune cells and subsequent resistance to ICB must be explored for devising new and effective combinatorial treatment strategies.

9. Therapeutic implications of tumor heterogeneity and plasticity

As described above, research is beginning to unravel diverse mechanisms by which EMT/cellular plasticity is linked with immune escape. One intriguing perspective raised by these studies is the possibility that such non-genetic and genetic changes give rise to new therapeutically exploitable weaknesses within the tumor. Future investigations are needed to determine the extent to which these mechanisms are associated with primary and/or acquired resistance. Some cancer clones within the EMT spectrum may be intrinsically resistant, and in other more sensitive ones, stress-induced phenotypic changes may emerge following therapy induced selection pressure.

Hypoxia is associated with non-genetic, conceivably reversible on the one hand, and more stable genetic changes in the other hand, both driving tumor heterogeneity and linked in many instances. In light of the fact that these processes are presumably linked in many instances, it is reasonable to speculate that specific targeting of hypoxia could be a beneficial approach to reduce tumor heterogeneity, prevent emergence of immune escape mechanisms, potentiate the host anti-tumor immunosurveillance, and trigger immune response. One challenge impeding specific targeting of hypoxia has been the lack of specific and potent HIF inhibitors. The recent development of improved HIF2 inhibitors has fueled the hope to target hypoxia and HIF-1α specifically [110]. A few HIF-1α inhibitors have already demonstrated interesting properties in preclinical studies. HIF-1α inhibitor PX-478 enhanced the anti-tumor effect of gemcitabine by inducing immunogenic cell death in murine pancreatic ductal adenocarcinoma [111]. Interestingly, hyperbaric oxygen therapy (HBOT) of a triple negative MDA-MB-231 breast cancer model attenuated EMT marks (N-cadherin, AXL), and hyperoxia was shown to reduce the metastatic potential the cancer cells [112]. One complicating factor in these studies is the difficulty to recapitulate in mouse models the complex heterogeneity often seen in human tumors. Moreover, an open question is when to target hypoxia. We assume that early targeting, such as in the neoadjuvant or adjuvant setting, may be particularly beneficial to restrain tumor heterogeneity and its evolution.

Less specific agents have been used alone or in combination to block hypoxia or particular targets of HIFs such as VEGF, with interesting benefits in cancer treatment. The rationale is first to normalize tumor vessels to improve drug delivery, and reduce immune suppression and resistance mediated by the TIME. Various compounds are currently being evaluated in combination with immunotherapy in solid tumors [113]. It will be interesting to assess the potential benefits in cases where tumor VEGFA expression associates with a high tumor diversity and aggressiveness [52]. To improve patient stratification, it would be appropriate in these studies to assess the value of hypoxic gene signatures in patients' tumor biopsies or high resolution Imaging approaches, as surrogate markers of hypoxia extent [113].

Based on the established tight association between hypoxia and EMT, approaches to target TGF-β have been a source of excitement recently [114]. TGF-β regulates both tumor plasticity, and the immunosuppressive microenvironment. Moreover, it could regulate genomic instability in some tumors [11]. Experimental evidence exists that inhibition of TGF-β receptors or TGF-β-blocking can potentiate anti-tumor immunity and response to ICB therapy [115,116].

The apparent multi-resistant phenotype conferred by AXL signaling is intriguing. Our recent work suggests that pharmacological inhibition of AXL using bemcentinib can enhance lymphocyte-mediated killing of highly resistant mesenchymal NSCLC cells [45]. In another NSCLC model in which AXL mediates EGFR inhibitor resistance, AXL targeting blocked clonogenicity, disrupted the autophagic flux and induced immunogenic cell death (ICD) in cells [117]. We would like to emphasize the potential of agents inducing ICD as it could give an opportunity to re-sensitize even the most aggressive cancer cell populations often characterized by lower immunogenicity [118]. The study of Aguilera et al. showed in mammary tumor models that Axl targeting in mice restored radiosensitivity in resistant tumors coinciding with CD8+ T-cell response that is largely enhanced in combination with ICB [119]. Likewise, Guo and colleagues, using lung tumor models outlined the potential of combining Axl targeting with anti-PD1 to prevent the emergence of resistance mechanism [120]. AXL inhibitors and anti-AXL antibodies are currently being explored in combination with ICB for various indications in patients with solid tumors including ovarian, urothelial and NSCLC (reviewed by Horn LA et al.[114]).

Thus, it is tempting to speculate that if one could warm up the hypoxia-induced immune-suppressive TIME, and target some of the hallmarks of the mesenchymal cancer cells as described above, one could increase the efficacy of ICB also in these tumors.

Increased PD-L1 expression on mesenchymal or CSC-like cells provide a rationale to test anti-PD-1/PD-L1 agents in highly heterogenous disease [61]. However, it is worth noting that the effects of these ICB on altering tumor heterogeneity is yet to be addressed. Evidence is missing whether cancer cell populations with substantial phenotypic adaptability are effectively affected by immunotherapeutic regimens. The study of Hugo et al. in melanoma may indicate they are not [69]. Single cell analysis of various cancer types is required to address this question.

Multiple approaches have been proposed to target CSCs or carcinoma cells with stem-like properties [121]. Moreover, these slow cycling immune privileged cells should express CSC antigens potentially targetable by immunotherapy [122]. Clinical trials are ongoing or planned to investigate CD47 blockade either alone or in combination with ICB to boost immune response in relapsed and refractory diseases. IL8 and the developmental/EMT-associated factor Brachyury also seem good candidates for immunological intervention [116,123].

A better understanding of the epigenetic mechanisms also raised the possibility to target epigenetic modifiers with “epidrugs” in combination with current immunotherapeutic approaches (reviewed in [124]). HBOT, hypoxia targeting drugs that are capable of inducing DNA damage (like Evofosfamide), hypoxia alleviating drugs in combination with demethylating agents (azacytidine) and ICB may therefore enhance patient benefit when compared to a single-agent inhibition.

Further research is needed to determine which patients are more likely to respond these treatments and if intratumoral heterogeneity influences response. Finally, increasing evidence suggest the involvement of WNT/β-catenin pathway activation in tumor immune escape, and thus it would be interesting to explore this new therapeutic avenue [125–127].

10. Conclusions and perspectives

Molecular paradigms and transcriptional programs engaged in the control of tumor heterogeneity and tumor plasticity seem to converge to drug resistance, and new evidence highlights their importance in the immune escape of tumors. As suggested by experimental and transcriptomic data, hypoxia may play a central role in the regulation of these coordinated programs, while modulating anti-tumor immunity. However, efficient tools are urgently needed to determine the involvement of hypoxic stress at the global and at the single cell levels within tumors. Certainly, one aspect that might confuse the issue is that hypoxia can manifest in different forms and contexts, in particular hypoxia may vary in spatial (levels, gradients) and temporal (acute vs chronic) appearance in tumors, which in turn may alter the cellular manifestation of the hypoxic footprint. Additionally, both genetic and non-genetic changes could be driven by hypoxia to generate tumor resistant variants, that depending on the selection pressure, may represent a very small fraction of the total tumor burden. Technological advances combined with cellular analyses may aid in resolving these issues. Moreover, developing therapies targeting tumor hypoxia, hypoxia-induced clonal heterogeneity or cellular plasticity have the potential to provide new therapeutic solutions to improve the response rates in combination with ICB regimens. It is encouraging to see a number of targets and compounds in early development, and a few being tested in clinical trials in combination with ICB. The rationale for these combination therapies is to reduce hypoxia-driven heterogeneity and tumor

plasticity on one hand, while targeting the immune escape properties of these cancer clones on the other hand. How these combinations affect the polarization and the intricate communication between cancer cells and immune cells remain to be elucidated. Rigorous evaluation of the efficacity, optimal sequences, as well as potential toxicities of these combinatorial approaches will determine their eventual implementation in clinical practice.

Declaration of Competing Interest

Authors declare no conflict of interest

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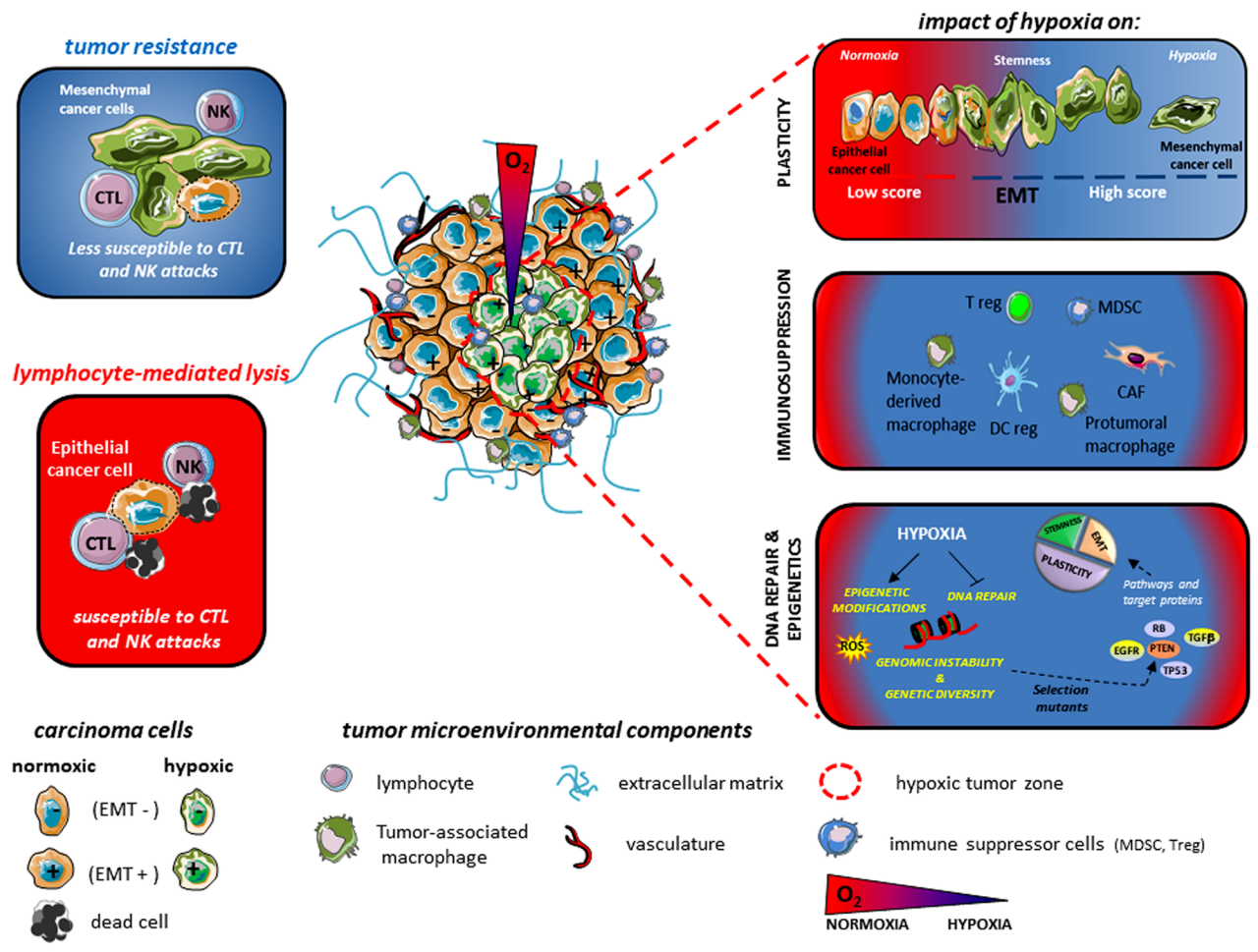
Figure 1: Schematic model for hypoxic-driven mechanisms promoting intratumoral heterogeneity. Tumor hypoxia can mediate genetic and non-genetic changes in cancer cells as well as remodeling of the tumor microenvironment. Non-genetic changes such as phenotypic switches observed during EMT, owing to increased plasticity, can result in the emergence of immune resistant cancer clones less susceptible to lymphocyte-mediated cell death. Furthermore, tumor hypoxia often leads to the establishment of an immunosuppressive TME able to shut down immune responses. Epigenetic and genetic modifications mediated by hypoxia may further lead to increase genomic instability, intratumor heterogeneity concurrent with the acquisition of new identities via increased plasticity and stemness. Again, this could result in the emergence of cancer clones more adept at growing and progressing towards aggressive and therapy resistant stages. In fact, all these coordinated processes might interact with each other's during cancer evolution to drive innate and acquired resistance to immunotherapy (adapted from [34])


tumor resistance
Mesenchymal cancer cells
NK
CTL
Less susceptible to CTL and NK attacks
lymphocyte-mediated lysis
Epithelial cancer cell
NK
CTL
susceptible to CTL and NK attacks
O2
impact of hypoxia on:
PLASTICITY
Normoxia
Stemness
Hypoxia
Epithelial cancer cell
Mesenchymal cancer cell
Low score
EMT
High score
IMMUNOSUPPRESSION
T reg
MDSC
Monocyte-derived macrophage
DC reg
CAF
Protumoral macrophage
DNA REPAIR & EPIGENETICS
HYPOXIA
EPIGENETIC MODIFICATIONS
DNA REPAIR
ROS
GENOMIC INSTABILITY & GENETIC DIVERSITY
STEMNESS
EMT
PLASTICITY
Pathways and target proteins
RB
TGFβ
EGFR
PTEN
TP53
Selection mutants
carcinoma cells
normoxic
hypoxic
(EMT -)
(EMT +)
dead cell
tumor microenvironmental components
lymphocyte
Tumor-associated macrophage
extracellular matrix
vasculature
hypoxic tumor zone
immune suppressor cells (MDSC, Treg)
O2
NORMOXIA
HYPOXIA