Molecular Pathways: Hypoxia response in immune cells fighting or promoting cancer

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ABSTRACT

Both malignant and stromal components in tumors are influenced by the physiological conditions of the microenvironment. Hypoxia is a prominent feature of solid tumors as a result of defective vascularization and intense metabolic activity. The gene expression control mechanisms that adapt tissues to hypoxia are exploited by tumors to promote angiogenesis and vasculogenesis. Infiltrating immune cells (macrophages and lymphocytes) and other stromal components are also influenced in their functions by limited O₂ supply. Hypoxia-inducible factors (HIF) are the main molecular transcriptional mediators in the hypoxia-response. Degradation and activity of HIF-1α and HIF-2α are tightly controlled by the fine-tuned action of oxygen-sensing prolyl and asparagyl hydroxylase enzymes (PHDs and FIH). Recent evidence indicates that hypoxia can modulate differentiation and function of T lymphocytes and myeloid cells, skewing their cytokine-production profiles and modifying the expression of co-stimulatory receptors. This conceivably includes tumor-infiltrating lymphocytes (TILs). Hypoxia not only directly impacts tumor-infiltrating leukocytes, but also exerts effects on tumor cells and vascular cells that indirectly cause selective chemokine-mediated recruitment of suppressive and pro-angiogenic T cell subsets. This review focuses on changes induced by hypoxia in immune cells infiltrating solid malignancies and which might be either promoting or fighting cancer and are important for immunotherapy.
Background.

**HIF response to hypoxia shapes the tumor microenvironment.**

O$_2$ supplied by haemoglobin from the blood stream is intracellularly present in normal tissues at around 10% and even less in metabolically active tissues (1). Under these normoxic conditions there is sufficient O$_2$ to ensure correct function of ATP synthesis in mitochondria. In tissue culture experiments, immune cells are placed under normoxic conditions overlooking and ignoring the fact that immune cells must work under hypoxia (0.1 to 3 % O2) when performing their functions in infected tissues, tissues undergoing autoimmunity or inside solid tumors (2). Even in secondary lymphoid organs such as the spleen, the O2 concentration is below 5% (3).

Hypoxia-inducible factor 1 (HIF-1α) is the main regulator of the adaptation to a shortage of O$_2$ (4). It was originally discovered in cells regulating erythropoietin production(5). As a way to preserve cell metabolism most genes under the control of HIF-1α and HIF-2α are related to glucose transport and metabolism, diverting aerobic to anaerobic ATP production. In addition, the HIF-1α response induces soluble enhancers of vascularization such as VEGF (6) and adrenomedulin (7).

Figure 1 shows that O$_2$ concentration-sensitive hydroxylase enzymes govern the molecular machinery which regulates the function of HIF-1α (8). Under normoxic conditions, prolyl-4-hydroxylase isoforms
(PHD1, 2 and 3) act on prolyl residues of HIF-1α (Pro402 and Pro564) defining two oxygen-dependent degradation domains (ODDD) in a reaction co-catalyzed by iron (Fe$^{2+}$) and 2-oxoglutarate (2-OG) (9). Once HIF-1α is prolyl-hydroxylated, a protein complex containing the Von-Hippel Lindau tumor-suppressor protein (pVHL) acting as an E3 ubiquitin ligase can build up ubiquitin chains linked to HIF-1α (Figure 1). Lysine-48 poly-ubiquitination in HIF factors leads to rapid proteolytic degradation by the proteasome (9). If O$_2$ is below a certain threshold (around 3% but the exact figure is probably dependent on cell type and species) the PHDs become unfuctional (figure 1). HIFs are then not efficiently degraded and translocate to the nucleus to dimerize with HIF-β. HIF-1α or HIF-2α forming dimers with HIF-β find short nucleotide sequences referred to as hypoxia responsive elements (HRE) on which these proteins act as transcription-promoting factors (10). The crucial function of HIF transcription factors is illustrated by the lethal phenotypes of HIF knockout mice which die as embryos from vascularization failures (11).

Experimentation to clarify the role of the different molecular elements in the pathway has required tissue lineage restricted conditional knock-out mice of HIF and the three PHDs (12).

Hydroxylation on asparaginyl residues (Asn-804) located on the C-terminal transactivation domain (CTAD) of HIF-1α by the Factor Inhibiting HIF (FIH) constitutes another level of regulation (13). FIH activity is also dependent on O$_2$, Fe$^{2+}$ and 2-OG. When oxygen is
available, hydroxylation on Asn-804 blocks the interaction of HIF-1α with the nuclear transcriptional co-activator protein p300 (14) (Figure 1). Non-overlapping functions of all these mechanisms are supported by recent studies that rely on gene-modified animals selectively deficient for PHD1, 2, 3 or FIH (12).

HIF-2α has important functions mainly in erythropoiesis and vascularization (15, 16), and on macrophages (17) because expression of HIF-2α mRNA is cell type-dependent (18). As the focus of this review is immune cells undergoing hypoxia, we will concentrate on responses mediated by HIF-1α and refer to HIF-2α only in relation to macrophages.

HIF-1α does not only control gene transcription directly and a number of alternative functions and mechanisms of the protein are being revealed. These include: i) protein-to-protein association with other transcription factors such as RAR-related orphan receptor-γt (RORγt) (4) and activator protein-1 (AP-1) (19) (Figure 1), ii) induction of miRNAs such as miR-210 (20), iii) induction of the expression of transcription factors which in turn control other genes and/or epigenetic mechanisms such as histone acetylation involving p300/CBP (21).

HIF-1α can also decrease the levels of gene transcription by different mechanisms (4). Surprisingly, HIF-1α can also induce the degradation of proteins targeting them to polyubiquitination as has been recently observed with the FOXP-3 transcription factor in CD4+ T lymphocytes (4) (Figure 1). The PHDs that hydroxylate HIF have also
been discovered to prolyl hydroxylate members of the canonical NF-κB pathway (22) that are critically involved in inflammation.

**The innate immune system and inflammation under hypoxic conditions.**

Tumor stroma contains vascular cells, myeloid cells such as tumor associated macrophages, tumor infiltrating lymphocytes and activated fibroblasts associated with the tumor. Vascular cells including endothelium and pericytes have been extensively studied in tumors and undergo functional changes due to the hypoxic microenvironment (23). Moreover, components of tumor stroma have been shown to outperform malignant cells in the production of VEGF and thus play a key role in angiogenesis and aberrant vascularization of tumors (Figure 2) (24, 25).

Myeloid cells are highly represented in intratumoral environments and macrophages are frequently the most abundant of these cells that usually invade the intratumoral hypoxic environment. Indeed, hypoxic tumor areas induce tumor-homing of bone marrow-derived CD45+ myeloid cells (12). These effects have been reported to be in part dependent on the production by hypoxic tumor cells of stromal cell-derived factor α (SDF1α) which binds to CXCR4. These cells are also sensitive to IL-8 (CXCL8)-guided chemotaxis (26) and IL-8 is abundantly produced in the tumor at least in part in response to hypoxia.
Tumor associated macrophages (TAMs) normally contribute to tumor progression (Figure 2) (27). Indeed, while the cytotoxicity of macrophages in the early immune response contributes to the death of tumor cells, the presence of macrophages has been also correlated with a poor prognosis for patients. Macrophages recruited to inflammatory sites undergo distinct forms of activation depending on the cytokines signals encountered in the surrounding milieu. For example, cytokines or bacterial compounds that usually accumulate during bacterial infection such as lipopolysaccharide (LPS) and helper type-1 (Th1) cytokines (e.g., IFN-γ) induce classic macrophage polarization (M1)(28). This M1 polarization is chiefly characterized by enhanced intracellular bacterial killing, production of nitric oxide (NO), tissue destruction but also with resistance to tumor progression. By contrast, helper type-2 (Th2) cytokines such as IL-4 as well as other signals induce alternative macrophage polarization (M2) mainly oriented towards immunoregulation, tissue remodeling and tumor promotion (28-30).

Recent evidence suggests possible contrasting roles for HIF-1α and HIF-2α in macrophage M1 and M2 polarization. In this regard, HIF-1α is activated in response stimuli that induce M1 polarization, but is not altered under conditions in which macrophages undergo M2 polarization in response to IL-4 (31, 32). Such HIF-1α upregulation seems to be dependent on a specific increase in HIF-1α mRNA levels which involves NF-κB transcription factor (17, 33, 34). In addition, HIF-1α induces
markers of M1 polarization such as inducible nitric oxide synthase (iNOS). Moreover, loss of HIF-1α represses M2 markers such as macrophage scavenger receptor 1 (35, 36).

In contrast, a recent study have indicated that HIF-2α is induced specifically during M2 polarization and also show that HIF-2α is a critical regulator of arginase 1 gene expression, a molecular marker of M2 polarization which reduces the intracellular L-arginine pool required to produce NO (31, 37). This could suggest opposite roles of the HIF1 and HIF2 systems in NO generation and potentially in M1/M2 polarization. However, controversy surrounds the role of HIF-2α in M2 polarization and arginase expression, probably reflecting the different conditions of the in vitro models employed (38).

A recent study in an adenocarcinoma model showed that hypoxic TAMs show signs of M2 polarization. Interestingly, the majority of TAMs resembling a M1 phenotype are located in normoxic areas while M2 are in the hypoxic regions, suggesting that the proposed hypoxia-HIF2α-M2 polarization axis could dominate in the intratumoral hypoxic environment (30). In this regard, immunohistological studies have found strong HIF-2α expression in TAMs (39). Moreover, genetic studies in myeloid HIF-2-deficient mice show altered migratory properties of HIF-2-deficient macrophages that were associated with reduced tumor progression (38). It is also important to consider that HIF activity of TAMs not only alters intratumoral endothelial cells but also regulates other cell types acting as
immunosuppressors of T cells such myeloid derived suppressor cells (40, 41) (Figure 2)

Importantly, metastatic niche formation has recently been reported to depend on HIF-1α, which induces multiple members of the lysyl oxidase (LOX) family that facilitates bone marrow-derived cell recruitment prior to metastatic colonization by malignant cells (figure 2) (42).

It is not yet known what effects hypoxia exerts on dendritic cells (Figure 2), but hypoxia has been found to favour the immune and inflammatory response, thereby presumably enhancing their ability to prime and activate T cells (43). Moreover, dendritic cells cultured with hypoxic glioblastoma tumor lysates are more efficient at priming effector T cells if compared to antigen uptake from normoxic tumor-cell lysates (44).

**Tumor-infiltrating lymphocytes under hypoxia.**

T lymphocytes in tumors come in different flavours (Figure 2). Some of these are potentially endowed with tumoricidal properties that are restrained, while others are suppressive of the functions of effector T cells and NK cells. Such immunosuppressor lymphocytes are named regulatory T cells (Tregs) (45). Among effector T lymphocytes, CD8 and CD4 can be found in the tumor stroma. Tumor-infiltrating lymphocytes enter tumors from blood vessels. A variety of factors in the tumor repress their functional activity although there is evidence for tumor antigen
recognition by TILs. The balance of regulatory and effector T cells in the stroma correlates with the prognosis of patients and with the response to immunotherapeutic interventions (44).

T cells when entering tumors reach progressively hypoxic regions as they penetrate deeper into the tumor. Nonetheless, T cells infiltrating tumors tend to remain associated with vessels in subregions that ought to be less hypoxic. It is well known that areas of solid tumors can become completely anoxic and undergo necrosis. Very few T cells are found around these necrotic areas.

Unfortunately little research has been carried out into the activation of T cells under hypoxia and the differences with activation under normoxic conditions. Some pioneer studies seem to suggest a less efficient activation by T Cell receptor (TCR)-derived signals and costimulation (46-48). Moreover, it has been reported that HIF-1α-deficient T cells respond with higher intensity to stimulation of the antigen receptor. A further instance is bacterial sepsis where it has been reported that there is a functional advantage for T cells upon conditional deletion of the HIF-1α gene in T cells in the clearance of disseminated bacteria in mice (49). The CD4 and CD8 HIF-1-deficient T cells of these mice show more intense ability to proliferate and to produce IFNγ (50). Therefore it is concluded that HIF-1α is a repressor of T cells and hypoxia should be an intrinsic immunosuppressive condition for T cells including the ability to kill TCR-recognized targets (50) (51). In contrast, a recent study suggests
that HIF-1α deficiency in T cells does not affect proliferation and Th-1/Th-2 polarization (4). It has also been shown that hypoxia leads to less efficient target cell killing by CTLs (51). Overall, the T-cell HIF1α response to hypoxia can be considered a factor that contributes to immunosuppression in the tumor microenvironment. At the present point of time there is a conspicuous lack of information on how hypoxia affects Treg functions at the tumor microenvironment or even in ex-vivo culture studies.

The mechanisms involved in the modulation of effector T cells by hypoxia include the release to the extracellular milieu of adenosine which acts on A2R inhibitory receptors (50), production of oxygen free radicals and HIF-1α control of genes that down-modulate the immune response (Figure 1).

Dying cells release ATP that is metabolized to adenosine by the coordinated extracellular enzymatic functions of CD73 and CD39 which are also up-regulated by hypoxia (40) (Figure 1). Soluble adenosine in turn acts on surface receptors of T cells (A2Rs) to augment intracellular cAMP, a second messenger known to repress T cell functions (52) (Figure 1).

Under hypoxic conditions the respiratory chain in the mitochondria becomes less efficient to the point of releasing reactive oxygen species (ROS) that are produced both by tumor and stromal cells. There is evidence that ROS can inhibit NF-κB nuclear translocation and thereby
switch off cytokine production. In addition, this oxidative stress has been shown to promote the selective degradation of components of the TCR-CD3 complex such as CD3ζ (53).

Very recently it has been discovered that HIF-1α favors the differentiation of Th17 cells (54) via direct transcriptional induction of RAR-related orphan receptor γt (RORγt) and indirectly IL-17 (4). For these functions HIF-1α partners with phosphorylated signal Transducer and Activator of Transcription-3 (STAT-3), STAT-3 enhances the transcription of HIF-1α and cooperates with HIF-1α at the RORγt promoter (Figure 1). Moreover, CD4 T cells starting their differentiation towards FOXP-3+ Treg cells experience another surprising effect of HIF-1α which involves degradation of the forkhead box P3 (FOXP-3) protein (Figure 1). Amazingly, HIF1-α binds protein-to-protein to FOXP-3 and as a result recruits the VHL E3 ubiquitination machinery to mark FOXP-3 for degradation (4).

We have observed that HIF-1α can modulate the expression of costimulatory receptors such as CD137 (Palazon et al submitted). Indeed, T cell activation under hypoxia leads to a much more readily up-regulation of surface CD137 upon antigen stimulation through a mechanism dependent on HIF-1α. This explains why tumor infiltrating lymphocytes in tumors are brightly CD137+ including effector and regulatory T cells. Importantly, CD137 is a receptor that if targeted with immunostimulatory monoclonal antibodies leads to a therapeutic enhancement of antitumor
immunity (55). In the absence of any agonist ligand expressed by neighbouring cells, CD137 is useless for the antigen primed T cells. However, if immunotherapeutic agonist antibodies are given then a strengthened function of the TILs ensues and tumor eradication becomes a frequent outcome. It is of note that recent findings indicate that CD137 expression on TILs inside human lymphomas has an important impact as a prognostic factor (54).

**Indirect mechanisms of hypoxia on T lymphocytes.**

In addition to affecting T cells directly, hypoxia can modify other components of the tumor tissue that in turn dictate the behaviour of T cells. Hypoxic tumor cells produce high levels of CC-chemokine ligand 28 (CCL28). This chemokine has been shown to selectively attract regulatory T cells that express CXCR10 which also induce antigen tolerance and promote angiogenesis (56). Hypoxia acting on vascular endothelium in tumors gives rise to surface CD137 expression (57). If such molecule is activated proinflammatory changes take place that increase T-cell infiltration (57).

It has also been observed that tumor cells under the effect of hypoxia activate autophagia and that autophagia protects malignant cells from being killed by cytotoxic T cells (51).
The overall picture is that tumor-infiltrating lymphocytes are restrained in tumors by the fact that there is a partial deprivation of oxygen, which leads to HIF-1α stabilization and extracellular adenosine accumulation (50).

**Clinical translational advances**

We must rethink tumor immunotherapy taking local tumor hypoxia into consideration. Innate and adaptive immune cells are profoundly modified under hypoxia (Figure 2). The response to hypoxia as mediated by HIF-1α in T cells and macrophages is entangled with key transcription factors that modify the intensity and quality of the immune/inflammatory response such as STAT-3, NF-κB and RORγt (58).

Two immunotherapy strategies currently stand out given their potential benefit in the clinical arena, namely, adoptive T cell transfer (59) and immunostimulatory monoclonal antibodies (60). The impact of local tumor hypoxia on these therapies should be re-examined. In the case of the most clinically effective form of adoptive T cell therapy, TILs are taken from tumors reinvigorated and expanded to be given back to a host who has been preconditioned by chemotherapy and total body irradiation (59). TILs need to find their way back to the tumor and destroy it. The exact state of hypoxia at the tumor lesions modulating all these phenomena (i.e. T cell activation, memory differentiation, migration, extravasation, etc) needs to be experimentally addressed.
In the case of immunostimulatory monoclonal antibodies (60), we know virtually nothing of the function of costimulatory receptors of the TNF Receptor and immunoglobulin superfamilies when the concentration of O₂ drops. As in the case of CD137, even the expression of the targets for the immunotherapeutic antibodies can be modified by the hypoxia response.

If hypoxia were found to be a guilty accomplice of pernicious immunoregulatory networks in the tumor microenvironment, interventions with antioxidants, pharmacological adenosine inhibitors and drugs acting on the HIF response pathway, including proteasome inhibitors such as bortezomib, could be interesting adjuvants for a variety of immunotherapies. In any case, the bottom line is that immunomodulatory mechanisms controlled directly or indirectly by the hypoxia response certainly do exist and that such mechanisms offer real opportunities for intervention.

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Figure legends.

Figure 1. **O₂ sensing and molecular response to Hypoxia in cells of the immune system.**

Schematic representation of the O₂ control of HIF stability and direct and indirect effects of HIF to control the expression of other gene/proteins. Hypoxia exerts effects mainly by controlling the functions of HIF-1α but also by enhancing the concentration of oxygen free radicals and adenosine. HIF-1α levels are controlled mainly by proteasomal degradation and to a lesser extent by transcriptional induction. Degradation of HIF-1α depends on Lysine-48 polyubiquitination by the Von Hippel Lindau (VHL) E3-Ubiquitin ligase complex. Degradation only takes place under normoxic conditions because HIF-1α is targeted to ubiquitination by prolyl hydorxilases (PHDs) that are only operational in the presence of O₂. Under hypoxic conditions, stabilized HIF-1α relocates to the nucleus and exerts direct and indirect transcription-promoting activities partnering with the constitutively expressed HIF-1β protein. Recent evidence has also shown a role for HIF-1α at targeting the FOXP-3 protein for ubiquitination and proteasomal degradation. This is extraordinarily important because FOXP-3 is critical for the differentiation of the regulatory T cells (Treg cells).

Figure 2. **Direct and indirect effects of hypoxia on stromal and malignant cells**

Schematic representation of hypoxia effects on the different cell subpopulations found in the tumor microenvironment. Literature references pertaining the indicated phenomena is provided in the scheme.
Hypoxia and IFN-Gamma Signaling in Cancer Immunotherapy

HIF-1α expression is regulated by both hypoxia and IFN-γ. In hypoxia, HIF-1α is stabilized and translocates to the nucleus, where it binds to HRE (Hypoxia Response Element) and activates the expression of HIF-1α-controlled genes. In normoxia, HIF-1α is degraded by the proteasome. IFN-γ and IFN-γR signaling activates STAT3, which can induce HIF-1α expression.

Indirect regulation of proteins involved in:
- Metabolism
- Inflammation
- Immune response

Processes induced by HIF-1α-controlled genes:
- Migration
- Invasion
- Angiogenesis

VHL E3 Ub Ligase

Prolyl hydroxylase
Asparagine hydroxylase

AMP Adenosine
Adenine nucleotide translocator
Proteasomal degradation

HIF-1α gene
HIF-1α-controlled genes
HRE

cAMP
Immune suppression

CD137
CD73
Adrenomedullin

IL-6
IL-17
Proteasomal degradation

LPS
IFN-γ
TLR4
IL-6R

NF-κB
STAT3

HIF-1α mRNA

Fe²⁺ 2-OG
O₂

Pro564
Pro402

Pro564
Pro402

OH
OH

OH
OH

Asparagine hydroxylase
Prolyl hydroxylase

NORMOXIA
HYPOXIA

CD137
CD73

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