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The Ever Expanding Role of HIF in Tumour and Stromal Biology

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Abstract

Low oxygen tension (hypoxia) is a hallmark of cancer that influences cancer cell function, but is also an important component of the tumour microenvironment as it alters the extracellular matrix, modulates the tumour-immune response and increases angiogenesis. Here we discuss the regulation and role of hypoxia and its key transcriptional mediators, the hypoxia inducible factor (HIF) family of transcription factors, in the tumour microenvironment and stromal compartments.

Introduction

A wealth of clinical evidence indicates the prevalence of hypoxia in solid tumours[1]. Hypoxia arises due to a combination of excessive oxygen consumption by cells in the tumour, and the leaky and disorganized tumour-associated vasculature, which leads to both acute fluxes in oxygen tension and diffusion-limited regions of low oxygen levels within the tumour[2]. Tumour hypoxia is associated with increased genetic instability, disease progression and metastasis, and can inhibit tumour response to cytotoxic and targeted therapies[1]. Its profound clinical implications have led to an intensive effort to characterize the cellular response to hypoxia, and to modulate it for therapeutic benefit.

Tumours are composed of a heterogenous mix of malignant and stromal cell populations, the latter including cancer associated fibroblasts (CAFs), immune cells, endothelial cells (ECs) and pericytes[3]. Stromal cells are intimately associated with malignant cells and participate in paracrine signaling, metabolite exchange, extracellular matrix remodeling and regulation of immune surveillance in the tumour[3]. Microenvironmental cues, including hypoxia, synergistically modulate the behaviour of tumour cells and associated stromal cells to potentiate tumour progression[4]. Central in the cellular responses of both the tumour and stromal compartments to hypoxia, is the HIF family of transcription factors. In this review, we discuss the regulation of HIF signaling and the role of the hypoxic microenvironment in shaping the tumour-stromal cell interface.

Canonical regulation of HIF signaling

HIF-1α, HIF-2α, and the lesser studied HIF-3α constitute a family of oxygen sensitive basic helix-loop-helix transcription factors that direct the transcriptional response to hypoxia[5]. HIF-1α is ubiquitously expressed in human cells, whereas HIF-2α expression is restricted to

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specific tissues and cell types[6]. Transcriptional activation of HIF target genes requires assembly of a heterodimer between HIF-1α or HIF-2α and their obligate binding partner, the aryl hydrocarbon nuclear translocator (ARNT, also known as HIF-1β)[7]. Under normoxic conditions, α-ketoglutarate-dependent prolyl hydroxylases (PHDs) catalyze the hydroxylation of proline residues within oxygen-dependent degradation domains (ODD) of HIF-α, which are recognized by the Von Hippel-Lindau (VHL) E3 ubiquitin ligase complex, leading to HIF-α ubiquitination and subsequent degradation (Fig. 1A)[8,9]. Owing to its crucial role in HIF-α degradation, loss of function mutations in the VHL gene result in constitutive activation of HIF signaling and are characteristic of several cancer syndromes, including clear cell renal cell carcinoma[10]. In addition to regulation by prolyl hydroxylation, oxygen-dependent hydroxylation of a key asparagine residue by Factor Inhibiting HIF (FIH) disrupts binding of the p300 transcriptional co-activator to HIF, thereby inhibiting its transcriptional activation potential[11]. Oxygen-dependent hydroxylases provide an elegant oxygen sensing mechanism that directs the transcriptional response to hypoxia.

Non-Canonical regulation of HIF signaling

In addition to the canonical oxygen-dependent regulation of HIF-α, non-canonical regulation of HIF signaling has been demonstrated in many cell types including both malignant and stromal cells. Activation of PI3K-mTOR signaling increases cap-dependent translation of HIF-α mRNA, resulting in increased expression of HIF-α protein[12–14] (Fig. 1B). In cancer cells, frequent activation of the PI3K-mTOR axis stimulates HIF-α activity and promotes tumour angiogenesis[15,16]. In Peutz-Jeghers syndrome, dysregulation of mTOR signaling downstream of LKB1 loss leads to metabolic reprogramming by a HIF-dependent mechanism[17,18]. The RNA- and DNA-binding protein YB-1, which is upregulated in sarcomas, can also regulate cap-dependent translation of HIF-1α, but not HIF-2α, and has separately been reported to indirectly regulate HIF-1α by transcriptional repression of Foxo3a[19,20].

Activation of mTOR signaling, and subsequent HIF-α stabilization and activation, also occurs downstream of TCR signaling in T cells, and is imperative for their function, because it drives metabolic reprograming and prolongs survival of peripheral T cells under hypoxia[21,22] (Fig. 2A). HIF-α signaling in lymphocytes is also modulated by pro-inflammatory cytokines like IL-6, which signal through the JAK/Stat3 pathway to increase transcription of HIF-α mRNA[23,24]. In the innate immune system, Toll-like receptor (TLR) signaling induces HIF-α expression in myeloid cells through increased NF-κB-dependent transcription of *Hif1a* mRNA, highlighting an important link between innate immunity and the HIF-pathway[25–27] (Fig. 1B, 2A). These findings indicate that additional, oxygen-independent mechanisms in normal and malignant cells drive activation of HIF-signaling, to sustain the function of these cell types.

Mitochondrial regulation of HIF activity

Reprograming of cellular metabolism towards increased glycolysis and suppressed oxidative phosphorylation is a major adaptation mechanism to hypoxia. HIF-α transcription factors are central regulators of metabolism, and several HIF target genes encode metabolic enzymes. In turn, a wide range of cellular metabolites can modulate HIF-signaling.

Numerous reports indicate that mitochondria, the major site of cellular oxygen consumption, are important for HIF-α stabilization under hypoxic and non-hypoxic conditions. One hypothesis is that mitochondrial respiration regulates HIF-α stability by increasing intracellular hypoxia, thereby impairing PHD-mediated HIF-α hydroxylation. Indeed, high resolution imaging indicates that mitochondria reside in regions of lower oxygen, compared to other cellular compartments[28], and mitochondrial ETC inhibitors interfere with hypoxic stabilization of HIF-α, presumably by preserving intracellular oxygen for PHD-mediated hydroxylation[29]. Furthermore, PGC-1α, a central regulator of mitochondrial biogenesis, stabilizes HIF-α by stimulating mitochondrial oxygen consumption and increasing intracellular hypoxia[30,31]. HIF was recently shown to repress PGC-1α expression in clear cell renal cell carcinoma, suggesting that a regulatory loop exists between HIF-α and PGC-1α, linking oxygen sensing to mitochondrial biogenesis[32].

Aberrant metabolism and oncogene activation frequently induces reactive oxygen species (ROS) accumulation, which positively and negatively influences tumourigenesis[33]. *In vitro* models blocking mitochondrial ROS production indicate that ROS are an important mediator of HIF-stability, acting by inhibiting PHD function[34–37]. However, the tight coupling between ROS production and oxygen consumption in the mitochondria have made it difficult to discern whether mitochondria regulate HIF through consumption of oxygen or production of ROS. Whether ROS production in the mitochondria is increased or decreased under hypoxic conditions remains unclear and adding to the controversial role of ROS as mediators of HIF signaling, recent reports indicate that the activity of FIH, but not the PHDs, is sensitive to peroxide radicals[38,39].

Influence of intracellular metabolites on HIF-α stability

Loss of function mutations to genes encoding the succinate dehydrogenase (SDH) complex and fumarate hydratase (FH) lead to accumulation of succinate or fumarate respectively, metabolites that inhibit the activity of the PHDs and result in HIF-α stabilization (Fig. 2B)[40]. Other intracellular metabolites, including pyruvate, lactate and oxaloacetate can also modulate HIF-signaling through PHD inhibition[41]. Isocitrate dehydrogenase isoforms (most commonly IDH1 and IDH2) are also frequently mutated in multiple cancer types[42]. These mutations result in neomorphic IDH activity, with mutant IDH1/2 producing 2-hydroxyglutarate (2-HG) instead of α-ketoglutarate, which is an essential co-substrate of the PHDs[43]. The impact of 2-HG on HIF-stability is hotly contested, with reports suggesting that 2-HG can either activate or inhibit PHD-mediated hydroxylation of HIF-α in an enantiomer specific manner[44]. Further complicating matters, IDH3α expression has been reported to repress or activate HIF-stability in CAFs and malignant cells by modulating intracellular α-ketoglutarate levels[45,46]. How IDH3α can increase or decrease α-ketoglutarate levels to modulate HIF signaling in different contexts remains unclear.

HIF function can also be modulated by non-catalytic functions of metabolic enzymes. The gluconeogenic enzyme fructose-1,6-bisphosphatase (FBP1) binds to the inhibitory domain on HIF, thereby preventing HIF nuclear translocation and transactivating potential, and inhibiting ccRCC tumorigenesis[47]. In contrast, the PKM2 isoform of pyruvate kinase can act as a HIF-1α coactivator by recruiting p300[48]. Dimeric PKM2 can also activate Stat3, which

is known to coordinate expression of genes involved in inflammation and the hypoxic response together with HIF-α, suggesting that PKM2 may act as a metabolic sensor integrating these two gene expression programs[49]. Together, these studies indicate that both metabolites and metabolic enzymes can directly modulate oxygen sensing.

Hypoxia and HIF-signaling in the Tumour Microenvironment

Tumour-associated stromal cells are exposed to similar, often harsh, microenvironmental cues as tumour cells. HIF signaling promotes adaptation to these microenvironmental conditions, and in doing so, induces changes in both tumour and stromal cells that potentiate tumourigenesis. Tumour hypoxia promotes the recruitment of endothelial cells and pericytes to stimulate angiogenesis and facilitates the recruitment of bone marrow derived cells. Recruited stromal cells enhance tumourigenesis through extracellular matrix remodeling, growth factor signaling and evasion of the anti-tumour immune response (Fig. 3)[3].

The hypoxic tumour microenvironment spurs adaptive metabolic changes that complement the metabolic flux of normoxic tumour regions to sustain metabolic fitness in both compartments in a process termed “metabolic symbiosis.” For example, the lactate secreted by highly glycolytic hypoxic cells, is consumed by normoxic cells for ATP production via oxidative phosphorylation[50] (Fig. 3). In addition to poor oxygenation, a consequence of the malformed tumour-associated vasculature is limiting nutrient supply. Byproducts of hypoxic cell metabolism may alter the function and viability of a wide range of stromal cells by increasing tissue acidity and by depriving stromal cells of access to essential nutrients[51]. Augmented HIF-signaling and oncogene activation in tumour cells improves metabolic fitness, allowing tumour cells to outcompete stromal cells for vital metabolites such as glucose and glutamine[52]. This dynamic competition for nutrients between tumour cells and their stroma has recently been implicated in the exhaustion of tumour infiltrating lymphocytes, and indicates that metabolic competition promotes immune suppression in the tumour microenvironment[53,54].

Vascular Endothelial Cells and Pericytes

The contribution of angiogenesis to tumour progression is well established[55]. Although early in tumourigenesis the resident tissue vasculature supplies sufficient oxygen to the burgeoning tumour, larger tumours exhibit significant regions of hypoxia that limit further tumour growth. Hypoxic cancer and stromal cells secrete soluble factors, such as vascular endothelial growth factor (VEGF), that facilitate angiogenesis and thereby oxygen and nutrient delivery, to promote tumour growth under hypoxic conditions. Two major vascular cell types are the endothelial cells (ECs) that line blood vessels and pericytes which surround blood vessels, and hypoxia and HIF signaling are known to influence both.

Vascular endothelial cells

Apart from their direct impact on the tumour microenvironment by regulating oxygen and nutrient supply, ECs are a major structural component of blood vessels and consequently regulate tumour cell extravasation and the recruitment of circulating cells to the tumour[56]. ECs respond to VEGF secretion from cancer and stromal cells by sprouting from the

existing vasculature to form new vessels during angiogenesis. Sprouting vessels culminate in specialized vascular ECs (tip cells) that direct vessel formation. As these tip cells reside far from functional vessels, they become hypoxic and must mount an adaptive response to hypoxia[57]. Thus, during vessel sprouting, ECs exhibit unique patterns of cellular metabolism, with high rates of glycolysis and a dependence on fatty acid oxidation for nucleic acid synthesis and proliferation[58,59], despite the fact that hypoxia suppresses fatty acid oxidation in a wide range of cell types and tissues[60–63]. These findings indicate that ECs may exhibit unique metabolic behaviours that could be targeted therapeutically to block tumour angiogenesis.

Early evidence of the role of HIF signaling in ECs came from studies in which conditional deletion of HIF-1α in ECs impaired vascularization and tumour growth[64]. Surprisingly, deletion of endothelial HIF-2α enhanced tumour angiogenesis, but the resulting vasculature was highly disorganized and resulting tumours were more hypoxic[65,66]. Additionally, heterozygous loss of PHD2 (*Egln1*) resulted in increased HIF-α stabilization, normalized tumour vasculature, increased oxygenation and diminished metastasis[67]. These findings indicate that HIF signaling in ECs plays an essential and complex role in functional angiogenesis.

Tumour associated ECs also represent an important barrier to intravasation of invading tumour cells. HIF-1α and HIF-2α play opposing roles in regulating the barrier function of ECs: whereas loss of endothelial HIF-1α impairs tumour cell migration through endothelial layers, endothelial HIF-2α deficiency enhances tumour cell migration and metastasis[68]. These contradictory roles for HIF-signaling in ECs may be explained by diminished production of iNOS in HIF-1α-deficient endothelial cells, compared to enhanced iNOS production under HIF-2α deficiency, however, more work is needed to elucidate these contrasting roles of HIF-1α and HIF-2α.

Pericytes

Vascular endothelial cells are tightly associated with pericytes, which line the outside of blood vessels[69]. Pericytes are an important component of functional vasculature, contributing to vessel contractility and permeability to ensure efficient nutrient and oxygen delivery to healthy tissues. During angiogenesis, pericytes respond to PDGF to induce neovascularization. Whereas VEGF promotes EC proliferation and migration, it disrupts pericyte coverage of nascent blood vessels, resulting in pericytes being more weakly attached to tumour-associated vasculature[70,71]. Pre-clinical models of pericyte deficiency indicate that decreased pericyte vessel coverage drives increased hypoxia, tumour aggressiveness and metastasis[72,73]. The role of pericytes in tumour biology may fluctuate at different stages of tumour progression: whereas pericyte depletion at early stages of tumour growth can decrease growth and metastasis, their depletion at later stages enhances intratumoural hypoxia and has the converse effect[72,73].

Hypoxia and anti-angiogenic therapy

A major focus of targeted cancer therapy has been the inhibition of angiogenesis through VEGF blockade[74]. Although these therapies often induces acute tumour shrinkage,

increased hypoxia resulting from vessel pruning can promote tumour aggressiveness and metastatic dissemination[69]. Concomitant treatment with Sema3A during VEGF blockade can prevent invasion and metastasis by reducing hypoxia and HIF-stabilization, indicating that modulation of the hypoxic response may enhance therapeutic efficacy of anti-angiogenic agents[75]. Metabolic reprogramming is a key adaptation to anti-angiogenic therapies, and several lines of evidence point to increased dependency on lipid metabolism during and after treatment with VEGF inhibitors[76,77]. These preclinical studies indicate that understanding the dynamic response of hypoxia and HIF-signaling to anti-angiogenic therapies may contribute to enhancing their efficacy.

Although prolonged VEGF blockade enhances tumour hypoxia and promotes tumour aggressiveness, recent studies indicate a therapeutic window during which tumour vasculature appears to normalize[78,79]. Although vessel normalization is counterintuitive to the initial efforts of anti-angiogenic therapies, restored tumour oxygenation may enhance the delivery and efficacy of cytotoxic chemotherapies since increased interstitial pressure and hypoxia are known to decrease drug delivery and efficacy[69].

Cancer-Associated Fibroblasts

CAFs directly contribute to virtually every stage of tumourigenesis[80]. CAFs are a heterogenous mix of myofibroblast-like cells that arise from various cell types including normal fibroblasts, endothelial cells, adipocytes, or bone marrow derived stromal cells[81–83]. Relative to normal fibroblasts, CAFs exhibit heightened metabolic activity and enhanced ECM remodeling, properties that promote metabolic symbiosis and facilitate metastatic dissemination. CAFs secrete a variety of growth factors, cytokines and chemokines that stimulate cancer cell proliferation and recruit bone-marrow-derived cells to the tumour site.

Hypoxic tumour cells secrete paracrine signaling factors including TGF-β, PDGF, CXCL2 and Endothelin that promote conversion of precursor cell types into CAFs, indicating the importance of hypoxia to CAF function in tumours[84]. Although hypoxia mediates CAF recruitment and activation through tumour cell intrinsic HIF-signaling, the direct effects of hypoxia on CAFs remain elusive. Fibroblast-specific loss of HIF-1α induces vascular normalization, decreases hypoxia and enhances tumourigenesis in a mouse model of breast cancer, indicating that HIF-signaling in CAFs may inhibit tumourigenesis[85]. Indeed, hypoxia or loss of PHD2 leads to a decrease in CAF-induced ECM remodeling and diminished metastasis[86,87]. These findings indicate the complex role of hypoxia and HIF-signaling in CAF function. Below we highlight three major mechanisms through which CAFs modulate tumourigenesis and discuss how hypoxia influences these pathways.

Extracellular matrix remodeling

The ECM is composed of fibrous proteins including collagen and proteoglycans that provide the structural fabric of tissues. During tumour growth, extensive ECM remodeling releases paracrine growth factors that stimulate tumour growth and enables tumour migration and metastasis. To facilitate ECM remodeling, CAFs express enzymes including collagen prolyl- and lysyl-hydroxylases and lysyl oxidases that catalyze crosslinking of collagens to elastin and other ECM molecules, resulting in increased matrix stiffness[88]. Hypoxia is an important

stimulus of this process, driving increased expression of remodeling enzymes including prolyl-4-hydroxylases and lysyl oxidases leading to increased tumour stiffness and enhanced metastasis[84].

Reprogrammed cellular metabolism

Compared to normal fibroblasts, CAFs exhibit increased glycolysis, which is critical for their ability to promote tumourigenesis at least in part, by activating HIF-signaling[89]. The metabolic byproducts of CAF metabolism can be taken up by tumour cells to feed anabolic metabolism and tumour cell proliferation, suggesting that metabolic symbiosis is a key mechanism by which CAFs support tumour growth[90]. Gain of function studies in fibroblasts indicate that expression of HIF-1α, but not HIF-2α, regulates aerobic metabolism and tumour promoting effects of CAFs[91]. In addition to exhibiting enhanced glucose uptake and glycolytic flux, cancer cells also take up significant amounts of lactate by expressing the lactate transporter MCT1[92,93]. CAFs produce and secrete large amounts of lactate, which can be taken up by tumour cells to feed the tumour's requirement for carbon metabolism[94]. Tumour cells alter ROS signaling in the microenvironment which may promote HIF-dependent and independent metabolic reprograming in CAFs, thus potentiating metabolic symbiosis between these cell types[95]. These results indicate a dynamic interplay between the metabolism of CAFs and tumour cells and suggest that targeting CAF metabolism could interfere with metabolic symbiosis in the tumour microenvironment and thus impair tumour growth.

Paracrine signaling

CAFs are an important source of signaling factors that act on tumour and stromal cells to potentiate paracrine signaling within the tumour microenvironment. CAFs exposed to hypoxia secrete SDF-1 (CXCL12), which promotes tumour growth through paracrine mechanisms[96]. Hypoxia also upregulates expression of the SDF-1 receptor, CXCR4, in numerous cell types, thus potentiating the paracrine signaling between CAFs and malignant cells[97]. In prostate cancer, androgen ablation therapy enhances tumour hypoxia, stimulating increased CXCL13 secretion from tumour-resident myofibroblasts, leading to disease progression[98]. Indeed, several factors involved in the transformation of CAFs from precursor cells, including TGF-β, PDGF-B, and bFGF are directly or indirectly regulated by HIF, indicating the importance of hypoxia in regulating paracrine signaling in CAFs[99–101]

Paracrine signaling by hypoxic CAFs also contributes to the immunosuppressive nature of the tumour microenvironment, by inducing secretion of arginase II (ArgII). ArgI and ArgII are important drivers of immune suppression in the tumour microenvironment because they promote conversion of L-arginine to ornithine, leading to T cell anergy and limited anti-tumour immune response[102]. In pancreatic cancer, CAFs localized to hypoxic tumour regions express high levels of ArgII, indicating an important role for hypoxia in CAF-mediated immunosuppression[103].

Immune cells in the tumour microenvironment

Tumour infiltrating immune cells play an important role in disease progression, and anti-tumour immune therapies have been the subject of intense research for some time. Traditionally these approaches have involved augmenting intrinsic anti-tumour immunity with cytokines such as interleukin-2, or tumour-antigen vaccines. However, the tumour microenvironment is functionally immunosuppressive, and intrinsic HIF signaling drives resistance to immune-mediated tumour cell lysis, limiting the efficacy of these therapies in all but a subset of patients[24,104]. Consequently, a better understanding of how the microenvironment limits the effectiveness of immunotherapy could improve the efficacy of this treatment modality.

Tumour infiltrating immune cells encompass a diverse array of cell types, including tumour associated macrophages (TAM), myeloid derived suppressor cells (MDSC), T cells, neutrophils, and others[3]. In most tumours, immune cells are recruited from the circulation by tumour-secreted cytokines and chemokines[105]. Hypoxia is known to directly or indirectly modulate the function of virtually all immune cell types, thereby facilitating tumour progression[106]. Indeed, myeloid-specific loss of the prolyl hydroxylase PHD2 has been reported to impair tumour growth and metastasis, suggesting an important role for myeloid cell oxygen sensing in tumorigenesis[107]. Hypoxic regions within solid tumours offer distinct niches that fine-tune the tumour-immune system interface and enhance pro-tumorigenic phenotypes of tumour-infiltrating lymphocytes. Below, we discuss the role of hypoxia and HIF-signaling in innate and adaptive tumour immunity.

Cells of the innate immune system

Innate immune cells can potentiate the anti-tumour activity of tumour-infiltrating lymphocytes, leading to remarkable immune-mediated tumour regression in a subset of cancer patients. However, tumour microenvironmental factors, including hypoxia, limit the ability of the innate immune system to activate adaptive immunity, and instead subvert the function of the innate immune system to promote tumourigenesis.

Tumour-associated macrophages

Macrophage density correlates with poor prognosis in various cancer types, indicating an important role for macrophages in tumour progression[108,109]. Hypoxia in the tumour microenvironment is a potent stimulus for macrophage recruitment through the induction of chemoattractant secretion by tumour cells (Fig. 3). Semaphorin 3A (Sema3A), is such a factor, and attracts macrophages through Nrp1/Plexin signaling[110]. Hypoxia also induces secretion of chemokines, such as endothelial-monocyte-activating peptide II (EMAP II), endothelin-1 and endothelin-2 (ET-1 and ET-2) that recruit macrophages to the tumour[111,112]. In ovarian cancer, hypoxia drives macrophage recruitment by stimulating the production of pro-inflammatory leukotrienes, a process that can be reversed by pharmacologic targeting of leukotriene production[113].

Macrophage activation includes the classically activated M1 phenotype, which is characterized by nitric oxide synthase 2 (iNOS) expression, pro-inflammatory cytokine

production and tumour suppression. In contrast, alternatively activated (M2) macrophages express arginase 1 (*Arg1*), promote angiogenesis, secrete anti-inflammatory glucocorticoids and effectively dampen the anti-tumour immune response. Consequently, M1 macrophages oppose, whereas M2 macrophages promote tumour progression and metastasis[114].

Macrophage polarization within the tumour microenvironment is heterogeneous and dynamic, and hypoxia plays an important role in this process (Fig. 4A). Blockade of Sema3A-stimulated macrophage recruitment to hypoxic tumour regions skews macrophages towards the M1 phenotype, indicating that therapeutic blockade of Sema3A may promote anti-tumour activity of TAMs[110]. Preclinical studies further indicate that vascular normalization after low dose irradiation decreases tumour hypoxia and promotes M1 polarization of TAMs, an effect that may be exploited to enhance anti-tumour immunity[115].

Hypoxia can directly impact macrophage polarization by inducing M2-like gene expression in TAMs[116]. HIF-1α and HIF-2 appear to play opposing roles in macrophage polarization, with HIF-2α specifically induced in response to Th2 cytokines in macrophages[117]. HIF-1α and HIF-2α are nevertheless essential for macrophage function, as loss of HIF-1α results in defective macrophage maturation and function[118]. Furthermore, genetically engineered mouse models indicate that expression of HIF-1α and HIF-2α are essential for macrophage infiltration and immune suppression within tumours and loss of either isoform in macrophages diminishes tumour growth[119,120]. Elevated lactate levels in tumours promotes M2 polarization in a HIF-1α dependent manner, indicating that hypoxic metabolism by tumour cells also impacts macrophage polarization[121]. These findings indicate that a combination of microenvironmental hypoxia and metabolic symbiosis between tumour cells and macrophages skew their polarization and shape the anti-tumour immune response[122,123]. In addition to mediating immunosuppression, M2 macrophages are important drivers of angiogenesis, indicating an intricate cross talk between hypoxic tumour cells, macrophages and endothelial cells that dictates oxygen availability in the tumour, cellular metabolism, and the anti-tumour immune response[124]. Thus, TAMs have a significant and direct impact on hypoxia in the tumour microenvironment, creating a feedback loop between oxygen availability and recruitment/polarization of TAMs.

Myeloid Derived Suppressor Cells

Myeloid derived suppressor cells (MDSCs) are bone marrow-derived cells that suppress the anti-tumour immune response. MDSCs exposed to hypoxia induce HIF-signaling and upregulate HIF targets that enhance MDSC function[125]. Hypoxia enhances MDSC suppressor function through a mechanism that is partially dependent on the HIF-regulated miRNA, mir-210 and increased expression of Arg1[125,126]. Tumour hypoxia also influences seeding of MDSCs in the pre-metastatic niche by stimulating increased secretion of lysyl oxidase[127–129]. This process drives ECM remodeling in the metastatic niche and suppresses natural killer anti-tumour immune responses[127]. The enhanced MDSC suppressor function in hypoxia indicates an additional mechanism by which the hypoxic microenvironment suppresses anti-tumour immunity.

Tumour-associated neutrophils

Tumour associated neutrophils (TANs) can have both pro- and anti-tumorigenic properties[130]. Hypoxia in the tumour microenvironment promotes neutrophil recruitment by changing the adherence properties of ECs to neutrophils[131]. In a preclinical model of uterine carcinoma, hypoxia-mediated neutrophil recruitment impeded tumour growth, indicating a tumour suppressive role for neutrophils in this cancer type[132]. Hypoxia prolongs neutrophil survival, and HIF-dependent increases in glycolytic metabolism are important for neutrophil function in hypoxia[118,133]. Similar to macrophages, neutrophil function can be classified into N1 and N2 polarization, with N2 neutrophils displaying tumour promoting properties analogous to the role of M2 macrophages in tumour progression[134]. TGF-β in the tumour microenvironment promotes N2 neutrophil polarization, thereby enhancing their tumour promoting function[135]. Despite the association between HIF-α signaling and TGF-β, whether hypoxia directly modulates neutrophil polarization remains unclear.

Cells of the adaptive immune system

Cells of the adaptive immune system, such as tumour-infiltrating lymphocytes, have the potential to effectively eliminate tumour cells from the body by recognizing tumour-associated antigens. However, the hypoxic tumour microenvironment limits their anti-tumour activity by promoting immune checkpoint activation, limiting access to key nutrients and recruiting a variety of immunosuppressive cell types to the tumour site, thereby reducing the effectiveness of the anti-tumour immune response.

Tumour-infiltrating lymphocytes

Despite frequent infiltration of T-cells into solid tumours, anti-tumour immunity is often limited by features of the tumour microenvironment, including hypoxia[136]. Hypoxic cancer cells and macrophages secrete chemokines and cytokines including CCL22, CCL28, and IL-10, to recruit a CD4+, $CD25^{high}$, Foxp3+ subpopulation of T cells known as regulatory T cells (Tregs) from the circulation, thereby blunting the T-cell mediated anti-tumour response[137,138] (Fig. 4B). Hypoxia also stimulates production of extracellular adenosine by Tregs, which inhibits effector T-cell function through activation of cAMP signaling[139,140]. Surprisingly, while hypoxia promotes critical Treg functions, constitutive activation of HIF-1α, achieved through Treg-specific VHL deletion, disrupts Treg function and drives interferon-γ mediated tissue inflammation, indicating that physiologically appropriate levels of HIF-signaling are important for Treg differentiation[141].

Whereas Tregs are functionally immunosuppressive, Th17 cells are pro-inflammatory CD4+ T cells that play a complex and controversial role in anti-tumour immunity[142]. Hypoxia promotes the differentiation of naïve CD4+ T cells into Tregs or Th17 cells by modulating expression of Foxp3 and RORγt, essential transcription factors for these respective cell types[23,143,144] (Fig. 4B). Induction of Th17 development is accompanied by increased glycolysis driven by mTOR-HIF-1α signaling[145]. In contrast, Tregs cells rely primarily on β-oxidation for their metabolic function. More work is needed to understand the complex and seemingly contradictory effects of HIF-signaling on Th17/Treg development, including

whether different mechanisms of HIF-activation (hypoxia versus TCR signaling) result in different T cell fate decisions.

Promising results from clinical trials have dramatically increased interest in targeting immune checkpoints in cancer therapy. To date, most attention has focused on targeting the receptors, PD-1 and CTLA-4 and their ligands, PD-L1/PDL-2 and CD80/CD86, respectively, but the number of targets is likely to increase as knowledge of immune checkpoint regulation expands[146]. Recent studies revealed direct regulation of PD-L1 in MDSCs and tumour cells by HIF-1α, indicating that hypoxia transcriptionally modulates immune checkpoints[147]. Similarly, *in vitro* studies in human and canine cell lines indicate that PD-L1 is induced in a HIF-1α dependent manner upon exposure to hypoxia, and that this induction inhibits T-cell mediated lysis[148].

Conclusions

Unlike genetic aberrations, which directly modulate tumour cell function, microenvironmental factors such as hypoxia influence both tumour and stromal cells. Hypoxia promotes genomic instability in cancer cells, and also recruits vascular endothelial cells and regulates pericyte function to promote angiogenesis, thereby increasing nutrient and oxygen supply and facilitating metastatic dissemination. In CAFs, hypoxia promotes symbiotic metabolism, ECM remodeling and secretion of paracrine factors that support primary tumour growth and metastasis. Hypoxia also drives recruitment of various immune cells and promotes an immunosuppressive microenvironment to limit anti-tumour immunity. On a global level, low oxygen levels trigger HIF-dependent and –independent adaptive responses in both tumour and stromal cells that increase tumour aggressiveness and metastasis.

Tumour hypoxia fluctuates during tumour growth and in response to cytotoxic and targeted therapies, with profound impacts on disease progression and therapeutic efficacy. As our understanding of the hypoxic response in stromal and tumour cells increases, developing cancer therapies against hypoxia response pathways is emerging as an attractive option. Delivery of such therapies could be timed to coincide with the increased hypoxia observed with VEGF blockade, to prevent the hypoxia-associated increased aggressiveness upon VEGF inhibition. Another potential therapeutic approach, would be to directly target HIF-transcriptional activity, for example by exploiting potential small molecule binding pockets revealed by the elucidation of the ARNT-bound HIF-1α and HIF-2α crystal structures[149,150] A better understanding of the effects of hypoxia and HIF-mediated responses in CAFs and immune cells, including macrophages, T cells, and neutrophils, could also allow their therapeutic manipulation to stimulate anti-tumour immune responses. Likewise, elucidating the role of HIF signaling in controlling immune checkpoint signaling components, could be useful in cancer immunotherapy.

Given their potential to inhibit tumour promoting pathways in both malignant and stromal cells, the development of therapeutic approaches targeting HIF-signaling directly, or by inhibiting downstream effector pathways holds great promise for cancer treatment.

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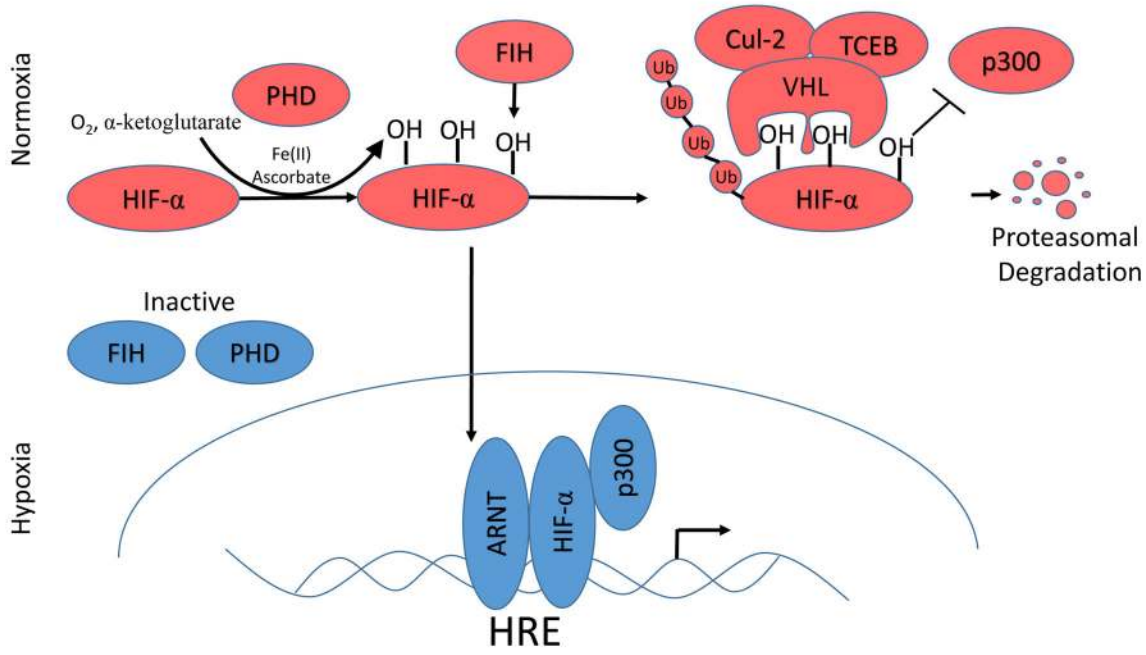
Figure 1A – Oxygen-dependent regulation of HIF levels and activity



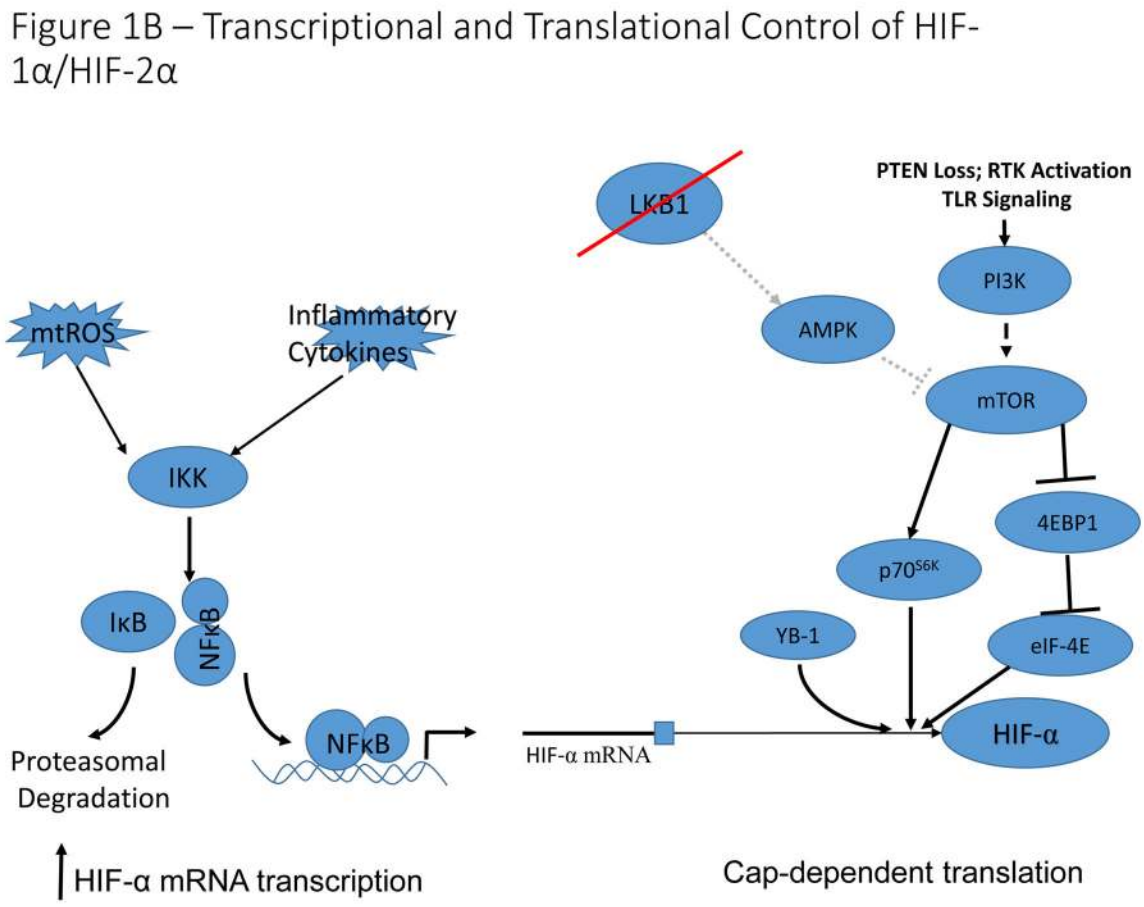


Figure 1. Oyxgen-dependent and -independent regulation of HIF-signaling

A) *Oxygen-dependent regulation of HIF-1α/HIF-2α.* Under conditions in which sufficient oxygen is present (normoxia), prolyl hydroxylases (PHD) catalyze the hydroxylation of two key proline residues within the oxygen dependent degradation domains of HIF-1α and HIF-2α. The hydroxylation reaction catalyzed by PHDs utilizes molecular oxygen and α-ketoglutarate (which is converted to succinate in the reaction) as co-substrates and ferrous iron (Fe(II)) and ascorbate as cofactors. These hydroxylation events form a binding site for the E3 ubiquitin ligase, VHL, which catalyzes ubiquitination and subsequent proteasomal degradation. An additional asparagine residue in the C-terminal activation domain is hydroxylated by factor inhibiting HIF (FIH). Hydroxylation of this asparagine residue disrupts binding of p300 to HIF-α, thereby inhibiting HIF-transcriptional activation potential. Under hypoxic conditions, PHD and FIH activity is inhibited and unhydroxylated HIF-1α and HIF-2α translocate to the nucleus, form a complex with ARNT and p300, and activate transcription of HIF-target genes. ***B).*** *Transcriptional and translational control of HIF-1α/HIF-2α expression*. Reactive oxygen species and pro-inflammatory conditions in the tumour microenvironment stimulate NF-κB-dependent transcriptional activation of HIF-1α, leading to increased expression under normoxic conditions. Activation of the mTOR signaling pathway by PI3K activation or LKB1 loss of function, results in increased cap-dependent translation of HIF-α mRNA. Additionally, the RNA/DNA binding protein, YB-1 is induced in multiple cancer types, and can bind to HIF-1α mRNA to stimulate cap-

dependent translation. This leads to increased normoxic expression of HIF- α in cells with constitutively active PI3K signaling.

Figure 2A: Oxygen-independent HIF-signaling in the immune system

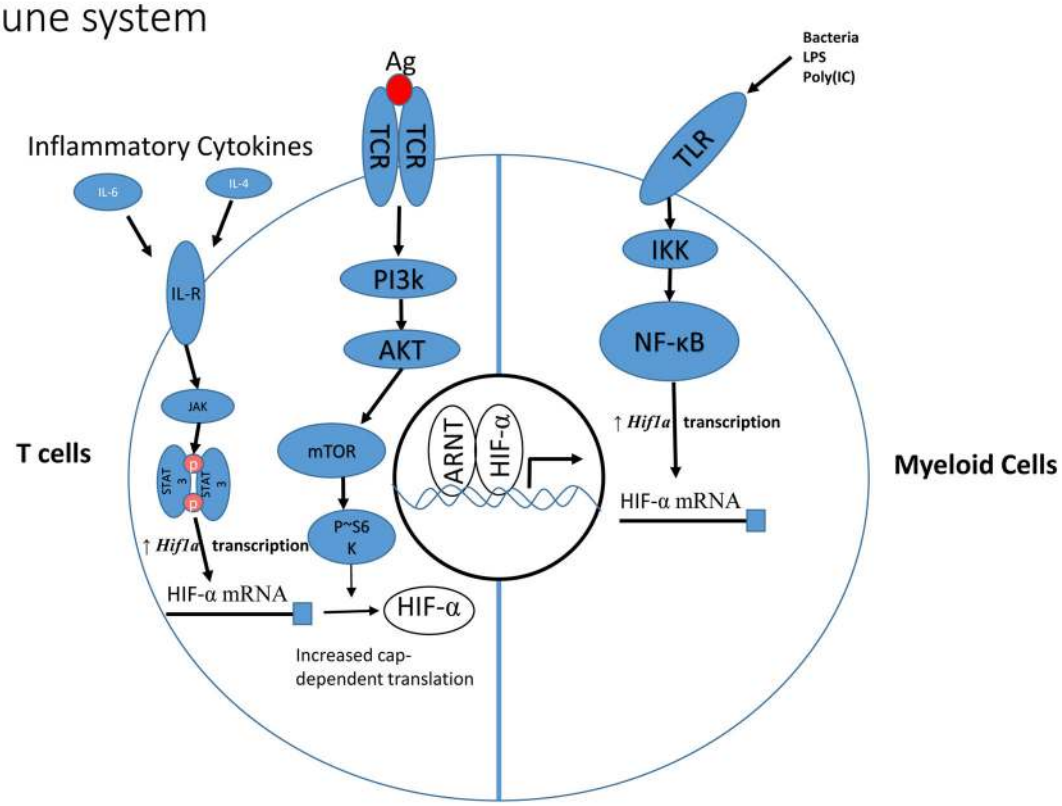


Figure 2B – Metabolic Regulation of HIF-α

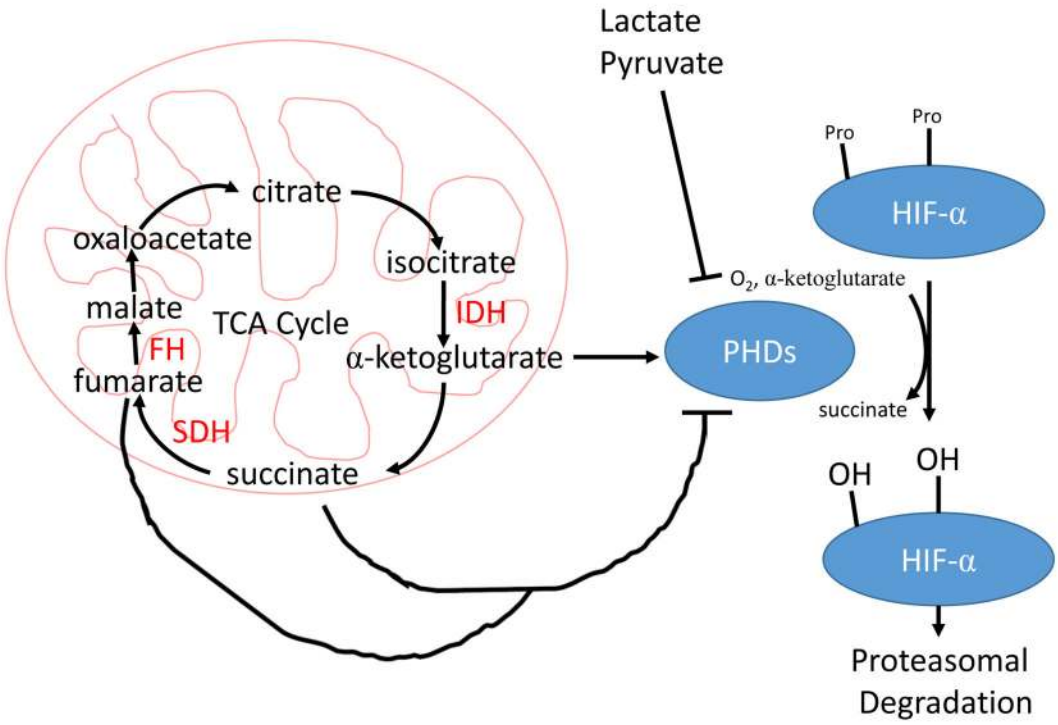


Figure 2. Non-canonical regulation of HIF-signaling by cell signaling and metabolism

A). In T cells, antigen-dependent activation of the T cell Receptor (TCR) signals through the PI3K pathway to stimulate increased translation of HIF-α mRNA. Additionally, inflammatory cytokines signal through the JAK/STAT3 pathway to increase transcription of HIF-α mRNA. These pathways are essential for induction of HIF-signaling in hypoxia and during T cell activation. In myeloid cells, toll-like receptor (TLR) signaling increases NF-κB-dependent transcription of HIF-α mRNA in response to a variety of stimuli including bacterial infection leading to increased HIF-signaling.

B). The hydroxylation reaction catalyzed by PHDs utilizes α-ketoglutarate as a co-substrate, producing succinate in the process. Loss of function mutations to succinate dehydrogenase (SDH) and fumarate hydratase (FH) result in accumulation of succinate, which inhibits PHD activity and stabilizes HIF-α in normoxia. The enzymes labelled in red (SDH, FH, and IDH) are frequently mutated in a variety of malignancies resulting in inhibition of PHD activity and stabilization of HIF-α subunits. Increased concentrations of lactate and pyruvate have also been demonstrated to promote stabilization of HIF-α, but the mechanism by which this stabilization occurs remains unclear.

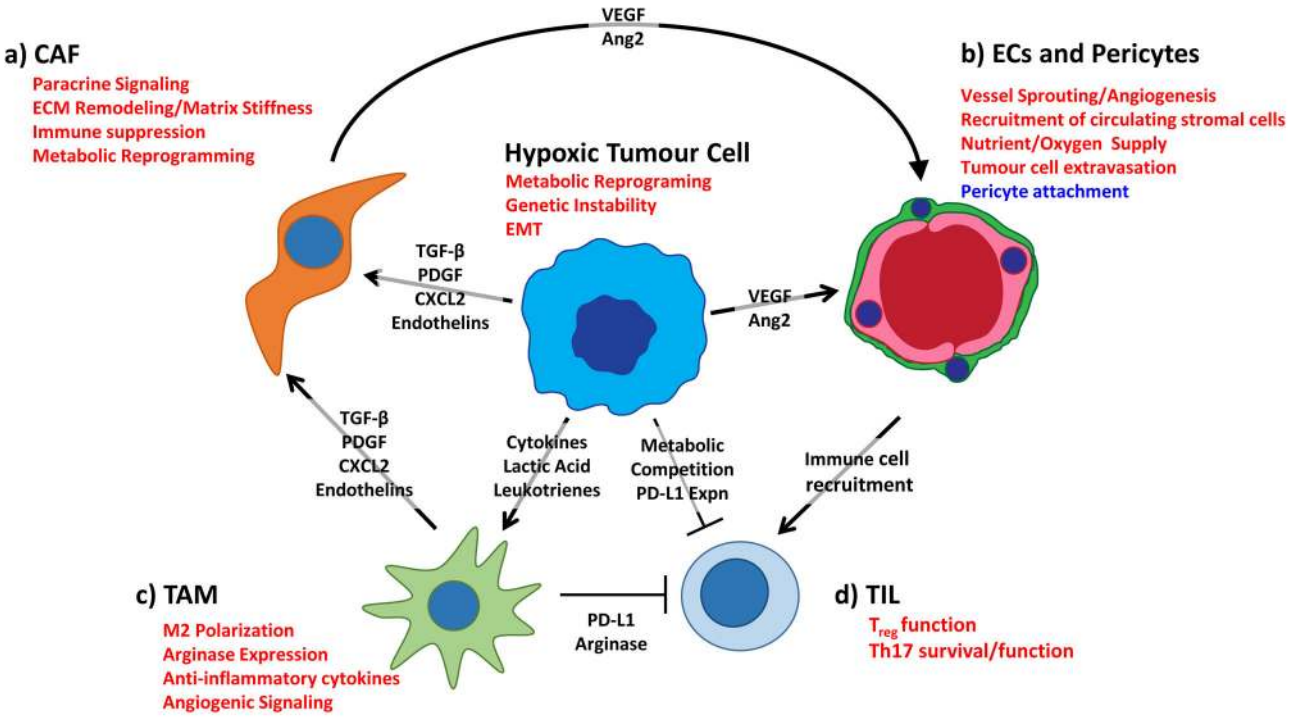
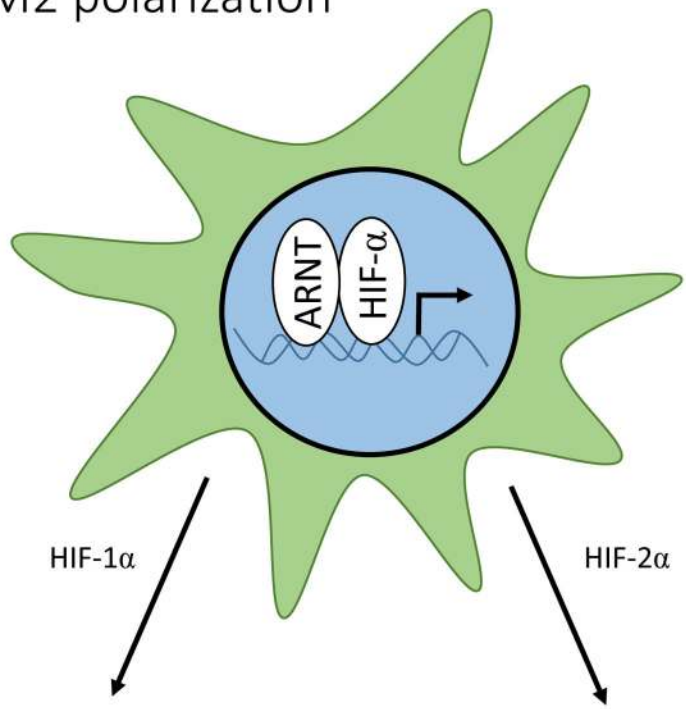


Figure 3. Tumour hypoxia co-opts the stroma to potentiate tumourigenesis

Hypoxic tumour and stromal cells initiate paracrine signalling to stimulate angiogenesis and recruit stromal cells from the circulation, creating an immunosuppressive microenvironment. ***A).*** Hypoxia stimulates tumour cells and TAMs to secrete paracrine factors (TGF-β, PDGF, CXCL2, Endothelin) that promote activation of cancer-associated fibroblasts. In CAFs, hypoxia stimulates extracellular matrix (ECM) remodelling, which promotes increased tumour aggressiveness through stiffening of the ECM. Hypoxic CAFs also synthesize and release factors that drive angiogenesis and immune cell recruitment to the tumour site. ***B).*** Hypoxia drives tumour and stromal secretion of vascular endothelial growth factor (VEGF) and other pro-angiogenic factors that recruit endothelial cells and pericytes from the surrounding vasculature. Hypoxia directly effects vascular barrier function by decreasing the association between pericytes and endothelial cells, thereby facilitating tumour cell extravasation and recruitment of stromal cells from the circulation. ***C).*** Hypoxia stimulates recruitment of circulating macrophages and promotes alternative (M2) activation by increasing expression of macrophage chemotractants and lactate levels. Hypoxic M2 macrophages create a functionally immunosuppressive microenvironment by increasing expression of arginase and immune checkpoint ligands. ***D).*** Hypoxia promotes a functionally immunosuppressive microenvironment by stimulating Treg cell function and increasing expression of immune checkpoint molecules such as PD-L1 and CTLA4 on tumour cells. Hypoxia also drives metabolic reprogramming in tumour cells, allowing them to out-compete T cells for key metabolites critical for T cell function. Hypoxia prolongs the survival of TH17 cells, though the role of this helper T cell subtype in tumourigenesis remains controversial. Pathways in red are induced in hypoxia, while pathways in blue are suppressed under hypoxic conditions. Ang2, angiopoietin 2; CAF, cancer associated

fibroblast; CTL, cytotoxic T cell; EC, endothelial cell; EMT, epithelial to mesenchymal transition; TAM, tumour-associated macrophage.

Figure 4A – Tumour hypoxia stimulates macrophage recruitment and M2 polarization



M1

- iNOS expression
- Tumour promoting inflammation

M2

- Arginase expression
- Immune suppression
- Pro-Angiogenic (VEGF, bFGF, CXCL8)
- ECM Remodeling (MMP9)

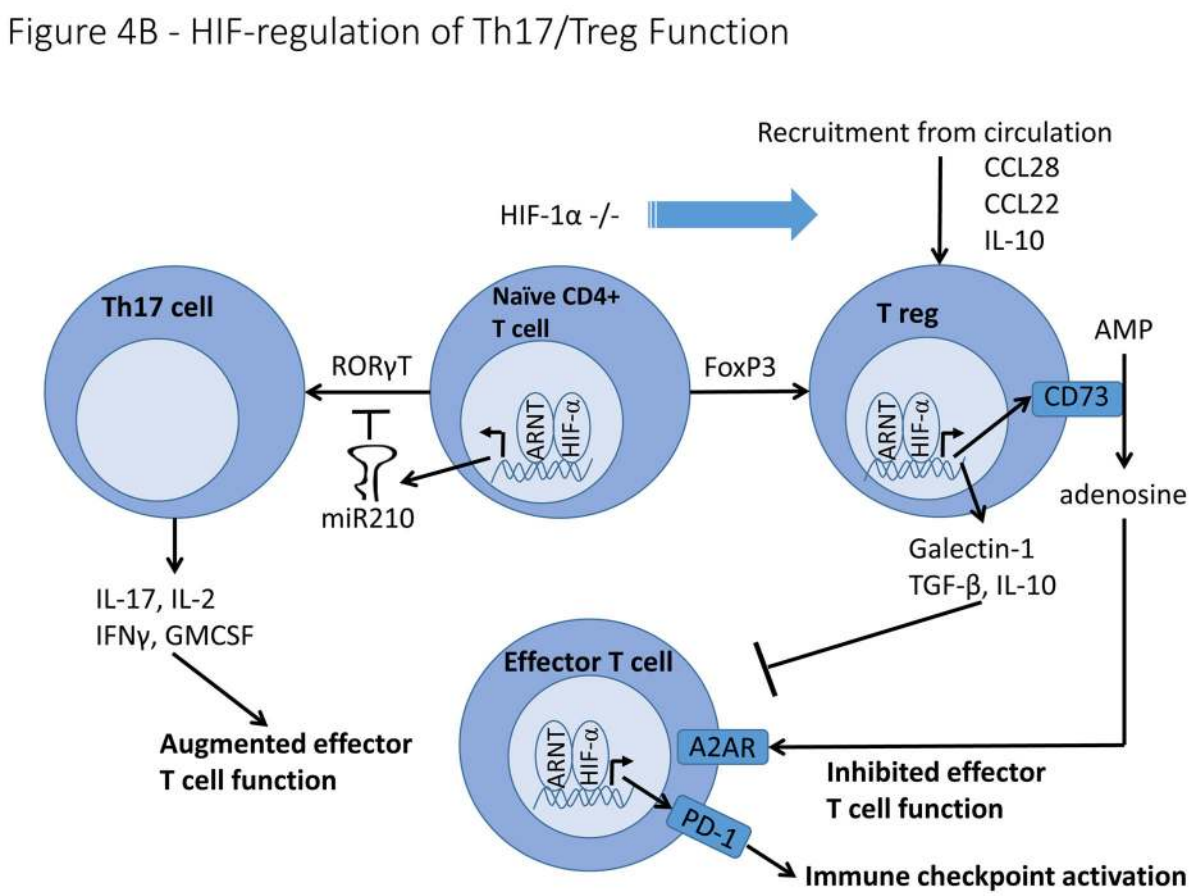


Figure 4. Hypoxia and HIF-signaling effects on immune cells in the tumour microenvironment

A). Hypoxia regulates macrophage polarization by controlling expression of genes involved in the function of M1 and M2 macrophages. HIF-1α promotes expression of the M1 gene, *iNOS*, while HIF-2α promotes expression of the M2 gene, *Arg1*. Hypoxia induces macrophage production of genes involved in angiogenesis (VEGF, bFGF, CXCL12), ECM remodeling (MMP9), and immune suppression (Arg1). ***B).*** HIF signaling plays a controversial role in determining the differentiation of naïve CD4+ T cells into either Th17 pro-inflammatory T cells or FoxP3+ T reg cells with reports suggesting that HIF either induces or inhibits the formation of both cell types. In contrast, overwhelming evidence indicates that hypoxia stimulates the secretion of a number of cytokines and chemoattractants from cancer cells and tumour associated macrophages that recruit T regs from the circulation. On a cell intrinsic basis, hypoxia stimulates T reg production of CD73, thereby increasing adenosine levels in the tumour microenvironment, resulting in inhibition of effector T cells. Hypoxia also stimulates T reg production of other immune suppressive molecules, including Galectin-1, TGF-β and IL-10.