Hypoxia-inducible factors not only regulate but also are myeloid-cell treatment targets

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Abstract
Hypoxia describes limited oxygen availability at the cellular level. Myeloid cells are exposed to hypoxia at various bodily sites and even contribute to hypoxia by consuming large amounts of oxygen during respiratory burst. Hypoxia-inducible factors (HIFs) are ubiquitously expressed heterodimeric transcription factors, composed of an oxygen-dependent α and a constitutive β subunit. The stability of HIF-1α and HIF-2α is regulated by oxygen-sensing prolyl-hydroxylases (PHD). HIF-1α and HIF-2α modify the innate immune response and are context dependent. We provide a historic perspective of HIF discovery, discuss the molecular components of the HIF pathway, and how HIF-dependent mechanisms modify myeloid cell functions. HIFs enable myeloid-cell adaptation to hypoxia by up-regulating anaerobic glycolysis. In addition to effects on metabolism, HIFs control chemotaxis, phagocytosis, degranulation, oxidative burst, and apoptosis. HIF-1α enables efficient infection defense by myeloid cells. HIF-2α delays inflammation resolution and decreases antitumor effects by promoting tumor-associated myeloid-cell hibernation. PHDs not only control HIF degradation, but also regulate the crosstalk between innate and adaptive immune cells thereby suppressing autoimmunity. HIF-modifying pharmacologic compounds are entering clinical practice. Current indications include renal anemia and certain cancers. Beneficial and adverse effects on myeloid cells should be considered and could possibly lead to drug repurposing for inflammatory disorders.

KEYWORDS
hypoxia-inducible factor, hypoxia, innate immunity, myeloid cells

1 | INTRODUCTION

Myeloid cells consist of granulocytes, mostly neutrophils, and monocytes. Once released from the bone marrow, these cells circulate in the blood and are recruited to inflammatory sites where they execute functions that protect the host from infectious and noninfectious challenges. Myeloid cells perform efficiently even under hostile conditions, such as extreme temperatures, mechanical and osmotic stress, and low oxygen concentration. Hypoxia describes low oxygen availability at the tissue level that is further categorized into hypoxicemic, anemic, circulatory, and histotoxic hypoxia.1 During their life span, myeloid cells encounter a wide range of oxygen partial pressures. Bone marrow is a rather hypoxic niche with 13 mm Hg mean oxygen tension.2 Measurements in healthy humans indicate a large variation in partial oxygen pressures with 100 mm Hg in arterial blood and 8 mm Hg in the epidermis (reviewed in Ortiz-Prado et al.3). Kidneys exhibit a large...
gradient with >70 mm Hg in the cortex and 10 mm Hg in the medulla. Importantly, hypoxia is also characteristic of inflamed tissues, where activated myeloid cells migrate against a low oxygen supply.

With reduced oxygen supply, mitochondrial oxidative phosphorylation is strongly decreased, and most ATP is provided by the conversion of pyruvate into lactate. Hypoxia-inducible factors (HIFs) are ubiquitous transcriptional regulators of gene expression in response to low oxygen availability. HIFs help myeloid cells to cope with low oxygen conditions by modifying several metabolic and inflammatory aspects. Currently, drugs that either activate or inhibit HIF-mediated effects are being explored in clinical studies. We discuss the HIF system with its implications for myeloid cell functions together with the potential effects of HIF-directed treatments.

2 | HISTORIC PERSPECTIVE OF HIF DISCOVERY

The scientific interest in hypoxia dates back to Paul Bert, who identified the cause of altitude sickness in the second half of the 19th century. Over decades, hypoxia-mediated effects on erythropoiesis became a main research focus that more recently extended to inflammation. However, it took more than a century until molecular hypoxia mechanisms were characterized, the term HIF was introduced and pharmacologic HIF modulators were developed along a timeline outlined in Fig. 1.

In 1906, Carnot reported a serum factor extracted from anemic rabbits that stimulated erythropoiesis in recipient animal bone marrow and termed this putative factor "hémopoïétine." Yet, the nature of this factor remained elusive for several decades. In the 1950s, Jacobson suggested that erythropoietin (EPO) was secreted by the kidneys. The investigators observed that bilateral nephrectomy abrogated the erythropoietic effect of CoCl₂ in rats and rabbits. Later, the liver was identified as an additional extrarenal source of erythropoietin. Most clinical observations supported the importance of the kidneys for erythropoiesis with complete erythroblastopenia in anuric renal failure patients and after nephrectomy, and polycythemia in patients with renal pathologies, such as renal cysts, hypernephroma, or hydropneumosis. In the 1970s, EPO was isolated from the urine of anemic patients by cloning and recombinant expression. The hormone causes the body to make more red blood cells and is now the pivotal drug to treat anemia caused by cancer therapy, dialysis, and kidney disease. Erslev made rabbits anemic by injecting their anemic plasma into normal rabbits, the rabbits increased production of red blood cells and as the number of red blood cells increased so hematocrit increased. In contrast, injection of normal plasma into normal rabbits did not lead to an increase in red blood cells. Erslev concluded that a hormone (EPO) was responsible for the increase in red blood cells. Yet, the molecular mechanisms of hypoxia-regulated EPO transcription and, as it later turned out, many additional genes were still unknown.

In the late 1980s, Goldberg et al. suggested a ferroprotein as the oxygen sensor. The investigators used metals (i.e., manganese, nickel, and cobalt as CoCl₂) that interact with protoporphyrin structures and compete with iron in heme prosthetic groups to induce EPO. Locking heme-bearing proteins in a deoxy conformation with these metals induced EPO mRNA and protein. Hypoxia was not synergistic with this deoxy state, whereas carbon monoxide, which created an oxy state of hemoglobin, reduced the EPO-enhancing effect of hypoxia. Together, these experiments led to the reasonable assumption that the cellular oxygen sensor is a heme protein. However, as it turned out later, the free iron was bound to a nonheme protein that was yet to be discovered.

In the 1990s, reporter assays unmasked cis-acting elements responsive to hypoxia. Transgenic mice carrying the human EPO gene produced nuclear factors selectively binding to 3′ flanking sequences of the human EPO transgene. Consecutive mutational analysis of a 50 nt 3′ flanking sequence of the human EPO gene revealed a proteinaceous DNA binding that the authors termed HIF-1. HIF-1 was characterized as a protein complex generated in hypoxic cells that binds to a DNA sequence crucial for hypoxic activation of EPO transcription. More than 10 yr passed until prolyl-hydroxylase domain containing enzymes (PHD) were finally identified as the long-assumed sensor of cellular oxygen tension that regulate HIF abundance. Although the earlier suggested iron-binding domain was confirmed, the implied heme-involving mechanism was not. In 2019, William G. Kaelin Jr., Sir Peter J. Ratcliffe, and Gregg L. Semenza received the Nobel Prize for explaining how cells sense and adapt to different oxygen concentrations.

3 | MOLECULAR HIF PATHWAY COMPONENTS

3.1 | HIFα subunit isoforms and dimerization with aryl hydrocarbon receptor nuclear translocator (ARNT)

HIFs are transcription factors with an N-terminal basic helix-loop-helix (bHLH) followed by a Per-ARNT-Sim (PAS) domain and C-terminal transcription activation domains. HIFs function mostly as heterodimers consisting of HIFβ, formerly named ARNT, and one of three HIFα subunits. Similar to other members of bHLH-PAS transactivators, the basic domain is indispensable for DNA binding by recognizing the consensus core sequence of hypoxia-response elements (HRE): 5′-TACGTG-3′, whereas the HLH and PAS domains promote α and β heterodimerization. The PAS-A domain also enhances DNA binding of the HIF heterodimer illustrating the synergistic interplay of elements from the entire bHLH-PAS region. Studying fusion and chimeric proteins of HIFα monomers revealed additional domains, namely two oxygen-dependent degradation domains (ODD) as well as two transactivation domains, N terminal transactivation domain (N-TAD) and C terminal transactivation domain (C-TAD). HIF structure details are illustrated in Fig. 2.
3.2 | Oxygen-dependent HIF regulation by proteasomal degradation

The HIFα isoforms and HIF-1β are all constitutively transcribed and translated. The cellular abundance of the former, but not the latter, is controlled by oxygen concentration. Under normoxic conditions, the HIFα proteins reside in the cytoplasm where they interact with heat shock protein (Hsp)90 through the bHLH-PAS domain.\(^\text{73–75}\) However, under normoxia, HIFα proteins are continuously degraded in the proteasome mediated by the von-Hippel-Lindau (pVHL) protein.\(^\text{76}\) pVHL serves as a substrate-recognizing subunit of an E3 ubiquitin ligase complex\(^\text{54,77,78}\) and forms a ternary complex with elongins B and C\(^\text{79}\) thereby recruiting Cul-2 and Rbx-1.\(^\text{80–82}\) The resulting multimeric complex acquires E3 ligase activity and, in concert with the E1 ligase Uba and the E2 ligases Ubc5a, Ubc5b, and Ubc5c,\(^\text{83}\) leads to oxygen- and iron-dependent ubiquitination and subsequent proteasomal degradation of the HIFα subunits.\(^\text{57,84,85}\) Mass spectrometry established that hydroxylation of proline residues within the HIF ODD was indispensable for pVHL recognition of degradation-designated HIFα subunits under normoxic conditions. Subsequently, new dioxygenase isoforms were identified that were responsible for posttranslational oxygen-dependent HIFα hydroxylation.\(^\text{55}\) Thus, the cellular oxygen sensor was finally characterized as PHD. \textit{C. elegans} expresses a HIF system that is homologous

![FIGURE 1](image1.png)

**FIGURE 1**  Timeline and milestones of hypoxia-inducible factors (HIFs) discovery

- 1878: hypoxemic hypoxia in altitude sickness
- 1906: isolation of human urinary EPO from anemic patients
- 1957: cloning of the human EPO gene
- 1970: cloning of the human EPO gene
- 1957: isolated heme protein as cellular oxygen sensor suggested
- 1985: HIF purification and characterization
- 1985: hypoxemia in altitude sickness
- 1986: human EPO gene assigned to 7q11
- 1988: “hémopoïétine” in anemic rabbits
- 1991: human EPO gene
- 1995: hypoxia response element identification in the EPO gene
- 2001: identification and characterization of the PHDs as oxygen sensor
- 2019: Nobel Prize in Physiology or Medicine for how cells sense and adapt to oxygen availability

![FIGURE 2](image2.png)

**FIGURE 2**  Schematic of hypoxia-inducible factor (HIF)-1α and HIF-2α protein structure and hydroxylation sites at proline and asparagine residues. The basic submotif and the helix-loop-helix domain (bHLH) are located close to the N terminus, followed by the Per-ARNT-Sim (PAS) domain. The PAS domain comprises repetitive amino acid sequences PAS-A and -B. The oxygen-dependent degradation domain (ODD) overlaps with the N terminal transactivation domain (N-TAD), followed by the C terminal transactivation domain (C-TAD). Hydroxylation of proline residues within the ODD and of asparagine residues within the C-TAD of HIF-1α and HIF-2α are highlighted. The nonequilibrium hydroxylation by the prolyl-hydroxylases (PHD) and the asparagine hydroxylase factor inhibiting HIF-1 (FIH-1) including substrates and products is depicted exemplarily for two of the three hydroxylation sites of HIF-1α.
FIGURE 3  Hypoxia-inducible factor (HIF) pathway components and regulating mechanisms in myeloid cells. HIF\(\alpha\) and HIF\(\beta\) subunits are constitutively transcribed and translated. The HIF-1\(\alpha\) antisense strand transcript \textit{ahif} decreases HIF\(\alpha\) protein expression. In hypoxia, nonhydroxylated HIF\(\alpha\) dimerizes with HIF\(\beta\) and binds to hypoxia-response elements (HRE) enhancing target gene transcription upon recruitment of co-transactivators, including CREB-binding protein (CBP) and p300. Under normoxia, prolyl-hydroxylase domain-containing enzymes (PHD) hydroxylate HIF\(\alpha\) subunits, thus enabling von-Hippel-Lindau gene product (pVHL) binding to HIF\(\alpha\) subunits and subsequent proteasomal degradation. Factor inhibiting HIF-1 (FIH-1)-mediated HIF\(\alpha\) hydroxylation under normoxia prevents HIF\(\alpha\) association with indispensable co-transactivators for target gene transcriptional enhancement. Phosphorylated receptor of activated protein kinase C (RACK1) induces HIF\(\alpha\) proteasomal degradation, which is inhibited by endothelin-receptor-dependent calcineurin activation. NO inhibits PHD activity. Hypoxia, infectious (gram-positive bacteria as blue circles, gram-negative bacteria in red) and noninfectious (endothelin in violet and ET receptor) stimuli activate the HIF pathway in myeloid cells.

Despite cellular hypoxic adaptation by the HIF pathway, HIF abundance and target gene expression are also under the control of oxygen-independent mechanisms. HSC70, LAMP2a, and Cezanne\(^{94}\) concert HIF-1\(\alpha\) lysosomal degradation by cyclin-dependent kinase regulated chaperone-mediated autophagy.\(^{95,96}\) The HIF subunit specific E3 ubiquitin ligases hypoxia-associated factor\(^{97}\) and mammary tumor integration site 6 (Int6)\(^{98}\) initiate HIF-1\(\alpha\), and HIF-2\(\alpha\) proteasomal degradation irrespective of oxygen tension and pVHL, respectively.

3.3  Oxygen-dependent HIF regulation by transcriptional inhibition

In addition to directing HIF degradation, oxygen controls the transactivation efficacy of HIF heterodimers by factor inhibiting HIF-1 (FIH-1).\(^{99}\) FIH-1 is an asparaginyl-hydroxylase belonging to the same oxygen- and 2-oxoglutarate-dependent dioxygenase superfamily as the PHDs.\(^{100}\) However, FIH-1 activity persists even under hypoxic conditions of 1% oxygen when PHD2 activity is abolished.\(^{101}\) Under normoxic oxygen tensions, FIH-1 hydroxylates Asn803 of HIF-1\(\alpha\), and Asn851 of HIF-2\(\alpha\), respectively.\(^{102}\) These hydroxylation sites are located within the C-TAD. Their hydroxylation prevents indispensable
co-transactivator recruitment that initiate target gene transcription. Fig. 3 depicts important components of the HIF pathway.

3.4 | HIF-regulated target genes and signaling pathways

CREB-binding protein (CBP) and p300 bind to HIF-α/β heterodimers with nonhydroxylated asparagine residues in the C-TAD. All HIFα isoforms recognize the same HRE 5′-TACGTT-3′ motif, but result in unique differential target gene expression with the N-TAD determining target gene selectivity. Nonetheless, comparison of cell-specific target gene regulation highlighted the importance of the cell type for the HIF-controlled transcriptome. HIF-1α primarily controls metabolic pathways, including adaptation to anaerobic energy supply by up-regulating glycolysis and the hexose monophosphate pathway. These effects facilitate cell survival in low-oxygen conditions. In addition, HIF-1α regulates apoptosis-related genes, for example, members of the B-cell lymphoma 2 (BCL-2) family, and proinflammatory genes including IL-1α, IL-6, and IL-8. HIF-1α also prevents excessive cellular reactions to hypoxia by up-regulating PHD transcription.

In contrast, HIF-2α fine-tunes embryonic development and cellular differentiation. Despite the initial discovery of HIF-1 in the context of EPO expression, we and others have shown predominant EPO transcriptional control by HIF-2.

4 | HIF-CONTROLLED MYELOID CELL FUNCTIONS IN HUMANS

Human neutrophils express all PHD isoforms and do not express HIF-1α protein under normoxia. However, neutrophil HIF-1α protein is induced at low oxygen tensions. Some but not all studies described HIF-2α expression in human granulocytes under normoxia with preserved response to hypoxia. Possibly, differences are explained by the use of different culture media as for example the presence of the NO donor GEA3162 inhibits PHD activity. In human monocytes, hypoxic induction of the HIF-1α natural antisense transcript ahi f contributes to a negative feedback mechanism on HIF-1α activity.

Evolutionary adaptation of high-altitude populations, as well as monogenetic mutations affecting the HIF pathway, provide insight in HIF-controlled mechanisms that help myeloid cells to cope with low oxygen concentrations and to maintain their functions. Additional information comes from human individuals or isolated myeloid cells exposed to hypoxia.

4.1 | Adaptive genetic variations in HIF pathway components provide an opportunity to study consequences for myeloid cell functions

Despite living above 4000 m, Tibetan communities, in contrast to communities residing at similar altitudes in the Andes, have mostly normal red-blood-cell and hemoglobin values. A missense mutation in the EGLN1 gene results in a PHD2 variant with a lower $K_m$ and higher $V_{max}$ value for oxygen. Consequentially, HIF hydroxylation is facilitated even under hypoxia. Other studies in Tibetans correlated SNPs in EPAS1 (HIF-2α) with hemoglobin levels and SNPs in the EGLN3 (PHD3) and PPP1R2P1 (protein phosphatase 1 regulatory inhibitor subunit 2) genes with altitude polycythemia. However, these Tibetan adaptations of the HIF pathway provide an interesting opportunity to study myeloid cell functions, immunity, and inflammatory disorders.

Chuvash polycythemia, named after the Chuvash republic in Russia, is another endemic genetic variation of the HIF pathway accompanied by elevated EPO and VEGF plasma levels. The C598T base exchange in the third VHL exon causes a missense mutation (R200W) that stabilizes predominantly HIF-2α over HIF-1α. Th1 (IL-2, IL-12, IFNγ, TNFα, GM-CSF) and Th2 cytokine (IL-4, IL-5, IL-10, IL-13) plasma levels in affected individuals were found to be elevated together with decreased CD4+ T-cell frequency and reduced CD4/CD8 ratio. Transcriptome analysis in PBMCs from Chuvash polycythemia patients showed up-regulated HIF target genes involved in the inflammatory response (TNFα, IL-1α, TLR4) as well as in myeloid cell differentiation, phagocytosis, and bacterial defense (FCGR2A, HCK, GAB2, ITGB1). Pro-apoptotic genes (CASP8, CASP2) and TCR elements were down-regulated. The reasons for the apparent discrepancy between TCR down-regulation found in this and increased Th1 and Th2 cytokines in the other study are not clear. Myeloid cell functions in the Chuvash polycythemia cohort have not been investigated.

4.2 | Genetic diseases highlight the interplay of metabolism and HIF pathway components

Patients with VHL syndrome harbor heterozygous germline VHL mutations predisposing to hemangiomas, paragangliomas, and renal carcinomas. Neutrophils from these patients showed decreased spontaneous apoptosis as well as increased phagocytic activity against bacteria. Hypoxia further enhanced these functions in both VHL neutrophils and cells from healthy controls. Thus, VHL neutrophils showed a partial hypoxic phenotype under normoxic conditions indicating that HIF indeed regulates neutrophil functions. However, whether or not enhanced neutrophil function contributes to the clinical phenotype of patients with the VHL syndrome remains unclear.

Glycogen storage disease Ib (GSD1b) is characterized by a nonfunctional glucose-6-phosphate transporter, neutropenia, and recurrent infections. Myeloid cells from GSD1b patients comprise a defective energy metabolism leading to endoplasmic reticulum stress with Hsp induction and elevated ROS. In some of the GSD1b patients, constitutive neutrophil HIF-1α stabilization, attributed to the Hsp90 and ROS increase, was observed. Nevertheless, the metabolic impairment in GSD1b neutrophils led to accelerated constitutive apoptosis, reduced respiratory burst, phagocytosis, and chemotaxis despite stabilized HIF-1α. As expected, HIF stabilization improves cellular energy supply that is indispensable for neutrophil survival and...
functioning. In fact, HIF-1α target genes, including peroxisome proliferator-activated receptor γ (PPARγ), were up-regulated in GSD1b neutrophils. PPARγ up-regulation contributed to neutrophil dysfunction because PPARγ inhibition improved chemotaxis and the respiratory burst. Accordingly, neutrophils isolated from healthy controls mimicked GSD1b-associated neutrophil dysfunction upon pharmacologic HIF stabilization and PPARγ activation by rosiglitazone. These data suggest that HIFs control myeloid cell functions not only by providing cellular energy supply but also through PPARγ activation.

Succinate is a powerful PHD inhibitor inasmuch as it is an end product of the hydroxylation reactions mediated by PHDs. Patients with heterozygous germline mutations of succinate dehydrogenase B (SDHB) had elevated succinate levels in neutrophils and their effects on the HIF pathway were analyzed. Neutrophil glycolytic activity and HIF-1α protein expression did not differ from healthy controls under normoxic culture conditions but were increased with hypoxia. However, neutrophils from patients with SDHB deficiency demonstrated a reduced apoptotic rate and a lower intracellular ROS stress under both normoxia and hypoxia that could be mimicked in control neutrophils by selective inhibition of SDHB. These observations suggest that the neutrophil phenotype was caused by the metabolic consequences of the SDHB mutation and not by HIF pathway activation. The observations obtained in neutrophils from patients with these Mendelian diseases underscore the importance of HIF-dependent effects on neutrophil survival and functions. However, these studies reveal additional HIF-independent metabolic pathways that modify HIF-dependent actions.

### 4.3 Exposure of human individuals to hypoxia enhances myeloid cell performance

Several studies investigated myeloid cells isolated from healthy volunteers who were exposed to hypoxic conditions before blood donation. Following hypoxic donor exposure neutrophil phagocytosis increased and decreased with normoxia. More mechanistically, low oxygen tension increased cytokine-induced expression of phagocytosis receptors on the neutrophil surface, including Fcγ receptors CD32w, CD16, CD64, and complement receptor CD35. Other phagocytic receptors (C5aR, CD16b) and adhesion molecules (LFA-1, L-selectin) were also up-regulated.

Blood donor hypoxia increased stimulated respiratory burst, chemotactic motility, and degranulation in isolated neutrophils. However, plasma neutrophil elastase (NE), IL-1, IL-6, and IL-8, and ROS concentration in resting blood neutrophils were not increased.

Together, these experiments indicate that hypoxia exposure of humans, which leads to HIF stabilization, enhances inflammatory myeloid cell functions. However, these observations cannot establish that HIFs play a causal role in this process. Another caveat is that although the blood donors were exposed to hypoxia, isolated myeloid cells were studied under normoxia. Conceivably, normoxia led to rapid HIF degradation of in vivo stabilized HIFs, whereas HIF-induced effects on transcription and metabolism may have persisted.

### 4.4 Myeloid-cell exposure to hypoxia in vitro prolongs survival and increases activation responses

Several studies analyzed neutrophils that were isolated from normoxic donors and exposed to hypoxia in vitro. McGovern and coworkers found that hypoxic culture of human neutrophils did not affect the secretion of IL-6, IL-8, TNFα, or IL-10, whereas ROS-dependent bactericidal activity was reduced. Limited molecular oxygen leading to reduced NADPH oxidase-dependent respiratory burst was the possible explanation for the latter observation. In contrast, ROS-independent killing by hypoxic neutrophils was increased.

Hypoxia augmented the release of granule proteins from activated neutrophils as shown for NE, myeloperoxidase, lactoferrin, and matrix metalloproteinase-9 (MMP-9). Consequently, supernatants from activated hypoxic neutrophils caused more epithelial cell damage compared to normoxic neutrophils. The hypoxic degranulation increase was reduced by a selective PI3Kγ inhibitor that abrogated the hypoxic degranulation augmentation. The fact that pharmacologic HIF stabilization by PHD inhibitors did not mimic augmented degranulation and increased epithelial cell injury seen with hypoxic neutrophils, questions a causal role for HIF but does not exclude involvement of components upstream from HIF mediated by PHDs or FIH-1.

Hypoxic inhibition of constitutive neutrophil apoptosis in vitro was reported by several investigators possibly via HIF-1α mediated NFκB activation. By contrast, other groups reported HIF-1α stabilization to be downstream of NFκB or mammalian target of rapamycin activation. Anoxia also attenuated TNFα-accelerated neutrophil apoptosis in vitro. Moreover, hypoxic culture conditions abrogated the pro-apoptotic effect of synovial fluid from rheumatoid arthritis patients on healthy control neutrophils.

Hypoxia also has effects on monocytes and macrophages that, similar to neutrophils, express HIFs. Hypoxia up-regulated the LPS- and phytohemagglutinin-induced secretion of proinflammatory cytokines IL-8, IL-2, IL-4, IL-6, and IFNγ, whereas anti-inflammatory IL-10 was repressed. HIF-1α-mediated β2-integrin up-regulation enhanced monocyte adhesion to endothelial cells under hypoxia.

Hypoxia increased efferocytosis, the phagocytosis of apoptotic neutrophils by monocytes or macrophages. This effect was, at least in part, mediated by HIF-1α-dependent induction of the class B scavenger receptor CD36 and its ligand thrombospondin-1 conveying apoptotic material. HIF-mediated CD36 induction is supported by the observation that CD36 and HIF-1α expressing macrophages correlated in biopsies from patients with inflammatory bowel disease.

Altogether, these studies support the notion that hypoxia exposure in vitro prolongs myeloid cell survival and promotes proinflammatory responses that are important for host defense. The exact role of HIFs in these adaptive processes remains unclear and needs to be addressed in animal studies that allow HIF manipulations.
5 | HIF-CONTROLLED MYELOID CELL FUNCTIONS—LESSONS FROM ANIMAL EXPERIMENTS

5.1 | Hypoxic modulation of myeloid cells controls inflammation in animals

Various animal models were employed to study oxygen-dependent modifications of myeloid cell-driven inflammation. In rats, hypoxic preconditioning protected the animals from gastrointestinal ischemia-reperfusion injury, including bacterial translocation. Neutropenic rats lacked the protective effect suggesting a neutrophil-dependent mechanism. In agreement with this suggestion, neutrophils consumed oxygen during respiratory burst thereby creating a hypoxic environment for epithelial cells that promoted HIF stabilization and induction of HIF target genes. As a result, the epithelial barrier was increased. Neutrophils from NADPH oxidase gene-deficient chronic granulomatous disease (CGD) mice are unable to produce superoxide anions and therefore did not create a hypoxic environment. Consequently, CGD neutrophils did not increase the epithelial barrier and CGD mice displayed a more severe phenotype of chemical colitis. Further evidence supporting HIF driven epithelial barrier stabilization is provided by murine colitis models showing beneficial effects of PHD inhibitors.

5.2 | Inflammatory and mechanical challenges imitate oxygen-dependent HIF stabilization

Myeloid cell studies in animals elucidated HIF stabilizing mechanisms above and beyond hypoxia. Bacterial antigens from gram-negative and gram-positive species stabilized HIFs in myeloid cells. Mechanistically, combined PHD down-regulation and up-regulation of HIF transcription were suggested to increase HIF proteins. More recently, HIF induction by physical forces was reported in murine bone marrow-derived monocytes. Cylindrical hydrostatic pressure, for example, due to in- and expiration, activated the monocytic ion channel PIEZO1 leading to paracrine endothelin-1 secretion. Subsequently, endothelin receptor stimulation activated calcineurin that dephosphorylated receptor of activated protein kinase C (RACK1). Phosphorylated RACK1 competes with cytoplasmic Hsp90 for binding HIFα subunits and promotes their proteosomal degradation. Finally, mechanotransduced RACK1 dephosphorylation contributed to HIF-1α protein accumulation.

5.3 | HIF-1α improves myeloid cell functions in infectious and noninfectious inflammation models

Myeloid-specific HIF-1α gene deletion severely reduced intracellular ATP concentrations in murine macrophages and neutrophils leading to reduced intracellular bactericidal activity, adhesion, and motility of monocytes. HIF-1α gene-deficient murine neutrophils displayed decreased NE and cathepsin G activities that were restored by VHL deletion, hence, by constitutive HIF-1α stabilization. Likewise, pharmacologic HIF stabilization by PHD inhibition enhanced monocyte bactericidal properties in murine skin abscesses by inducing monocyte cathelicidin and IL-8 production. HIF-1α gene-deleted myeloid cells demonstrated decreased killing of Helicobacter pylori resulting in aggravated murine H. pylori gastritis. Biopsies from patients with H. pylori gastritis showing local macrophage HIF-1α stabilization strengthened the clinical significance of myeloid HIF-1α for providing antibacterial defense. We demonstrated HIF-1α and HIF-2α up-regulation in human psoriatic skin lesions. Compared to control mice, myeloid-specific HIF-1α gene deficiency caused ameliorated leukocyte skin infiltration in bacterial and chemical skin inflammation, alleviated acute pathology of chemical colitis with reduced macrophage infiltration, and reduced colonic TNFα, IFNγ, and IL-17 expression. Conflicting results were reported in ARNTflox/flox; LysM-Cre mice. These mice that were not able to form HIF heterodimers showed prolonged myeloid cell infiltration in a colitis model despite expected accelerated myeloid cell apoptosis. Pronounced numbers of infiltrating cells were possibly explained by colonic up-regulation of antiapoptotic factors serum amyloid A3 and leukotriene B4 in the inflamed colon sections. LPS-induced HIF-1α up-regulated proinflammatory cytokines IL-1, IL-4, IL-6, IL-12, and TNFα in macrophages resulting in increased mortality in murine sepsis. Acute hypoxic HIF stabilization at inflammation induction increased mortality in murine skin infection and pneumonia models. Complementarily, myeloid-specific HIF-1α gene-deletion reduced shock and hypothermia in murine sepsis and decreased mortality, whereas hypoxia prior to infection improved infection control. Hypoxic preconditioning for 1 wk induced a myeloid cell memory effect as bone marrow from these hypoxic mice reduced sepsis morbidity in normoxic recipients. Together, these results imply a temporal component of HIF activation that determines the outcome of infection and systemic inflammation. Hypoxic preconditioning prior to the infection reduced inflammation, whereas HIF activation at the onset of inflammation induced collateral damage.

In addition to infection, reparative healing processes after physical trauma also depend on the HIF pathway in myeloid cells. Myeloid HIF-1α gene deletion delayed macrophage-driven resorption of muscle necrosis leading to reduced revascularization of the regenerated muscle tissue. In contrast, myeloid-specific HIFα subunit stabilization in VHLflox/flox; LysM-Cre mice preserved myocardial muscle integrity in fully MHC-mismatched murine cardiac allotransplantation, at least in part, by myeloid HIFα stabilization-dependent production of anti-inflammatory IL-10. In addition, myeloid-derived suppressor cells reduced T-cell proliferation. Ultimately, HIF signaling reduced acute rejection and ischemia-reperfusion injury leading to prolonged allograft survival.

Synovial tissue from rheumatoid arthritis patients and experimental arthritis mice both up-regulated HIFs and myeloid-specific HIF-1α and HIF-2α gene deficiency alleviated inflammation in a murine rheumatoid arthritis model. Neutrophils isolated from rheumatoid arthritis patients showed enhanced PHD2 and PHD3 mRNA expression in line with induction of proinflammatory HIF target genes.
However, myeloid HIF pathway activation as well as myeloid HIF deficiency did not affect the rheumatoid arthritis-associated uveitis phenotype in an intravitreal LPS-induced mouse model. Conceivably, this discrepancy is due to the divergent inflammatory stimuli underscoring the importance of the inflammatory context.

5.4 | HIF-2α mitigates myeloid cell destructive capacity, but prolongs inflammation

In contrast to HIF-1α deletion, myeloid-specific HIF-2α gene deficiency in mice did not affect ATP generation in macrophages. However, macrophage motility and tissue infiltration were significantly diminished accompanied by down-regulation of chemokine receptor CXCR4 and fibronectin-1. Secretion of proinflammatory cytokines IL-1β, IL-6, IL-12, TNF-α, and CXCL2 following stimulation with IFN-γ or LPS was significantly decreased, whereas these mice up-regulated anti-inflammatory IL-10 upon LPS injection.

Studies in a murine LPS-induced acute lung injury model revealed differential effects of the HIF-α subunits during neutrophil-mediated inflammation. A HIF-2α gain-of-function mutation did not affect neutrophil effector functions such as oxidative burst and phagocytosis but decreased constitutive apoptosis similar to what was aforementioned for HIF-1α. Acute pulmonary inflammation predominantly induced neutrophil HIF-1α at an early stage, whereas HIF-2α induction was prominent during the resolution phase. Myeloid HIF-2α gene deficiency shortened and alleviated pulmonary inflammation, particularly in later stages of acute lung injury, presumably by increased neutrophil apoptosis. Inflammation models in zebrafish with HIF-2α gain-of-function mutation and myeloid-specific HIF-2α gene-deficient mice further underscored the fact that HIF-2α prolongs inflammation.

We previously demonstrated that HIF-2α controls EPO transcription. In addition to its role in erythropoiesis, EPO was proposed to have anti-inflammatory effects. In a murine peritonitis model, hypoxia induced EPO as well as EPO receptors (EPOR) on infiltrating macrophages. Macrophage EPOR signaling led to PPARγ activation thereby inducing anti-inflammatory cytokines, down-regulating proinflammatory cytokines, enhancing macrophage efferocytosis and phagocytosis. CGD mice that were unable to mount a respiratory burst and therefore did not consume oxygen failed to develop peritoneal hypoxia. Consequently, endogenous EPO was not induced. Exogenous EPO therapy restricted peritoneal inflammation. The authors discuss this observation as a consequence of HIF-1α, despite the fact that EPO is rather a HIF-2α target gene.

Tumor-associated macrophages and neutrophils (TAM and TAN) contribute to the progression of solid tumors. We showed previously that these cells also promoted chronic lymphatic leukemia in a murine disease model and that selective depletion of myeloid subpopulations retarded leukemia progression. TAM express HIF-2α and several observations suggest that HIF-2α restrains their anti-cancer effects. Thus, HIF-2α−/−/+ LysM-Cre mice developed fewer chemically induced colon carcinomas. In contrast, the number and size of chemically induced hepatocellular carcinomas were not reduced in HIF-2α-deficient mice, but both tumor entities demonstrated lower grading, delayed tumor progression, and decreased mitotic indices compared to wild-type (WT) mice. Moreover, tumor histology showed a significant reduction of TAM numbers in HIF-2α-deficient mice, in line with HIF-2α-dependent macrophage invasiveness mentioned earlier. Conceivably, HIF-2α-dependent macrophage cytokine secretion possibly accounts for the observed tumor cell proliferation reduction and lower grading in HIF-2α-deficient mice. More recently, a murine endometrial cancer model established the importance of the hypoxic tumor microenvironment for controlling TAM tumoricidal properties. Tumor hypoxia prevented neutrophil NADPH-oxidase-dependent ROS production and MMP-9 secretion, which both promoted tumor cell sloughing by detachment from the basement membrane. Hyperoxia reduced HIF in tumors and increased neutrophil antitumor actions. Mechanistically, ROS and MMP-9 were increased, whereas NE secretion was reduced resulting in diminished tumor cell proliferation. Thus, hyperoxia reversed neutrophil hibernation in hypoxic tumors enhancing myeloid cell antitumor effects independent of adaptive immune cells.

5.5 | Genetic PHD deletion controls myeloid cell metabolism survival, and myeloid cell-mediated inflammation

Myeloid-specific gene-deletion of PHD enzymes facilitated the investigation of the HIF pathway in innate immunity in vivo. PHD2 is the most critical regulator of HIFs. Deciphering PHD2 involvement in myeloid cell-mediated inflammation and immunity in vivo is complicated by the fact that homozygous PHD2 gene-deletion (PHD2−/−) is embryonically lethal. Myeloid-specific PHD2 deletion using PHD2floxflox LysM-Cre mice highlighted that PHD2 controls both neutrophil metabolism and inflammatory neutrophil responses. PHD2 gene-deleted neutrophils up-regulated HIF-1α protein, but not HIF-2α, delayed constitutive apoptosis, and increased chemotactic motility while phagocytic activity was unaltered. Absence of PHD2 enhanced typical HIF-1α target genes that increase glucose uptake, glycogen storage, and glycolytic flux, culminating in an augmented extracellular acidification rate by lactate generation and increased intracellular ATP levels. Pharmacologic glycolysis inhibition reduced neutrophil chemotaxis and survival, suggesting a mechanistic link between these metabolic changes and the neutrophil effector functions. Myeloid-specific PHD2 gene deficiency resulted in earlier and faster pulmonary neutrophil recruitment in acute lung injury models compared to WT controls. In addition, pulmonary inflammation persisted longer because of delayed neutrophil apoptosis rather than reduced efferocytosis. Prolonged neutrophil persistence due to HIF stabilization was also demonstrated in chemical colitis induced in heterozygous PHD2+/− mice. It was suggested that the delayed apoptosis in PHD2 gene-deficient neutrophils is mediated by IL-4 and the neutrophil IL-4 receptor. In WT mice, but presumably not in PHD2 gene-deficient mice, IL-4 treatment reverses hypoxia- and HIF-1α-mediated neutrophil apoptosis delay by PPARγ-dependent PHD2 up-regulation. Thus, IL-4-dependent PHD2 expression limited
inflammation and promoted its resolution. In another study, inducible PHD2 knock-down in mice, stabilizing both HIF-1α and HIF-2α, resulted in a lupus-like phenotype with antinuclear antibodies, leukocytosis, spontaneous weight loss, dermal lymphohistiocytic infiltration, splenomegaly, and lymphadenopathy. The phenotype depended largely on intact HIF-2α as combined PHD2 and HIF-2α shRNA-induced knockdown completely prevented the pathology. Hematopoietic cells were the main contributors to the phenotype as shown by bone marrow chimeric mice. Mechanistically, defective suppressive functionality of regulatory T cells (T_{reg}) explained the autoimmune phenomena. By contrast, others reported that dendritic cell HIF-1α was dispensable for T_{reg} induction and T_{reg} tissue recruitment by HIF-1α-controlled homing receptor expression. These observations suggest that a postnatal inducible model, but not constitutive myeloid-specific PHD2 gene-deletion resulted in a spontaneous autoimmune phenotype that was linked to HIF-2α controlling interactions of innate and adaptive immune cells. PHD1−/− mice were also protected from chemical colitis. PHD3−/− mice were also protected from chemical colitis.

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However, several murine genetic PHD3 deletion models revealed opposite effects with increased intestinal inflammation and decreased mucosal integrity. PHD3 inhibits the E3 ubiquitin-ligase Itch that orchestrates occludin-proteasomal degradation. Enterocyte-specific PHD3 gene-deleted (PHD3^{EC-KO}) mice developed spontaneous colitis without intestinal up-regulation of HIFα subunits, suggesting compensation by PHD2 or PHD1. PHD3 contributes to the mucosal barrier by securing epithelial occludin expression in the bowel independent of HIF stabilization. PHD3-dependent intestinal inflammation is further suggested by colonic biopsies from ulcerative colitis patients showing inverse correlation of local inflammation with PHD3 expression.

Under normoxic conditions, neutrophil inflammation was unaffected in PHD3−/− mice in LPS-induced acute lung injury or chemical colitis. Hypoxic conditions significantly reduced neutrophil lung and colon infiltration, possibly by hypoxia-induced PHD1 and 2 expression with subsequent HIF hydroxylation and degradation. PHD3−/− mice demonstrated normal white blood cell count and granulocyte functioning, but neutrophil apoptosis was increased due to up-regulated pro-apoptotic SIVA1 and suppressed antiapoptotic BCL-XL, thereby contributing to decreased numbers of infiltrating neutrophils. The role of HIFs was not explicitly established. In contrast to neutrophils, PHD3 gene deficiency severely affected murine monocyte functionality by enhancing migration and phagocytosis in zymosan-induced peritonitis also under normoxia. PHD3 gene-deficient macrophages demonstrated pronounced HIF-1α and NFκB activation polarizing macrophages toward a proinflammatory M1 phenotype. PHD3−/− mice as well as mice with PHD3 gene deficiency in hematopoietic cells only, were more susceptible to sepsis by LPS injection or cecal ligation with higher mortality rate compared to WT, PHD1−/−, and PHD2+/− haplodeficient mice. Moreover, monocyctic tissue infiltration and peripheral blood cytokines (TNFα, IL-1β) were increased whereas neutrophil infiltration remained unaffected.

Together, these studies indicate that PHD2 links innate and adaptive immunity. PHD2-dependent HIF-regulation is indispensable for limiting inflammation and autoimmunity. PHD1 and PHD3 reduce inflammation by preserving mucosal barriers with some of these effects being possibly HIF independent. PHD3 keeps specifically monocytes in check. Reasons for differential PHD effects remain ill-defined but could be related to cell type, HIFα subunit, and HIF-independent effects on additional pathways.

### 6 | PHARMACOLOGIC HIF MODIFIERS

Strategies to either stabilize or reduce HIFs are of major clinical interest and are currently explored in clinical studies. Given the profound HIF effects on myeloid cells, it will be important to carefully observe the effect of these pharmacologic substances on inflammation and immunity.

#### 6.1 | HIF stabilization

HIF stabilization is a new strategy currently tested in renal anemia patients. Various compounds were developed to inhibit PHD-mediated HIFα hydroxylation and subsequent degradation. PHD inhibitors that compete with the indispensable PHD co-substrates, including iron and 2-oxoglutarate are now available. These substances comprise roxadustat (FG-4592), vadadustat (AKB-6548), moldustat (Bay85-3934), daprodustat (GSK1278863), desidustat (ZYAN1), AKB-4924, and JNJ1935. Most of these compounds are currently investigated in phase 2 and phase 3 clinical trial programs for renal anemia treatment. Of note, roxadustat treatment was associated with an increased rate of upper respiratory infections compared to standard therapy with recombinant human EPO in phase 3 study in dialysis-dependent patients with kidney disease (18.1% vs. 11.0%).

Beyond the correction of renal anemia, preclinical evidence suggests that PHD inhibition offers novel opportunities for organ protection, an area of unmet clinical need. We showed potent PHD inhibition by 2-(1-chloro-4-hydroxyisoquinoline-3-carboxamido)acetate (ICA) with beneficial effects in murine models of kidney ischemia-reperfusion injury, allotransplantation, and chronic kidney disease. Tissue and organ protective effects of PHD inhibition have also been demonstrated in models of myocardial injury, brain injury, lung injury, and—as mentioned earlier—inflammatory bowel disease. AKB-4924 is evaluated for the treatment of inflammatory bowel disease (NCT02914262).

#### 6.2 | HIF inhibition

Cancer research incentivized the development of HIF inhibitors. Agents that inhibit HIF heterodimerization, DNA binding, or transactivation are classified as direct HIF inhibitors, whereas indirect HIF inhibitors reduce HIF de novo synthesis or increase proteasomal
Various compounds were reported in the literature (as reviewed in Bhattarai et al. and Ban et al., but only a few substances are currently available for clinical applications. PT-2385 is a direct HIF-2α inhibitor interfering with the HIF-2α-ARNT heterodimerization that is currently under investigation for treatment of renal cell carcinoma (NCT02293980, NCT03108066) and glioblastoma (NCT03216499). Furthermore, in vitro testing of the HIF inhibitor PX-478 in prostate carcinoma cells and a phase I clinical trial enrolling lymphoma and solid cancer patients (NCT00522652) were reported.

7 | CONCLUDING REMARKS

Human and animal data implicate HIFs as important regulators of myeloid cell metabolism, survival, and functioning. HIFs modify both the magnitude and the duration of the inflammation response. PHDs regulate HIF activity, preserve epithelial barrier function, and provide a bridge between innate and adaptive immunity thereby controlling autoimmunity. HIF pathway-modifying drugs are entering clinical medicine. Given the emerging evidence for the role of the HIF pathway in inflammation, patients should be monitored for inflammatory complications. At the same time, the opportunity may arise to repurpose HIF-modifying drugs for the treatment of inflammatory disorders.

AUTHORSHIP

L.K. and R.K. wrote the manuscript and designed the figures. R.K. conceived the project. K.-U.E. and A.S. supervised and reviewed the manuscript and figures.

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