

# Macrophage-mediated response to hypoxia in disease

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**Abstract:** Hypoxia plays a critical role in the pathobiology of various inflamed, diseased tissues, including malignant tumors, atherosclerotic plaques, myocardial infarcts, the synovia of rheumatoid arthritic joints, healing wounds, and sites of bacterial infection. These areas of hypoxia form when the blood supply is occluded and/or the oxygen supply is unable to keep pace with cell growth and/or infiltration of inflammatory cells. Macrophages are ubiquitous in all tissues of the body and exhibit great plasticity, allowing them to perform divergent functions, including, among others, patrolling tissue, combating invading pathogens and tumor cells, orchestrating wound healing, and restoring homeostasis after an inflammatory response. The number of tissue macrophages increases markedly with the onset and progression of many pathological states, with many macrophages accumulating in avascular and necrotic areas, where they are exposed to hypoxia. Recent studies show that these highly versatile cells then respond rapidly to the hypoxia present by altering their expression of a wide array of genes. Here we review the evidence for hypoxia-driven macrophage inflammatory responses in various disease states, and how this influences disease progression and treatment.

**Keywords:** macrophage, hypoxia, inflammation, cytokine

## Introduction

Macrophages are extremely versatile cells that adopt a distinct phenotype in response to a changing microenvironment. These cells are derived from circulating monocytes that exit the vasculature and invade into the surrounding tissues where they differentiate under the influence of local signals into resident tissue macrophages. Resident macrophages have a variety of roles; they patrol tissues for damaged or apoptotic cells, which they clear by phagocytosis, they identify and eliminate such invading pathogens as bacteria, fungi, and virally infected cells, they scavenge lipoproteins, and they are also responsible for regulating tissue oxygenation by influencing the formation of new blood vessels and modulating vascular permeability.<sup>1</sup> Although tissue macrophages are anatomically distinct from one another, and have different transcriptional profiles and functional capabilities, they are all required for the maintenance of homeostasis. However, these reparative and homeostatic functions can be subverted by chronic insults, resulting in a causal association of macrophages with disease states. One such insult is hypoxia, which usually occurs due to an inefficient blood supply or where there is an imbalance between the number of actively respiring cells and the supply of oxygen at a particular localized tissue site. Of course, in such diseases as tumors, both these things occur simultaneously. When macrophages experience hypoxia, they respond to it rapidly with altered gene expression, mediated, in part, via upregulation of the transcription factors,

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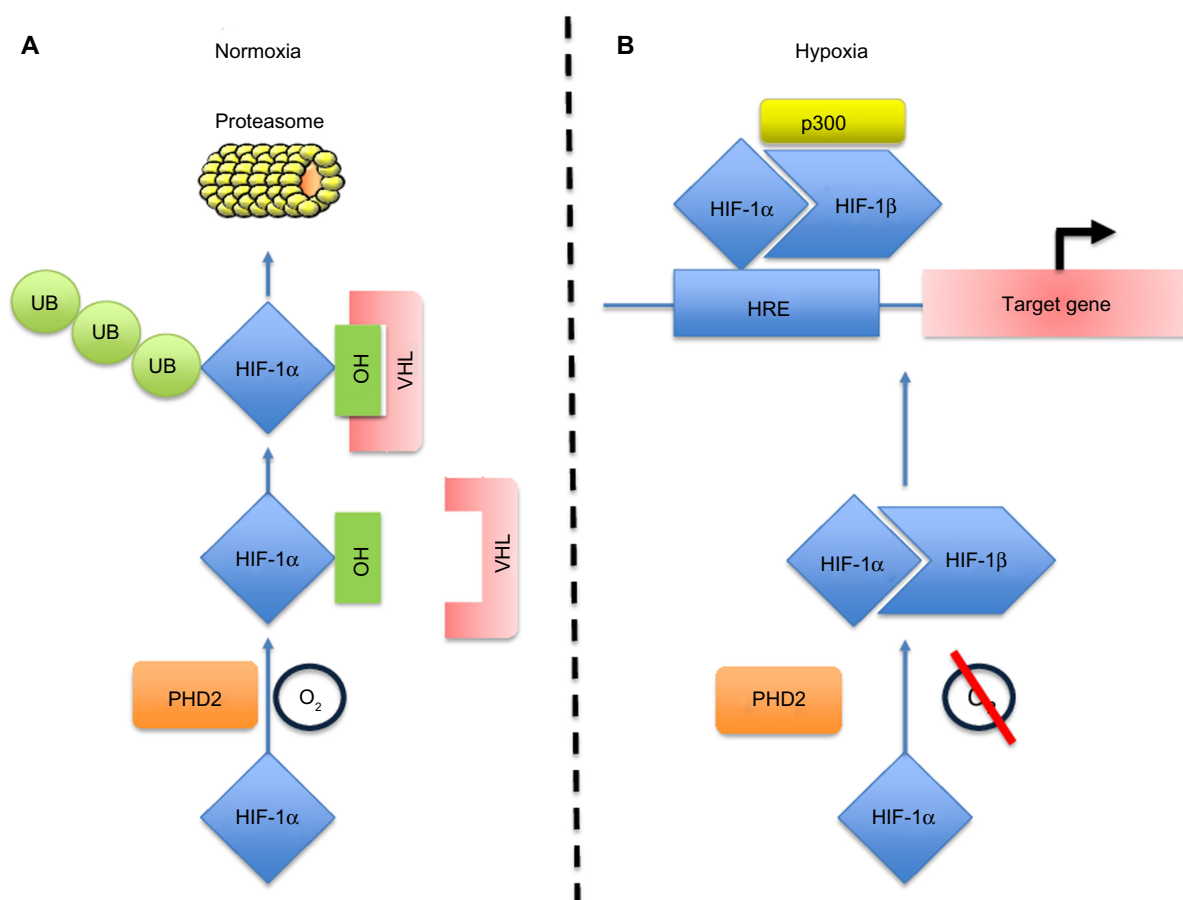
hypoxia-inducible factors 1 and 2 (HIF-1 and HIF-2).<sup>2,3</sup> These consist of a distinct hypoxia-inducible  $\alpha$  subunit and a common, constitutively expressed  $\beta$  subunit. HIF-1 $\alpha$  and HIF-2 $\alpha$  protein stability is negatively regulated by prolyl hydroxylase domain protein-(PHD) dependent hydroxylation, which leads to von Hippel–Lindau protein-(VHL) dependent ubiquitination and proteasome-dependent degradation under normoxic conditions.<sup>4,5</sup> HIF-1 $\alpha$  and HIF-2 $\alpha$  transactivation function is negatively regulated by factor inhibiting HIF-1 (FIH-1) through FIH-1-dependent asparaginyl hydroxylation, which blocks binding of the coactivators, CREB-binding protein (CBP) and p300.<sup>6,7</sup> Thus, the stability and activity of the HIFs are modulated by hydroxylation, such that changes in oxygen availability are transduced to the nucleus as changes in HIF activity (Figure 1).<sup>8</sup>

A number of studies have documented the distinct changes in gene expression that occur in macrophages when they experience hypoxia in vitro (see summary in

Table 1). These include upregulation of molecules required for macrophage survival, tissue revascularization (and thus reoxygenation of the ischemic site), matrix remodeling, and recruitment and activation of more macrophages and/or other inflammatory cells.<sup>2,8,9</sup> Importantly, a macrophage in a hypoxic tumor microenvironment is likely to express different genes than a macrophage in a hypoxic area in a wound, as other stimuli besides hypoxia will be present. In this review, we outline the responses of macrophages to hypoxia in a number of inflamed and/or diseased tissues. We then discuss the possible implications of these for their progression and treatment.

## Malignant tumors

One of the defining features of the tumor microenvironment is the presence of hypoxia. Within normal healthy tissue, oxygen levels are maintained within a typical range of 20–80 mmHg, due to a balance between supply and demand.



**Figure 1** Schematic diagram of the HIF-1 $\alpha$  pathway.

**Notes:** In normoxia (**A**), PHDs hydroxylate specific proline residues of HIF-1 $\alpha$ , leading to its proteosomal degradation, mediated by pVHL, an E3 ubiquitin ligase. During hypoxia (**B**), the lack of oxygen inhibits the actions of PHDs, and HIF-1 $\alpha$  is not ubiquitinated and then degraded. HIF-1 $\alpha$  builds up and translocates to the nucleus where it binds to HIF-1 $\beta$  and p300. This complex then acts as a transcription factor that binds to HREs to induce expression of hypoxia-regulated genes.

**Abbreviations:** UB, ubiquitin; HIF-1, hypoxia-inducible factor-1; OH, hydroxyl; VHL, von Hippel–Lindau protein; HRE, hypoxia response element; PHD, prolyl hydroxylase domain protein; p, phosphorylated.

**Table 1** Summary of select cytokines/proteases regulated by hypoxic macrophages

Gene	Functions	Up- or downregulated
<i>Glut-1</i>	Survival	Up
<i>VEGF</i>	Angiogenesis	Up
<i>TNF-<math>\alpha</math></i>	Inflammation, angiogenesis, apoptosis	Up
<i>MIF</i>	Multiple effects on inflammation including immobilizing macrophages	Up
<i>IL-1<math>\alpha</math></i>	Inflammation and angiogenesis	Up
<i>IL-1<math>\beta</math></i>	Proinflammatory, angiogenesis	Up
<i>IL-8 (CXCL8)</i>	Proangiogenic and chemoattractant for neutrophils	Up
<i>ANGPT2</i>	Angiogenesis and chemotactic for Tie2-positive monocytes	Up
<i>MMP7</i>	Wound healing, angiogenesis, metastasis	Up
<i>TIMP1</i>	Inhibits activity of most known MMPs. Stimulates proliferation in a wide range of cell types	Up
<i>GRO-<math>\alpha</math> and GRO-<math>\beta</math> (CXCL1 and CXCL2)</i>	Chemotactic for neutrophils	Up
<i>Rantes (CCL5)</i>	Chemotactic for T-cells, eosinophils, and basophils; recruits leukocytes to inflammatory sites	Up
<i>CSF2 (GM-CSF)</i>	Stimulates stem cells in the bone marrow to produce granulocytes and monocytes	Up
<i>TNFSF13B</i>	Cell proliferation	Down
<i>ORP150</i>	Proatherosclerotic	Up
<i>CCL2</i>	Chemotactic for monocytes	Down <sup>#</sup>

**Notes:** <sup>#</sup>Upregulated indirectly by hypoxia in atherosclerosis. This research was originally published in *Blood*. Adapted from: Fang HY, Hughes R, Murdoch C, et al. Hypoxia-inducible factors 1 and 2 are important transcriptional effectors in primary macrophages experiencing hypoxia. *Blood*. 2009;114(4):844–859. © by the American Society of Hematology.<sup>2</sup>

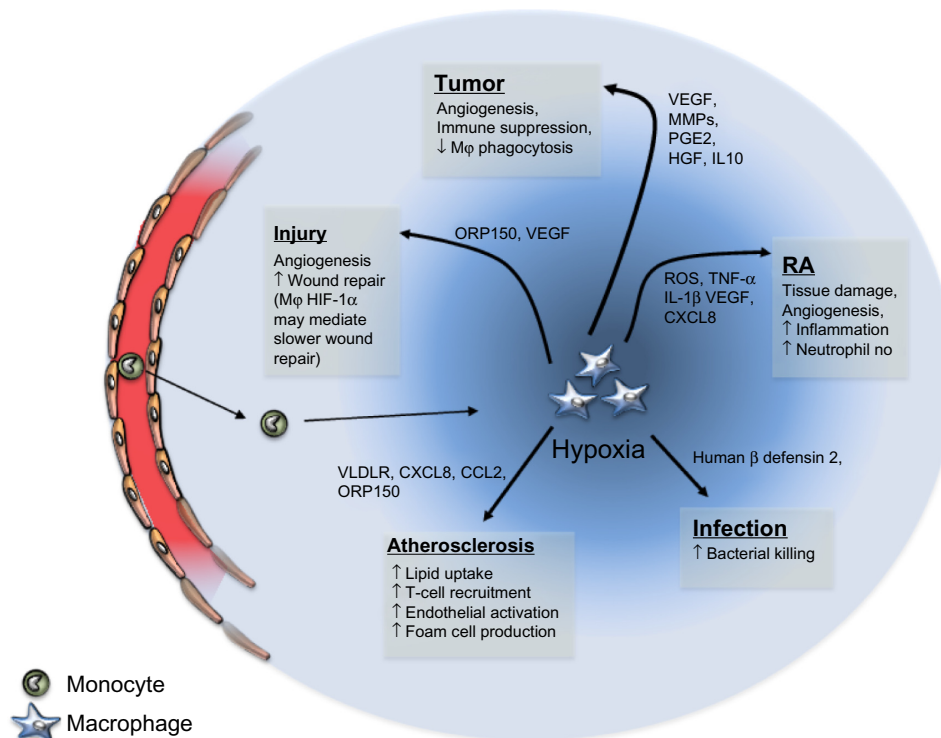
**Abbreviations:** Glut-1, glucose transporter-1; VEGF, vascular endothelial growth factor; TNF, tumor necrosis factor; MIF, macrophage inhibitory factor; IL, interleukin; ANGPT2, endothelial Tie2/Tek ligands angiopoietin-1; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase-1; GRO, growth-regulated oncogene; CSF, colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; TNFSF, tumor necrosis factor (ligand) superfamily; ORP, oxygen-regulated protein; CCL, chemokine (C-C motif) ligand.

In tumor tissues, this balance is disrupted by a combination of increased metabolic activity, due to rapidly dividing tumor cells, and a poor supply of oxygen from the poorly developed vasculature.<sup>10</sup> This leads to some tumor regions experiencing oxygen levels below 10 mmHg or <1.3% oxygen. Use of hypoxic cell markers, like pimonidazole, has demonstrated the presence of both transient (avascular, nonnecrotic) and

chronic (perinecrotic) areas of hypoxia in both human and experimental tumors.<sup>11,12</sup>

Tumor hypoxia is thought to play an important part in the education of tumor-associated macrophages (TAMs). Indeed, analysis of these hypoxic regions revealed a large infiltration of TAMs than has been observed in multiple tumor types, including breast, endometrial, ovarian, colorectal, prostate, and oral tumors.<sup>13–16</sup> The accumulation of TAMs in these hypoxic areas is mediated by several mechanisms, including active recruitment to the tumor and inhibition of macrophage escape (Figure 2). In response to reduced oxygen tension, tumors release such hypoxia-induced chemoattractants as vascular endothelial growth factor (VEGF) A, endothelins, endothelial monocyte-activating polypeptide-II (EMAPII), angiopoietin 2, and CXCL12 (stromal cell-derived factor 1 [SDF-1]).<sup>17</sup> For example, correlative studies have demonstrated that poorly vascularized areas of breast tumors express high levels of VEGF and have increased TAM numbers.<sup>18</sup> More recently, the hypoxia-induced tumor-derived chemoattractant CXCL12 was shown to be the main driver for TAM accumulation in experimental murine gliomas, where it also increased their survival.<sup>19</sup> CXCL12 binds to the macrophage cell surface receptor, CXCR4, which is upregulated through a HIF-1 $\alpha$ -dependent mechanism in both monocytes and macrophages. Therefore, the hypoxia-dependent CXCL12/CXCR4 axis is likely to be important for the chemotaxis of TAMs to hypoxic tumor sites.<sup>20</sup> Similarly, semaphorin 3A (Sema3A) also acts as an attractant for TAMs, by triggering VEGF-1 phosphorylation through the Neuropilin-1 (Nrp1) and PlexinA1/PlexinA4 receptors.<sup>21</sup> When Nrp1 levels are downregulated in the hypoxic environment, Sema3A continues to regulate TAMs in an Nrp1-independent manner, by eliciting PlexinA1/PlexinA4-mediated stop signals, which retain TAM inside the hypoxic niche. Interestingly, in a mouse model, where macrophages had a genetic deletion of Nrp1, it was shown that TAMs were retained in normoxic tumor sites, and this resulted in the prevention of tumor growth and metastasis.<sup>21</sup>

Since macrophages are phagocytes, they may also be attracted to hypoxic, perinecrotic areas via the trail of necrotic debris emanating from dead cells. Necrotic cells release high-mobility group box 1 (HMGB1) at sites of chronic tumor hypoxia, that has been shown to alter the phenotype of macrophages,<sup>22</sup> although it has yet to be shown that tumor-derived necrotic debris can mediate monocyte recruitment to tumors. Once monocytes/macrophages are recruited, the presence of hypoxia has also been implicated in their retention in these areas. Hypoxic macrophages



**Figure 2** Schematic representation of the role of macrophages following exposure to hypoxia in varying disease states.

**Notes:** Macrophages are recruited as monocytes from circulation. They enter the hypoxic environment of the diseased tissue and upregulate cytokines/proteases (black arrows) that mediate downstream biological functions (gray boxes) in each disease state. The up arrows correspond to an increase in expression or activity; the down arrows correspond to a downregulation in expression or activity.

**Abbreviations:** VEGF, vascular endothelial growth factor; MMP, matrix metalloproteinase; PGE2, prostaglandin E2; HGF, hepatocyte growth factor; IL10, interleukin 10; ORP150, oxygen-regulated protein 150; ROS, reactive oxygen species; TNF-α, tumor necrosis factor alpha; IL-1β, interleukin 1 beta; VLDLR, very low-density lipoprotein receptor; CCL2, chemokine (C-C motif) ligand 2; HIF-1, hypoxia-inducible factor-1; Mφ, macrophage; ORP150, oxygen-regulated protein 150; RA, rheumatoid arthritis.

upregulate mitogen-activated protein kinase phosphatase (MKP-1)<sup>23</sup> that, in turn, dephosphorylates the signaling enzymes MEK, ERK1/2, and p38 MAPK. This effectively prevents macrophages from responding to a multitude of chemotactic factors, including VEGF and chemokine (C-C motif) ligand (CCL) 2, that function through this signaling mechanism.<sup>24</sup> Additionally, hypoxia reduces the expression of the chemokine receptors CCR2 and CCR5, further preventing macrophages from responding to chemotactic signals outside the hypoxic environment.<sup>25,26</sup>

Once within the hypoxic tumor microenvironment, macrophages are induced to undergo a phenotype change toward what has been termed an M2, or alternatively activated state.<sup>27</sup> Exposure to hypoxia induces the build-up of the transcription factors HIF-1α and HIF-2α. These transcription factors bind to hypoxia response elements in a number of genes, increasing their transcription. Notably, in response to hypoxia, macrophages have been shown to induce such proangiogenic molecules as VEGF, fibroblast growth factor (FGF) 2, CXCL8, cyclooxygenase-2, hepatocyte growth factor (HGF), and matrix metalloproteinase- (MMP) 12.<sup>2,18</sup> There is now emerging data to show that tumor hypoxia also

establishes an immunosuppressive macrophage phenotype. The expression of such immunosuppressive factors as prostaglandin E2 (PGE2)<sup>28</sup> and interleukin (IL) 10<sup>29</sup> are increased in hypoxia, and their presence within the tumor microenvironment can downregulate the tumoricidal abilities of TAMs. In addition, PGE2 and IL-10 inhibit the functions of cytotoxic CD8+ T lymphocytes and other effector cells of the immune system,<sup>30</sup> which, combined with hypoxic inhibition of macrophage phagocytosis and antigen presentation, suppresses the triggering of an adaptive immune response toward tumor cells. Moreover, Doedens et al recently reported hypoxia- and HIF-1-dependent in vitro suppression of T-cell proliferation by macrophages.<sup>31</sup>

The hypoxia-induced skewing of macrophages toward an M2 state may not be entirely under the control of HIF-1α. Interestingly, Werno et al demonstrated that HIF-1α-deficient macrophages exposed to hypoxia were more M2-polarized, as assessed by their lower cytotoxicity toward tumor cells and the lack of a response to lipopolysaccharide (LPS) stimulation. In addition, the proangiogenic functions of these macrophages were decreased, compared to wild-type macrophages.<sup>32</sup> Taken together, these data show a clear role for hypoxia in

the promotion of tumors, by encouraging macrophages to support many aspects of tumor development.

## Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease characterized by a symmetrical, inflammatory arthropathy that results in destruction of bone and cartilage, leading to joint stiffness, significant pain, and loss of functional capacity.<sup>33</sup> Although the pathogenesis of RA is still not completely understood and treatment is non-curative, studies suggest that genetic and environmental factors can trigger joint inflammation.<sup>34-36</sup>

An early event in inflammatory arthritic joints is hypoxia-driven angiogenesis, where new blood vessels invade the synovial tissue, resulting in persistent infiltration of leukocytes into the synovial fluid and the dramatically increased proliferation of synovial fibroblasts.<sup>33</sup> Although these new blood vessels deliver oxygen to the inflammatory cell mass, the vascular network is dysfunctional, so the synovium remains a markedly hypoxic environment.<sup>37</sup> Hypoxia and angiogenesis are now recognized as seminal events in the perpetuation of joint destruction in RA.<sup>38-41</sup>

The presence of hypoxia in the human RA joint was initially confirmed using microelectrodes that measured oxygen tension indirectly in the synovial fluid.<sup>42,43</sup> More recent studies using a combined oxygen and temperature probe in patients with inflammatory arthritis showed that low synovial pO<sub>2</sub> levels (<20 mmHg) significantly correlated with joint inflammation,<sup>44</sup> lipid peroxidation, secretion of angiogenic factors, and the presence of T-cells and macrophages in the sublining layer of the synovium.<sup>45</sup> Animal models of RA also display pO<sub>2</sub> levels as low as 10 mmHg, and extensive hypoxia has been shown in the synovium, pannus, bone marrow, and articular cartilage chondrocytes in the arthritic joints of rats in *in vivo* models of the disease.<sup>38,46</sup> Repetitive cycles of hypoxia and reoxygenation, together with oxidants produced by macrophages and neutrophils, lead to the production of reactive oxygen species that result in significant tissue damage in RA joints.<sup>47,48</sup> Hypoxia also aggravates the synovial inflammatory lesion by increasing local production of such enzymes as COX-2 and MMPs by synoviocytes, monocytes/macrophages,<sup>49</sup> and fibroblast-like synoviocytes.<sup>50,51</sup> COX-2 catalyzes the conversion of arachidonic acid, which is cleaved from membrane phospholipids, to form proinflammatory eicosanoids such as PGE2 and prostacyclin I2 (PGI2). COX-2 expression is highly elevated in the RA synovium and has a direct relation to the degree of inflammation in synovial tissue.<sup>52</sup> PGE2 is involved in the erosion of cartilage and

juxta-articular bone,<sup>53</sup> stimulates the production of MMPs,<sup>54</sup> and contributes to angiogenesis,<sup>55</sup> all of which are important mediators of connective tissue degradation in the synovium.

A large proportion of macrophages have been shown to accumulate in the RA synovium, with most residing in the inflamed lining layer. In fact, Hollander et al reported that >90% of the cells in the RA synovial lining stained positive for the pan-macrophage marker CD68.<sup>56</sup> Immunohistochemical studies have revealed that both HIF-1 $\alpha$  and HIF-2 $\alpha$  are overexpressed in the synovial lining of patients with RA, compared to healthy joints.<sup>57</sup> Importantly, Hollander et al found that the CD68+ cells in RA joints also expressed HIF-1 $\alpha$  and were not detected in the synovium of normal or osteoarthritic (nonhypoxic) joints,<sup>56</sup> suggesting that hypoxic, HIF-1 $\alpha$ -activated macrophages are overrepresented in RA joints. In addition, Cramer et al showed that when arthritis was induced in the joints of mice bearing a targeted deletion of the HIF-1 $\alpha$  gene in myeloid cells (macrophages and neutrophils), the mice displayed a marked reduction in joint swelling and reduced synovial infiltration by macrophages, strongly implicating the hypoxic-induced responses of these cells in RA pathology.<sup>58</sup> A number of known hypoxia-regulated genes/proteins are also known to be expressed by macrophages in RA joints, including CXCL12,<sup>59</sup> CXCL8,<sup>60</sup> VEGF,<sup>61</sup> IL-1 $\beta$ , and tumor necrosis factor-(TNF)  $\alpha$ ,<sup>62</sup> which potentiate the inflammatory response and stimulate angiogenesis in RA. IL-1 $\beta$  and TNF- $\alpha$  are essential proinflammatory cytokines required for the progression of RA, because they are capable of inducing other proinflammatory cytokines and activating MMPs in autocrine and paracrine fashions.<sup>63</sup> Inhibitors of IL-1 and TNF- $\alpha$  cause a reduction in synovial inflammation, bone destruction, and macrophage infiltration in patients with RA.<sup>64,65</sup> A critical role of TNF- $\alpha$  and IL-1 during RA pathogenesis was confirmed by the recent development of new drugs targeting these molecules, that has revolutionized patient treatment.<sup>66</sup>

These data provide clear evidence that activation of HIF-1 $\alpha$  in macrophages and the subsequent expression of HIF-1 $\alpha$  target genes in these cells is an important contributor to the pathogenesis of RA. Whether HIF-1 $\alpha$  alone is sufficient to drive RA has not been demonstrated. HIF-2 $\alpha$ , a protein closely related to HIF-1 $\alpha$ , is also expressed in the RA synovium.<sup>57</sup> However, to date, there is little information on the role of HIF-2 $\alpha$ -expressing macrophages in hypoxic joints of RA patients.

Precisely how hypoxia regulates the activity HIF-1 $\alpha$  and HIF-2 $\alpha$  transcription factors in the RA joint has yet to be determined. In view of the abundance of macrophages in the

synovia of RA joints, and their increased activity of HIFs and subsequent expression of various proinflammatory HIF-target genes, it would be interesting to see whether inflammation in RA joints would be dampened by suppressing the expression/function of macrophage HIF-1 $\alpha$  or HIF-2 $\alpha$ . This could be achieved by injecting the synovial fluid of RA joints with a vector that bears short hairpin RNA (shRNA) for HIFs. More recently, highly selective antagonists have been developed that suppress HIF-1 $\alpha$  and HIF-2 $\alpha$  in vitro and in vivo, reducing the expression of HIF-related gene targets in tumor cells<sup>67,68</sup> and inhibiting tumor angiogenesis,<sup>66,69</sup> so, these could also be applied to RA. Such chemical tools provide a potential therapeutic opportunity to intervene in HIF-driven RA.

## Atherosclerosis

Atherogenesis is most commonly thought to occur at sites of vessel wall injury, where it is heralded by endothelial cell dysfunction. This results in lipid accumulation and inflammatory cell infiltration, including monocyte recruitment into the arterial intima.<sup>70</sup> These monocytes differentiate into macrophages that ingest such lipoproteins as oxidized low-density lipoprotein (LDL) and native LDL, resulting in cells laden with cytoplasmic lipid vacuoles, that are often termed “foam” cells.<sup>71</sup> Accumulating “foam” cells form the fatty streak characteristic of early atherosclerosis and remain present in the mature atherosclerotic plaque.<sup>72</sup> As the plaque develops, the arterial wall increases in volume, resulting in reduced oxygen diffusion and subsequent hypoxia. Bjornheden et al demonstrated arterial hypoxia at distances of 200–300  $\mu$ m from the endothelial surface of atherosclerotic plaques in rabbits, and observed large numbers of “foam” cells within these regions, indicating these macrophages experience hypoxia in atherosclerosis.<sup>73</sup>

Hypoxia significantly contributes to lipid metabolism in human macrophages in vitro. Bostrom et al demonstrated that human macrophages were capable of increasing triglyceride content in response to hypoxia, while Parathath et al observed an increase in cholesterol content within macrophages.<sup>74,75</sup> In vitro, hypoxia upregulates macrophage expression of 15-lipoxygenase-2 leading to increased LDL oxidation.<sup>76</sup> 15-lipoxygenase-2 expression also lead to increased expression of CXCL10 and CCL2, resulting in the increased T-cell recruitment observed in atherosclerosis.<sup>77</sup> Very low-density lipoprotein (VLDL) receptors are upregulated on macrophages experiencing hypoxia, and are also known to be upregulated on plaque macrophages in vivo,<sup>78</sup> suggesting a correlation between hypoxia and plaque macrophage expression of VLDL receptors. The VLDL receptor mediates

the uptake of such lipids as  $\beta$ -VLDL, chylomicron remnant, and lipoprotein Lp( $\alpha$ ), suggesting hypoxia also affects lipid metabolism through VLDL receptor-derived pathways.<sup>78–80</sup>

T lymphocyte recruitment, along with endothelial cell and smooth muscle migration and proliferation, is vital for the progression of atherosclerosis. CXCL8 is chemotactic for T lymphocytes,<sup>81</sup> and both chemotactic and mitogenic for endothelial<sup>82</sup> and smooth muscle cells.<sup>83</sup> Human foam cells taken from atherosclerotic plaques release increased levels of CXCL8, compared to cultured monocytes and macrophages,<sup>84</sup> while CXCR2-deficient mice demonstrated reduced atherosclerosis compared to wild-type mice.<sup>85</sup> These findings suggest CXCL8 aids atherosclerotic plaque development. Hypoxia itself has been shown to upregulate macrophage CXCL8 gene expression and foam cell production of CXCL8,<sup>86,87</sup> suggesting hypoxia prompts increased CXCL8 secretion from foam cells, leading to atherosclerotic development via interactions with T lymphocytes, endothelial cells, and smooth muscle.

Foam cells secrete a wide variety of other proteins in response to hypoxia. Foam cells from human atherosclerotic lesions have shown oxygen-regulated protein (ORP) 150 expression in response to hypoxia and the presence of oxidized LDL.<sup>88</sup> ORP150 expression by macrophages has been linked to subsequent increased VEGF expression and angiogenesis,<sup>67</sup> and it has been suggested that ORP150 plays an important role in preventing hypoxia-induced apoptotic cell death.<sup>90</sup> This, combined with its expression in areas of high oxidized LDL concentration, suggests that this factor has a protective role against environmental stressors in the atherosclerotic milieu. Macrophage inhibitory factor (MIF) is a protein that is highly expressed by human foam cells, and its expression is strongly correlated to neovascularization and other signs of atherosclerotic plaque instability.<sup>91</sup> Burger-Kentischer et al found treatment with a MIF antibody in an atherosclerotic-prone murine model resulted in reduction of a number of inflammatory markers typically associated with atherosclerosis, suggesting that MIF had a mediating effect on intimal inflammation.<sup>92</sup> In addition, MIF blockade in murine models has resulted in plaque regression and reduced levels of T lymphocytes and macrophages.<sup>93</sup> In addition, VEGF has been shown to be expressed in significantly greater amounts in hypoxic foam cells than nonhypoxic foam cells in vivo.<sup>75</sup> Other factors known to be induced by hypoxia are also expressed by foam cells, such as platelet-derived growth factor (PDGF),<sup>94</sup> TNF- $\alpha$ , IL-1,<sup>95</sup> CCL2,<sup>96</sup> MMP-1,<sup>97</sup> MMP-7,<sup>98</sup> though it is not yet directly known if foam cells vary their expression of these factor in a hypoxia-dependent manner.

One of the complications of atherosclerosis is myocardial infarction (MI), which results from plaque rupture, creating microemboli that can occlude blood vessels.<sup>99</sup> If this occurs in coronary arteries, it can cause chronic hypoxia in the myocardium. Regions of myocardial hypoxia have been demonstrated in canine models of MI, and macrophages have been found in significantly greater quantities in human myocardium from ischemic heart disease patients than in controls.<sup>100,101</sup> Macrophages recruited to the damaged tissue may be beneficial in heart recovery. Researchers have demonstrated that, after MI, these recruited macrophages express high levels of PR-39 (originally identified as an antibacterial peptide).<sup>102</sup> This research group went on to demonstrate that PR-39 had a proangiogenic effect on mice myocardium via inhibition of HIF-1 $\alpha$  degradation, resulting in increased HIF-1 $\alpha$  levels and, consequently, growth factor expression.<sup>103</sup> Evidence that elevated levels of PR-39 may be beneficial in MI has been shown with the use of recombinant adeno-associated virus, engineered to overexpress this gene. Use of this virus in a porcine model of MI resulted in decreased infarct areas in MI, creating the tantalizing possibility of utilizing PR-39 as a potential therapeutic agent in the future.<sup>104</sup>

## Wound healing

Ischemia is a common event in wounds that often leads to local tissue hypoxia. Wounds compromising the epidermis and entering the underlying connective tissue disrupt the local vasculature, leading to localized bleeding, blood vessel vasoconstriction, and clot formation. Hypoxia within the wound can then develop, due to both a reduction in local perfusion and increased oxygen demand of the cells within the healing tissue.<sup>105</sup> Oxygen tension measurements in rodent models decreased from 150 mmHg to 20–30 mmHg immediately after injury and, in some instances, these oxygen tensions decreased further to 5–7 mmHg 3–5 days post-injury.<sup>106</sup> This acute hypoxia is thought to be essential for tissue repair, by activating platelet aggregation and promoting release of such growth factors and cytokines as VEGF and TNF- $\alpha$ .<sup>107–109</sup> In addition to the typical laceration wound, hypoxia is also observed in burn wounds. Xing et al studied the expression of HIF-1 $\alpha$  along with the hypoxic marker pimonidazole, in order to examine the spatial and temporal course of hypoxia in a murine burn model. They found that the leading edge of the healing burn was hypoxic and that this level of hypoxia correlated positively with increased expression of HIF-1 $\alpha$ , as well as the proangiogenic factors VEGF and CXCL12.<sup>108</sup>

Inflammatory cell infiltration of wounds has long been accepted. Neutrophils are the first cells to be recruited to

wound sites, and these are followed by infiltration of monocytes that quickly differentiate into macrophages. Later, lymphocytes enter the wounds and, eventually, inflammatory resolution and matrix remodeling occurs.<sup>110</sup> The importance of macrophages in wound healing was clearly demonstrated by macrophage depletion studies. Wounded transgenic mice devoid of macrophages showed an increased expression of TNF- $\alpha$  but decreased levels of VEGF and tumor growth factor (TGF)- $\beta$  at the injured site. Interestingly, the altered expressions of these molecules were associated with a reduced rate of reepithelialization, impaired collagen deposition, angiogenesis, and decreased cell proliferation, suggesting that macrophages are important for wound closure and healing.<sup>111</sup> Furthermore, application of macrophages into experimentally induced cutaneous wounds was shown to accelerate wound repair.<sup>112</sup> Although a clear role for hypoxia and macrophages in wound healing is evident, the precise role of hypoxia-induced macrophages in this process is less understood. Macrophages at the site of tissue injury have been shown to upregulate and express many factors that are typically secreted by hypoxic macrophages, including VEGF,<sup>113</sup> PDGF,<sup>114</sup> MMP-1,<sup>115</sup> TNF- $\alpha$ , IL-1,<sup>116</sup> and FGF-2.<sup>117</sup> More specifically, the increased expression of these molecules in hypoxic macrophages is a direct result of HIF-1 $\alpha$  and HIF-2 $\alpha$  activation.<sup>2</sup> The importance of hypoxic macrophages in wounds has been suggested by Ozawa et al, who, using immunohistochemical staining of human wound granular tissue, found that CD68-positive macrophages coexpressed the proangiogenic factors VEGF and ORP150 at these sites. Overexpression of ORP150 by macrophages in a murine wound model increased the expression of VEGF by these cells, that accelerated neovascularization and wound healing.<sup>90</sup> Additional *in vitro* experiments using cultured macrophages under hypoxic and normoxic conditions have revealed a mechanism for the coexpression of these two factors. ORP150 is an endoplasmic reticulum chaperone that is encoded by a hypoxia-responsive gene. In hypoxic cultures, increased expression of ORP150 promoted secretion of VEGF by macrophages, while its inhibition caused retention of VEGF within the endoplasmic reticulum, thereby reducing the secretion of this angiogenic growth factor. This suggests that hypoxia-induced expression of ORP150 is important for the secretion of VEGF by hypoxic macrophages within healing wounds.<sup>90</sup> Similarly, in burn wounds, macrophages have been found in the deep, hypoxic regions of the injury, where they express VEGF.<sup>109</sup>

Taken together, these data suggest that macrophages experiencing hypoxia within wounds drive a proangiogenic

phenotype, with the expression of VEGF and other proangiogenic factors to revascularize the wound site. However, there are still a limited number of publications that have examined the presence of macrophages in hypoxic wound areas, and, more importantly, the effect that hypoxia-driven macrophage gene products have on wound healing remains speculative. Indeed, contradicting data regarding the role of hypoxic macrophages following tissue injury have been published. Owings et al showed that HIF-1 $\alpha$  expression in myeloid cells infiltrating wounds mediated a slower rate of wound repair. In their experiments, myeloid-specific HIF-1 $\alpha$  knockout mice showed a more rapid rate of wound closure when compared to wild-type controls, and this was not related to an alteration in angiogenesis, cell proliferation, or the number of recruited neutrophils or macrophages at the wound site.<sup>118</sup>

## Bacterial infection

Hypoxia is also a feature of tissues experiencing bacterial infection.<sup>119</sup> This hypoxic state is due to increased oxygen consumption by the proliferation of bacteria, the accumulation of phagocytes at the infected site, and/or the vasoconstriction of vessels in the area, which could impede delivery of oxygen to the site of inflammation. Therefore, macrophages are likely to experience hypoxia at sites of bacterial infection. However, there is currently little *in vivo* evidence that this directly alters/regulates macrophage antibacterial functions. Johnson's group used transgenic mice with a targeted HIF-1 $\alpha$  knockout in myeloid cells to demonstrate the importance of HIF-1 $\alpha$  in the antibacterial functions of macrophages, *in vitro* and *in vivo*, under both normoxic and hypoxic conditions.<sup>58</sup> It has been shown *in vitro* that macrophage behavior is altered by hypoxia in bacterial infections. Anand et al observed an increase in phagocytosis of *Escherichia coli* by murine macrophages in a p38- and HIF-1 $\alpha$ -dependent manner.<sup>120</sup> Curiously, a contradictory observation has also been made; Shirato et al found hypoxia resulted in HIF-1 $\alpha$  expression that was correlated with macrophage scavenger receptor 1 suppression and subsequent reduced phagocytic capacity on exposure to *Listeria monocytogenes*.<sup>121</sup> Hypoxia has also been observed changing the expression of factors by macrophages during bacterial infection. Nickel et al observed expression of human  $\beta$  defensin 2 and upregulation of the vitamin D-dependent antimicrobial pathway, exclusively in response to hypoxia in *Mycobacterium tuberculosis* infections. This, in turn, resulted in decreased intracellular growth of *M. tuberculosis*.<sup>122</sup>

It should be noted that HIF-1 $\alpha$  levels can be upregulated by rat and mouse macrophage cell lines in the

absence of hypoxia by LPS (which is present in bacterial cell walls).<sup>123,124</sup> Furthermore, mouse macrophages rapidly upregulate their expression of HIF-1 $\alpha$  when exposed to Gram-positive or Gram-negative bacteria.<sup>124</sup> Together, these findings indicate that HIF-1-mediated mechanisms play an important part in macrophage responses to bacterial infections and that these do not necessarily require activation by hypoxia.

## Conclusion

It is clear from the evidence cited above that macrophages accumulate in areas of severe hypoxia in various inflamed sites, where they respond to microenvironmental cues and contribute to hypoxia by consuming oxygen themselves. This raises the question of whether it might be possible to exploit our knowledge of how macrophages are recruited/trapped in these hypoxic/necrotic areas, to develop new treatment strategies devised at targeting these pathways. One such treatment may also be able to exploit the natural propensity of macrophages to home to hypoxic areas. We previously described how macrophages could be isolated from patients, treated *ex vivo* to carry hypoxically-activated therapeutic agents, and then reintroduced back into the body, systemically.<sup>15</sup> In addition, we recently demonstrated that the administration of macrophages "armed" with oncolytic virus 48 hours after treatment with the chemotherapeutic agent docetaxel, or following tumor irradiation, abolished the subsequent post-therapy regrowth of primary prostate tumors in mice.<sup>125</sup> Alternatively, new therapies involving the use of peptides or small molecule antagonists to inhibit macrophage receptors for specific chemoattractants produced by hypoxic areas of tumors may help to reduce macrophage accumulation in these sites, while leaving macrophage functions in healthy tissues unaffected. This would then remove their proangiogenic contribution under hypoxia and reduce the net proangiogenic activity of cells in such areas.

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## Disclosure

The authors report no conflicts of interest in this work.



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