



Molecular mechanisms regulating macrophage response to hypoxia

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Monocytes and Macrophages (Mo/M ϕ) exhibit great plasticity, as they can shift between different modes of activation and, driven by their immediate microenvironment, perform divergent functions. These include, among others, patrolling their surroundings and maintaining homeostasis (resident Mo/M ϕ), combating invading pathogens and tumor cells (classically activated or M1 Mo/M ϕ), orchestrating wound healing (alternatively activated or M2 Mo/M ϕ), and restoring homeostasis after an inflammatory response (resolution M ϕ). Hypoxia is an important factor in the M ϕ microenvironment, is prevalent in many physiological and pathological conditions, and is interdependent with the inflammatory response. Although Mo/M ϕ have been studied in hypoxia, the mechanisms by which hypoxia influences the different modes of their activation, and how it regulates the shift between them, remain unclear. Here we review the current knowledge about the molecular mechanisms that mediate this hypoxic regulation of M ϕ activation. Much is known about the hypoxic transcriptional regulatory network, which includes the master regulators hypoxia-induced factor-1 and NF- κ B, as well as other transcription factors (e.g., AP-1, Erg-1), but we also highlight the role of post-transcriptional and post-translational mechanisms. These mechanisms mediate hypoxic induction of M ϕ pro-angiogenic mediators, suppress M1 M ϕ by post-transcriptionally inhibiting pro-inflammatory mediators, and help shift the classically activated M ϕ into an activation state which approximate the alternatively activated or resolution M ϕ .

Keywords: low oxygen tension, inflammation, post-transcriptional regulation, post-translational regulation, M1 macrophages, M2 macrophages

INTRODUCTION

Many physiological and pathological processes (e.g., inflammation, wound healing, acute myocardial infarction, retinopathies, atherosclerosis, solid tumors, and more) are characterized by both low oxygen tensions (hypoxia) and presence of monocytes/macrophages (Mo/M ϕ). Mo/M ϕ are essential regulators of inflammation and central participants in hypoxia-driven processes, and the mediators they express and secrete recruit other cells and orchestrate their activity. The role of Mo/M ϕ and the effects hypoxia exerts on them were studied mostly in solid tumors, but the same molecular mechanisms apply in other clinical scenarios. We review here the different molecular mechanisms exerted by hypoxia, which regulate Mo/M ϕ functions. We elaborate on the hypoxia-induced transcriptional network in M ϕ , which is driven mostly by the hypoxia-induced factors (HIFs) and NF- κ B, the two master regulators of the hypoxic response. We also draw attention to additional post-transcriptional, translational, and post-translational mechanisms that enable hypoxia to activate or suppress gene and protein expression. Finally, we review the relatively few studies on the mechanisms and effects of hypoxia on differently activated Mo/M ϕ , and discuss how these mechanisms help shift the pro-inflammatory activated M ϕ toward an anti-inflammatory, pro-angiogenic phenotype.

We do not expand on how hypoxia-induced mechanisms affect the different M ϕ functions, as these aspects were extensively reviewed before (Lewis et al., 1999; Murdoch et al., 2004, 2008; Bosco et al., 2008; Martinez et al., 2008; Coffelt et al., 2009; Walsley et al., 2009; Qian and Pollard, 2010). Because the investigation of mechanisms is most often performed *in vitro*, we limit our discussion to these studies, and only briefly mention the increasing number of evidences pointing to the crucial role hypoxia plays *in vivo* in the pathophysiology of many diseases. Moreover, although hypoxia is physiologically and pathologically followed by re-oxygenation, we focus only on the isolated effects of hypoxic stress on Mo/M ϕ . Likewise, the effects of reduced pH, low glucose levels, or increased lactate, conditions that also accompany hypoxia and ischemia, are not discussed.

DIFFERENT MODES OF MACROPHAGE ACTIVATION

Monocytes (Mo) migrate into tissues and differentiate into Macrophages (M ϕ) to perform many functions needed in the tissue. These include resident M ϕ that patrol their surroundings and maintain homeostasis, M ϕ that combat invading pathogens and tumor cells and protect the tissue, M ϕ that orchestrate the process of wound healing, and M ϕ that resolve inflammation. To perform these multiple tasks, M ϕ phagocytose pathogens, secrete cytokines, chemokines, and growth factors, present antigenic determinants to

T cells and activate them, scavenge dead cells and necrotic debris and deposit matrix proteins. Since one cell cannot perform all these divergent tasks simultaneously, it was suggested that M ϕ can be differently activated, depending on the signals received from the microenvironment. Thus, M ϕ exhibit an enormous plasticity (Stout and Suttles, 2004), and dynamically shift from one form of activation to another according to the conditions in the changing microenvironment (Stout et al., 2009). This concept, as well as the evidences supporting it and the markers that characterize each M ϕ phenotype, has been extensively reviewed (Mosser, 2003; Martinez et al., 2008; Mosser and Edwards, 2008; Murdoch et al., 2008; Gordon and Martinez, 2010; Qian and Pollard, 2010), and will be only briefly mentioned here.

In the continuum of M ϕ phenotypes, two main sub-populations have been described. In one extreme, the classically activated M ϕ , or M1 M ϕ , is responsible for the multi-stage process of recognizing pathogens, phagocytosing, and degrading them to present their antigenic determinant to helper T cells in the context of the MHC class II molecules, whose expression is elevated (Mosser, 2003; Martinez et al., 2008). This process is accompanied by secretion of IL-12, which is necessary to support a Th1 response. M1 M ϕ are activated by ligands of different receptor families such as the toll-like receptor (TLR) ligands (e.g., lipopolysaccharides – LPS), and pro-inflammatory cytokines (e.g., interferon- γ – IFN γ , tumor necrosis factor- α – TNF α , and interleukin-1 β – IL-1 β). They secrete high amounts of pro-inflammatory mediators that kill the invading pathogens or tumor cells, such as the cytotoxic TNF α and nitric oxide (NO), the latter (and the enzyme producing it, inducible nitric oxide synthase – iNOS) serving as the hallmark of this M ϕ subset. They also secrete chemokines, which attract more neutrophils and Mo/M ϕ to the inflamed site, thus amplifying the pro-inflammatory response. Secretion of proteolytic enzymes, such as matrix metalloproteinases (MMPs) helps degrade components of the extracellular matrix (ECM) and allows migration of leukocytes to the inflamed tissue.

In the other extreme we find M ϕ that are activated by and secrete anti-inflammatory mediators (e.g., IL-10, IL-13, tumor growth factor beta – TGF β , and prostaglandin E $_2$ – PGE $_2$), which generate a microenvironment that suppresses the activity of M1 macrophage. They remove cellular debris and perform phagocytosis which is mediated by receptors different than those expressed by M1 M ϕ , such as the mannose receptor CD206; contribute to tissue remodeling by depositing ECM proteins; and express high levels of arginase-1, which produces ornithine, a precursor for the synthesis of the ECM protein collagen. Arginase-1 also competes with iNOS for their common substrate L-arginine and thus prevents NO production. Thus, these alternatively activated or M2 M ϕ are involved mainly in homeostasis and wound healing. M2 M ϕ also secrete proteolytic enzymes that help them move about, and as they are activated by IL-4 and IL-13, they are particularly suited to present helminthes antigens to helper T cells and initiate a Th2 response (Martinez et al., 2009), so that the term “anti-inflammatory” refers, in fact, to a different type of inflammation.

A third subset of M ϕ is responsible for immune regulation, and may include several subtypes. Some of these cells are activated by ligands of TLRs in combination with immune complexes, and some are activated by anti-inflammatory signals, such as

adenosine or phagocytosed apoptotic cells (Mosser, 2003; Mosser and Edwards, 2008). Immature M ϕ , which compose some of the myeloid-derived suppressor cells (MDSCs) population, also belong to regulatory M ϕ . MDSCs are triggered by a combination of IFN γ and IL-13, and secrete IFN γ , IL-13, IL-10, and TGF β , which help them suppress Th1 cell-mediated immune response, induce regulatory T cells and inhibit M1 M ϕ (Bronte, 2009; Gabrilovich and Nagaraj, 2009; Ostrand-Rosenberg and Sinha, 2009).

A different subset of M ϕ isolated at the resolution-phase of inflammation (resolution or rM ϕ) is often activated by mediators released from apoptotic cells, such as sphingosine-1-phosphate (S1P) or TGF β . These rM ϕ secrete predominantly the anti-inflammatory IL-10, very low levels of pro-inflammatory cytokines and chemokines and express arginase-1 and CD206, as well as the pro-inflammatory iNOS and COX-2 (Bystrom et al., 2008). Moreover, in a model of self-resolving peritonitis, rM ϕ were further divided into CD11b^{high} and CD11b^{low} cells (Schif-Zuck et al., 2011). CD11b^{high} rM ϕ engulf apoptotic neutrophils and stay at the inflammatory site, where they express markers of both M1 and M2 M ϕ (e.g., iNOS, arginase-1, COX-2, and MMP-9). However, once they engulf enough apoptotic cells (threshold determined at 7 cells), they convert into CD11b^{low} cells, induce the expression of the enzyme 12/15-lipoxygenase which produces pro-resolving lipid mediators, and emigrate to lymphoid organs to convey resolution signals to lymphocytes (Schif-Zuck et al., 2011).

Collectively, all these regulatory M ϕ , which secrete anti-inflammatory mediators designed to resolve inflammation, are positioned between M1 and M2 M ϕ , and share markers with these two sub-populations.

Malignant tumors are characterized by repeated cycles of hypoxia and reoxygenation, and several M ϕ subsets have been found located in different regions of the tumor (Lewis and Pollard, 2006). These include the tumor-associated macrophages (TAMs) and the Tie-2 expressing macrophages (TEMs), which support tumor growth and metastasis, and are both an important source of pro-angiogenic factors. Whereas some TEMs reside close to blood vessels (peri-endothelial; Venneri et al., 2007), TAMs infiltrate deeper into the tumor and are located in perinecrotic areas where oxygen tensions are very low. Regulatory MDSCs are present within tumors, as well as in lymphoid organs, such as the bone marrow and spleen, and expand proportionally to the tumor burden. All these subtypes share activation markers with both M2 and regulatory M ϕ , thus placing them between these two subsets (De Palma et al., 2007; Mosser and Edwards, 2008; Murdoch et al., 2008). Of note, the principle of M ϕ plasticity extends even to TAMs subset, as they exhibit divergent phenotypes (Lewis and Pollard, 2006), depending on the type of the tumor, the stage of its development, the interactions with both the ECM and other neighboring cells, and the level of oxygen tensions they are exposed to.

Moving from M ϕ to Mo, we find the monocytes to have similar but not identical classifications. Circulatory Mo are divided into three subsets according to their CD14 and CD16 expression (in humans) or Ly6C expression (in mice). The Classical or M1 Mo express high levels of CD14 and no CD16 (denoted either CD14⁺⁺CD16⁻ or CD14⁺CD16⁻ in human, and Ly6C^{high} in

mice), intermediate Mo, in humans only, express intermediate levels of CD14 and CD16 (CD14⁺⁺CD16⁺ or CD14⁺CD16⁺), and resident, M2 or non-classical monocytes express very low levels of CD14 and high levels of CD16 (CD14^{dim}CD16⁺ or CD14⁻CD16⁺ in human, and Ly6C^{low} in mice) (Ziegler-Heitbrock, 2007; van de Veerdonk and Netea, 2010). However, the function of these subsets as cells secreting either pro- or anti-inflammatory cytokines is still controversial. It is assumed that M1 and M2 Mo are the counterparts of M1 and M2 M ϕ , but this is not always accurate. For example, sorted Mo subsets that were stimulated *ex vivo* with LPS increased IL-10 secretion from CD14⁺CD16⁺ cells, whereas LPS-stimulated CD14^{dim}CD16⁺ cells showed increased TNF α with little IL-10 secretion (Skrzeczynska-Moncznik et al., 2008). In a different study, CD14⁻CD16⁺ cells showed patrolling characteristics with weak ability for phagocytosis and low production of ROS and cytokines when challenged by bacterial ligands of TLRs, but secreted high amounts of the pro-inflammatory TNF α and IL-1 β cytokines upon stimulation with viral ligands or nucleic acids (Cros et al., 2010). These results suggest that non-classical or M2 Mo can behave as M1 Mo under certain circumstances. Thus, Mo classification does not fully match the M1-M2 M ϕ taxonomy.

During inflammation differently activated Mo/M ϕ enter the tissue, first in order to eliminate pathogens or tumor cells (M1 Mo/M ϕ), and later to heal the damaged tissue and to restore homeostasis (M2 Mo/M ϕ and rM ϕ ; Nahrendorf et al., 2010). This requires precise signals that regulate this sequential migration. For example, the chemokines CCL2 and CX₃CL1 and their receptors, expressed on CD14⁺CD16⁻ and both CD16⁺ monocytes, respectively, were shown to mediate these responses (Geissmann et al., 2003; Auffray et al., 2009). Of note, this specific sequence of events may not always apply, and clinical scenarios which favor a Th2 response may differentially recruit non-classical Mo. Furthermore, whether classically activated Mo mature to M1 M ϕ in the tissue and are later skewed to become M2 M ϕ , or whether they die on site and are replaced by newly recruited M2 Mo, remains unclear, and the effects of the hypoxic microenvironment in this context must be further explored.

According to the danger model (Matzinger, 2002), the direction and magnitude of the inflammatory response depend not only on the nature of the stimulus (e.g., types of bacteria, site of entry, size of the bacterial inoculation), but also on the type and extent of expression of co-stimulatory molecules (e.g., CD80 vs. CD86) and specific signals received from the microenvironment, such as danger-associated molecular patterns (DAMPs). Necrotic and apoptotic cells that release their content into the microenvironment, are picked up by Mo/M ϕ , processed and presented to T cells in the context of MHC class II and co-stimulatory molecules. Thus, microenvironmental stimuli can affect Mo/M ϕ functions, directly or via their effect on other cells.

These examples reveal the high plasticity of Mo/M ϕ , and emphasize that their heterogeneity ranges beyond the M1–M2 polarization models, and extends to new phenotypes we may yet discover. Thus, Mo/M ϕ classification and the distinction between their different activation modes and differentiation status is an active area of study.

INFLAMMATION AND HYPOXIA

Low oxygen tensions (hypoxia) are relevant in many physiological and pathological conditions. For example, hypoxia may arise due to occlusion of a blood vessel (e.g., during myocardial infarction or pulmonary embolism), significant blood loss or dilated blood vessels which instigate reduced blood flow and lack of oxygen (e.g., in burns, trauma, and sepsis), or simply when oxygen demands are not met (e.g., physical exercise, high altitudes, tumors). Hypoxia has been demonstrated *in vivo* in a variety of acute and chronic inflammatory sites, including the synovium in RA patients, the arterial intima in atherosclerotic lesions, myocardial infarcts, wounds, and sites of bacterial infection (reviewed in Murdoch et al., 2005). Since even healthy tissues exhibit a wide range of oxygen tensions, no one value can represent hypoxia, and hypoxia is, therefore, functionally defined as the inability of oxygen delivery to meet oxygen demands of the tissue (Papandreou et al., 2005). According to this definition, hypoxia occurs during inflammation, as more cells that require oxygen infiltrate the site and increase oxygen consumption, but only few capillaries can supply it. Thus, different oxygen tensions can be measured in different areas of tumors, wounds, or in tissues affected by chronic inflammation, generating a gradient of hypoxia, where levels as low as 5–10 mmHg were measured (Crowther et al., 2001). Of note, other microenvironmental factors, such as increased lactate concentrations and reduced pH, are linked to hypoxia, as they result from the shift to anaerobic metabolism. Therefore, investigating the role of prolonged hypoxia actually looks into the combined effect of all of these stress factors. Most *in vitro* studies measure the percent oxygen in the atmosphere flowing into the hypoxic chamber, but usually even in anoxic atmosphere (0% O₂) some oxygen remains dissolved in the medium (20–30 mmHg).

Depending on its duration and severity, hypoxia can drive inflammation and aggravate cellular and tissue injury. For example, humans exposed to the scarce oxygen available in high altitudes or mice exposed to ambient hypoxia experience reduced arterial partial oxygen tensions, and develop pulmonary edema and increased release of pro-inflammatory cytokines (Hartmann et al., 2000; Grocott et al., 2009; Rosenberger et al., 2009). Hypoxia shifts cells toward anaerobic metabolism, leading to exclusive use of glycolysis as the means for ATP production, instead of Krebs cycle. As a result, lactate accumulates in the cells, causing cellular acidosis, production of ROS is increased, and lipids are peroxidated leading to membranal damage. This, combined with the lack of ATP, impairs the function of ion channels, increases Ca⁺² influx, and membrane permeability, leading to spillage of cellular content (including proteolytic enzymes, hydrolases, and increased lactate), thereby increasing acidosis and tissue damage (Minko et al., 2005; Behn et al., 2007). Necrotic cells and increased tissue acidosis recruit more leukocytes into the area, and trigger inflammation. Hence, hypoxia and inflammation are interdependent, as chronic inflammation is accompanied by hypoxia and prolonged hypoxia leads to inflammation (Eltzschig, 2011).

ROLE OF HIFs

Like other cell types, the macrophage response depends on the severity and the duration of the hypoxic insult. In recent years, the search for the way cells sense differences in oxygen tensions was

focused primarily on the prolyl hydroxylases (PHD)–HIF pathway outlined below.

Hypoxia-induced factors are heterodimeric transcription factors consisting of a constitutively expressed β subunit (aryl hydrocarbon nuclear translocator – ARNT), and an α subunit, which is constitutively transcribed but immediately degraded in normoxia. Three α subunits (HIF-1 α , HIF-2 α , and HIF-3 α) have been identified, which together with HIF-1 β compose three isoforms that are differently expressed in various tissues. HIF-3 α (also called inhibitory PAS domain protein – IPAS) is a transcriptional inhibitor and functions as a negative regulator of HIF-1 α and HIF-2 α , although its biology is still unclear (Heikkilä et al., 2011), whereas HIF-1 α and HIF-2 α were more extensively studied. HIF-1 α and HIF-2 α are homologous, especially in their DNA binding domains, allowing their binding to the same hypoxia response element (HRE).

The binding of HIF-1 and HIF-2 to many HRE-containing promoters can induce the expression of a myriad of genes, some with opposing effects. By means of over-expressing the HIF- α subunits, or by ablating them in knockouts or by siRNA knock-down, the role of each subunit in determining M ϕ phenotypes and response to hypoxic stress was established. Thus, HIF proteins were shown to regulate all aspects of M ϕ functions in response to hypoxia, including the shift to anaerobic glycolysis, mitochondrial impairment, angiogenesis, invasion, and immune suppression (Coffelt et al., 2009; Walmsley et al., 2009; Werno et al., 2010a). Moreover, HIFs are also induced in normoxia, and play a central role in immune recognition, phagocytosis, bacterial killing, and pro-inflammatory cytokine production (Cramer et al., 2003; Peyssonnaud et al., 2007).

HIFs EXPRESSION IN DIFFERENT M ϕ SUBSETS

The two HIF isoforms, HIF-1 α and HIF-2 α are differently expressed in Mo and M ϕ , as hypoxia could not induce the expression of HIF-1 α or HIF-2 α in human Mo, but did induce them in human monocyte-derived macrophages (hMDM; Elbarghati et al., 2008). Differentiated human and mouse MDM show different kinetics of HIFs expression in response to hypoxia, with higher stability for HIF-2 α expression (Elbarghati et al., 2008; Takeda et al., 2010). This suggests that HIF- α subunits could, at least partially, affect M ϕ activation modes. Furthermore, arginine metabolism was enhanced toward NO production in a HIF-1 α -dependent manner upon LPS or IFN γ stimulation, but NO production was reduced by IL-4 due to increase in HIF-2 α -induced arginase-1 activity (Takeda et al., 2010). Conflicting evidences show higher accumulation of HIF-1 α in hypoxic hMDM (Burke et al., 2002), or higher expression of HIF-2 α under similar conditions (Griffiths et al., 2000). One study showed almost undetectable HIF-2 α mRNA after prolonged exposure to hypoxia, but induced HIF-1 α mRNA that was even further elevated by LPS in thioglycollate (TG)-elicited peritoneal M ϕ (Acosta-Iborra et al., 2009), whereas another study showed constitutive HIF-2 α mRNA levels in these cells (Takeda et al., 2010). TAMs present in human breast carcinomas show high expression of HIF-2 α , which correlates with increased micro-vessel density and high tumor grade (Patel and Simon, 2008). TAMs in different tumors *in vivo* show high expression of both HIF-1 α and HIF-2 α (Lewis and Pollard, 2006). The

importance of exposure time is demonstrated in a study that compared acute (24 h) and chronic (5 days) exposures to hypoxia, and reported that hMDM subjected to chronic hypoxia increased HIF-1 α and HIF-2 α proteins relative to hMDM that were subjected to normoxia or to acute hypoxia (Staples et al., 2010). However, there is still no clear perspective regarding the role of the different HIFs in hypoxic M ϕ , and different periods of time of hypoxic exposure or different oxygen levels may have different effects on HIFs' expression.

REGULATION OF HIFs IN NORMOXIA AND HYPOXIA

Protein stability

Hypoxia-induced factor proteins are regulated primarily by protein stability. HIF- α subunits are constitutively transcribed and translated, but immediately directed for degradation in normoxia. This is achieved by the hydroxylation of proline residues (402 and 564 on HIF-1 α and 405 and 532 on HIF-2 α) by the three PHDs, which depend on oxygen, 2-oxoglutarate, Fe⁺², and ascorbate as substrates and cofactors for their activity. Hydroxylation recruits the von Hippel Lindau (VHL), which forms a complex together with elongin B, elongin C, cullin-2, and ring-box 1 that has an E3 ubiquitin ligase activity. This complex ubiquitinates HIF- α subunits and targets them for proteosomal degradation (Nizet and Johnson, 2009; Walmsley et al., 2009). Hypoxia inactivates PHDs due to the limited oxygen substrate, and therefore stabilizes the HIF- α subunits, allowing their heterodimerization with the HIF-1 β subunit. Hypoxia also impairs electron transport in the mitochondria, leading to increased production of ROS, which oxidizes Fe⁺² to Fe⁺³ and further inhibit PHDs. Additional proteins contribute to this regulation, and details on their activity are reviewed elsewhere (Yee Koh et al., 2008).

Although HIF- α subunits are typically induced in hypoxia, they can also be expressed in normoxia upon inflammatory stimulation. For example, high levels of succinate (the product of 2-oxoglutarate) or increased ROS production that oxidizes Fe⁺² may cause PHDs inactivation and HIF-1 α stabilization (Denko, 2008). Likewise, high concentrations of NO in normoxia can stabilize HIF-1 α by inhibiting the activity PHDs, probably by targeting their Fe⁺² catalytic site (Weigert and Brune, 2008). Ligation of TLRs, such as the binding of LPS to TLR4, stimulate HIF-1 α expression in normoxia through the activity of NF- κ B, and hypoxia in the presence of LPS shows a synergistic effect (Frede et al., 2006). Additional pathways can contribute to HIF-1 α expression, including the Phosphatidylinositol 3-kinase (PI3K), Mitogen-activated protein kinases (MAPK) and the Ca²⁺/calmodulin-dependent protein kinases (CaMKII) pathways, and their inhibitors reduce HIF-1 α accumulation as well as the expression of its targets (Westra et al., 2010). Thus, in addition to hypoxia, other stimuli, including bacterial infection, hormones, growth factors, cytokines, or RNS can stabilize and activate HIF-1 α in normoxia.

Translation and transcription of HIFs

The main known level of HIFs regulation is post-translational, as described above. However other possible regulatory checkpoints, such as transcription and translation, are under-investigated. Although HIF- α subunits are constitutively transcribed in many cell types, their mRNA levels are increased in hypoxia. HIF-1 α

promoter consists of Sp1 sites, several HRE sites (suggesting a positive feedback loop), as well as NF- κ B, Egr-1, and Stat3 binding sites that mediate increased transcription in hypoxia (Niu et al., 2008; Galban and Gorospe, 2009). Evidences show differences between Mo and M ϕ , as hypoxia inhibits HIF-1 α transcription in human Mo (Bosco et al., 2006), but increased HIF-1 α mRNA accumulation in hMDM by mechanisms other than increasing mRNA stability (Staples et al., 2010). Likewise, HIF-1 α mRNA was increased during differentiation of THP-1 cells or peripheral blood Mo into M ϕ (Oda et al., 2006). This suggests a negative feedback designed to control the systemic hypoxic response.

Translational regulation of HIFs is an important checkpoint in both normoxia and hypoxia, which was investigated mainly in cancer cells. Increased HIF-1 α protein levels in hypoxia can be observed without a parallel increase in the mRNA levels. This is mediated by the binding of the two RNA-binding proteins (RBP) polypyrimidine tract-binding protein (PTB) and human antigen R (HuR) to the 3'-UTR and 5'-UTR, respectively, as their knock-down through RNA interference inhibited HIF-1 α accumulation (Galban et al., 2008). Iron response proteins (IRP) bind to their elements located in the 5'-UTR region of the HIF-2 α mRNA and inhibit translation of the mRNA in normoxia, while hypoxia releases this binding and enhances HIF-2 α translation (Galban and Gorospe, 2009). Furthermore, specific microRNAs (the miR-17-92 cluster and miR-199a) target HIF-1 α mRNA in normoxia and inhibit its translation, whereas hypoxia reduces their levels and release HIF-1 α mRNA translation (Galban and Gorospe, 2009). The mechanisms for translational controls have not yet been described in Mo/M ϕ , and this warrants more investigation.

Regulation of HIFs' activity

Hypoxia-induced factors activity as transcription factors that bind to HREs and to the co-activators CBP/p300 is regulated by oxygen-dependent protein-protein interactions. Factor-inhibiting HIF-1 (FIH-1), another oxygen-dependent hydroxylase, can hydroxylate asparagine residues on HIF- α subunits (803 on HIF-1 α and 847 on HIF-2 α), resulting in proteins that cannot bind to the co-activators CBP/p300 (Walmsley et al., 2008; Nizet and Johnson, 2009). Additionally, VHL also acts as an adapter that facilitates the binding of FIH to HIF- α subunits in normoxia, while hypoxia disrupts this interaction (Li et al., 2011), allowing for the accumulation of HIFs and their binding to HRE-containing promoters. FIH-1 also plays a role in normoxia, as the cytoplasmic tail of MT1-MMP/MMP-14 provides a platform to which both FIH-1 and its inhibitor mint3/APBA3 bind. This limits the complex localization to the Golgi apparatus and allows mint3 to inhibit FIH-1 ability to hydroxylate HIF-1 α , resulting in enhanced accumulation of HIF-1 α in normoxia (Sakamoto and Seiki, 2010). It is yet unclear whether a similar mechanism operates on PHDs localization to render them inactive and inhibit HIFs degradation in normoxic M ϕ .

Post-translational modifications regulate stabilization, degradation, and activity of HIF- α subunits by affecting their protein-protein interactions, especially other transcription factors or components of the transcriptional machinery. These were investigated mostly in cancer cell lines and fibroblasts. For example, HIF- α is directly phosphorylated by the ERK1/2 MAPK, leading to its

transport to the nucleus and increased activity (Mylonis et al., 2006). Two components of the PI3K/Akt pathway were shown to oppositely regulate HIF-1 α . The mTOR kinase phosphorylates HIF-1 α in its oxygen-dependent degradation domain, leading to its increased stabilization and enhanced transactivation (Hudson et al., 2002), whereas GSK-3-mediated phosphorylation induces HIF-1 α destabilization and proteosomal degradation (Flugel et al., 2007). Recently, PHD3 was found to hydroxylate pyruvate kinase M2, leading to its enhanced interaction with both HIF-1 α and p300 in hypoxia and promoting their transactivation of HRE-containing target genes (Luo et al., 2011).

Effects of HIF-1 α S-nitrosylation are controversial, as it could increase or decrease its ability to interact with its co-activator p300 (Sumbayev et al., 2003; Cho et al., 2007; Li et al., 2007). However, this mechanism could be relevant only in normoxic induction of HIF-1 α , as iNOS is inactivated in hypoxia (see Intracellular Trafficking and Protein-Protein Interactions). Hypoxia was shown to induce SUMOylation of HIF-1 α , either promoting its binding to VHL and degradation (Cheng et al., 2007) or promoting its stabilization and transcriptional activity (Carbia-Nagashima et al., 2007). These apparent contradictions may reflect use of different cell types or different hypoxic regimes, and the effects of such post-translational modifications in hypoxic M ϕ should be studied.

ROLE OF NF- κ B

NF- κ B are a family of proteins consisting of NF- κ B1 (p105/p50), NF- κ B2 (p100/p52), RelA (p65), RelB, and c-Rel that can heterodimerize. The role of the NF- κ B family members as master regulators of many pro-inflammatory associated gene is widely accepted (Vallabhapurapu and Karin, 2009).

NF- κ B in different M ϕ subsets

Canonical NF- κ B activation is indispensable for M1 activation, although cooperation with other transcription factors such as Stat1 or the MAPK/AP-1 pathway is essential (Biswas and Lewis, 2010; Lawrence and Fong, 2010). NF- κ B also regulates anti-inflammatory and pro-angiogenic gene expression (e.g., IL-10, VEGF, COX-2, TGF β , and MMPs) and inhibits M1 M ϕ (Lawrence and Fong, 2010). In part, this is associated with over-expression of p50 and formation of p50:p50 homodimers, which inhibit transcription of pro-inflammatory cytokines while enhancing transcription of anti-inflammatory genes (Saccani et al., 2006; Biswas and Lewis, 2010; Mancino and Lawrence, 2010). Furthermore, deletion of IKK β in myeloid cells shifts M ϕ toward M1 activation, increasing the expression of IL-12, MHC class II and iNOS, and revealing the inhibitory role of IKK β on Stat1 (Fong et al., 2008).

Tumor-associated macrophages derived from human and murine tumors and cultured in normoxia demonstrate defective NF- κ B activation, high IL-10 expression and reduced IL-12 and TNF α production (Sica et al., 2000). However, as distinct regions can be identified within solid tumors, TAMs could be activated both as M1 and M2 M ϕ and fluctuate between them, depending on the type of the tumor, its stage, M ϕ localization within the tumor, and the signal received from the microenvironment, including hypoxia (Mancino and Lawrence, 2010). Therefore, the role of NF- κ B in specific M ϕ subsets is very complex.

Regulation of NF- κ B in normoxia and hypoxia – links to the HIF pathway

Activation of NF- κ B occurs in normoxia when TLRs or receptors for pro-inflammatory cytokines recruit the MyD88 adapter protein, which activates a complex signaling cascade that is based on protein–protein interactions and culminates in the phosphorylation, ubiquitination, and degradation of I κ B proteins and translocation of NF- κ B to the nucleus.

Hypoxia has long been shown to simulate NF- κ B signaling. *In vivo*, hypoxia activates NF- κ B expression in a tissue-specific manner, as transgenic mice harboring the luciferase gene under the control of NF- κ B expressed it in the hearts and lungs (Fitzpatrick et al., 2011). *In vitro* there are many examples for hypoxia-induced expression of pro-inflammatory genes. Hypoxic RAW 264.7 cells induced MIP-2 expression, and inhibition of the ERK1/2 or PI3K pathways decreased p65 activity (Zampetaki et al., 2004). Increased production of ROS due to hypoxia was demonstrated in Mo/M ϕ to be responsible for NF- κ B activation and for the synergetic effects of hypoxia and LPS (Chandel et al., 2000; Kim et al., 2010). Recently, PDHs were demonstrated as regulators of NF- κ B signaling, as their inhibition suppressed LPS-induced expression of TNF α in M ϕ (Takeda et al., 2009). IKK β was discovered as an additional substrate that PDHs can hydroxylate, leading to its activation and to I κ B phosphorylation (Cummins et al., 2006). Additionally, ankyrin repeats on both I κ B α and the p105 subunit of NF- κ B can be hydroxylated by FIH-1 (Cockman et al., 2006; Cummins et al., 2006; Frede et al., 2006), explaining the effect of ROS on NF- κ B activation. In fact, PDHs and FIH-1 involvement in NF- κ B regulation suggests that both HIF and NF- κ B proteins are redox-sensitive proteins regulated by the same oxygen sensors, in addition to their role as master regulators of inflammation and hypoxia.

Many hypoxia-induced genes are targeted by both NF- κ B and HIF proteins. Indeed, a link between the HIF and NF- κ B pathways is also provided by the presence of an NF- κ B binding site in the HIF-1 α promoter (but not HIF-2 α), and the ability of p50:p65 heterodimers to bind to it and drive its expression in hypoxia (Belaiba et al., 2007), while siRNA for p65 inhibits this expression (Fitzpatrick et al., 2011). For example, COX-2 was induced by hypoxia in a manner dependent on both HIF-1 and NF- κ B (Fitzpatrick et al., 2011), suggesting that the pro-inflammatory response to hypoxia is regulated by NF- κ B both by direct binding to promoters of relevant genes and by inducing HIF-1 α expression (Fitzpatrick et al., 2011).

Mice lacking IKK β show impaired accumulation of HIF-1 α both in hypoxia and upon bacterial infection (Rius et al., 2008), demonstrating that NF- κ B transcriptionally regulates HIF-1 α in both hypoxia and normoxia. In addition, a physical interaction between IKK γ /NEMO and HIF-2 α , but not HIF-1 α , was described to enhance HIF-2 α transcriptional activity in reporter assays (Patel and Simon, 2008), but the specific gene targets remain unknown. This link may be bidirectional, as prolonged hypoxia of hMDM increased the mRNA expression and phosphorylation of the several components in the NF- κ B pathway (e.g., IKK β , IKK γ , I κ B α , RelA/p65) in a manner that was dependent on both HIF-1 α and HIF-2 α , as demonstrated by knocking down their expression using siRNA (Fang et al., 2009).

INTERACTIONS WITH OTHER TRANSCRIPTION FACTORS

Hypoxia-induced factor and NF- κ B can cooperate not only with each other, but also with additional transcription factors in normoxia and hypoxia, to achieve better target specificity or maximal activity while forming a large protein complex at the promoters of target genes. Activation of many transcription factors is managed by the crosstalk between signaling pathways. For example, ligation of TLRs or pro-inflammatory cytokines can recruit the adapter protein MyD88 that activates both the IKK–NF- κ B and the MAPK pathways, leading to activation of NF- κ B and AP-1. The TRIF adapter protein may activate IRF3 and initiate delayed expression of IFN β , which activates Stat1 in an autocrine manner. Cooperation between NF- κ B, AP-1, Stat1, HIF-1, and HIF-2 is required for M1 M ϕ activation (Biswas and Lewis, 2010; Lawrence and Fong, 2010). In contrast, anti-inflammatory cytokines (e.g., IL-4, IL-10, IL-13) activate Stat3 and Stat6, which are needed to induce anti-inflammatory gene expression (e.g., arginase-1, suppressor of cytokine signaling 3 – SOCS-3), and inhibit Stat1 and NF- κ B.

The integration of all the different microenvironmental signals determines the balance between M1 and M2 activation modes, suggesting a complex interplay between the transcription factors. Thus, different combinations of transcription factors that cooperate on specific target promoters may determine the activation phenotype of the M ϕ (Biswas and Lewis, 2010; Lawrence and Fong, 2010). Signal transduction is transient, and different pathways can be activated with different kinetics, and may influence the complexity of the transcriptional network. This aspect warrants further study in differently activated M ϕ . **Table 1** details some of the interactions between HIFs/NF- κ B and several of the central transcription factors that are induced in hypoxia.

TRANSCRIPTIONAL INHIBITION

Generally, HIF and NF- κ B are considered activators of gene transcription, although the NF- κ B p50:p50 homodimers can be negative regulators, as mentioned above. Hypoxia can also selectively inhibit gene transcription by other means. For example, in human Mo hypoxia inhibited the constitutive expression of MCP-1/CCL2 mRNA and its induction by IFN γ and LPS by a dual mechanism that included inhibition of transcription and enhancement of mRNA destabilization (Bosco et al., 2004). As MCP-1/CCL2 is a central chemoattractant for Mo, its inhibition may represent a negative regulatory mechanism that controls recruitment of M1-activated Mo/M ϕ into inflamed tissues. In support of this finding, hypoxia can also inhibit gene expression of CCR5, cathepsin C, 2,5-oligoadenylate synthetase, and the Ras family member Rab7 (Bosco et al., 2006). TIMP-2, the endogenous inhibitor of MMPs, is constitutively expressed in Mo, and hypoxia down-regulates it through the involvement of Sp1 phosphorylation (Lahat et al., 2011).

Surprisingly, in several cases HIF-1 plays a role as a suppressor. In fibroblasts and epithelial cells HIF-1 α was implicated in the down-regulation of anti-angiogenic targets such as thrombospondin-1 (Laderoute et al., 2000) and the reversion-inducing cysteine-rich protein with Kazal motifs (RECK) together with histone deacetylase 1 (HDAC1; Lee et al., 2010). In M ϕ , the scavenger receptor MRS1 was down-regulated in hypoxia.

Table 1 | Transcription factors other than HIFs and NF- κ B, which are involved in hypoxic regulation of gene expression.

TF	General, function	Induction/activation	Hypoxia stimulates	Cooperation with
AP-1	Heterodimers of the c-Jun, c-Fos, and ATF families	TLR ligation via MAPK activation	Increased Fos-2, Fra-2. Activated by JNK	HIF-1, NF- κ B
Egr-1	Zinc-finger factor, regulates Mo differentiation and mitogenic responses	Growth factors	Ligation of RAGE receptor, involving PKC β II and JNK activation	HIF-1 and C/EBP α . Also binds to HIF-1 α promoter
Stat1	Regulates immune recognition (e.g., MHC class II, co-stimulatory molecules)	IFN α / β , IFN γ	Both increased and repressed expression were observed	NF- κ B and C/EBP β
Stat3	Regulates typical M2-M ϕ activation genes (e.g., arginase-1 and -2, SOCS-3)	IL-6, IL-10	Increased phosphorylation and activation	NF- κ B and HIF-1, but also inhibits IKK β and opposes NF- κ B; key player in M1 to M2-M ϕ shift
Stat6	Regulates typical M2-M ϕ activation genes (e.g., arginase-1, arginase-2, SOCS-3)	IL-4, IL-13	ND	ND
C/EBP β (NF-IL6)	Heterodimer with C/EBP α / γ / δ . Regulates genes of acute phase response, Mo differentiation, IL-12, iNOS, and arginase-1	LPS, IL-6, IFN γ . Constitutively expressed in M ϕ	ND	NF- κ B for IL-6 production
C/EBP α	Homodimer or heterodimer with C/EBP β / γ	Growth hormone, IGF-1 via ERK1/2 and GSK3	Suppressed expression by HIF-1 α	HIF-1 α and Egr-1. Also opposes HIF-1 α by competing with HIF-1 β for its binding

Studies involving the transcription factors indicated were conducted mostly in tumor cell lines and require confirmation in Mo/M ϕ .

References pertaining to the transcription factors mentioned are: AP-1 (Bandyopadhyay et al., 1995; Alfranca et al., 2002; Laderoute, 2005; Bosco et al., 2006; Mancino and Lawrence, 2010); Egr-1 (Liao et al., 2007; Sperandio et al., 2009; Xu et al., 2010); Stat1 (Lee et al., 2006; Ivanov et al., 2007; Sow et al., 2009); Stat3 (Sica et al., 2008; Hagemann et al., 2009; Noman et al., 2009; Grivennikov and Karin, 2010; Kang et al., 2010); Stat6 (Hagemann et al., 2009); C/EBP β (Dlaska and Weiss, 1999; Gorgoni et al., 2002; Albina et al., 2005; Elbarghati et al., 2008); C/EBP α (Liao et al., 2007; Janardhan, 2008; Yang et al., 2008).

TF, transcription factor; ND, not determined.

Over-expression of HIF-1 α suppressed MSR1 mRNA expression, and or its depletion by siRNA restored MSR1 expression, demonstrating the ability of HIF-1 to convey transcriptional inhibition (Shirato et al., 2009). Furthermore, hypoxia was able to suppress gene expression indirectly through HIF-1 α , as it induced the expression of the transcription repressor BACH1, which regulates the expression of heme oxygenase-1 in human primary monocytes (Bosco et al., 2006). However, the mechanisms that allow HIF-1 α to play a dual role as both an activator and suppressor are not yet known, and this needs to be further explored.

POST-TRANSCRIPTIONAL MECHANISMS OF HYPOXIC REGULATION

ALTERNATIVE SPLICING AND mRNA STABILITY

Like many other genes, the murine HIF-1 α mRNA is alternatively spliced, generating the HIF-1 α I.1 and HIF-1 α I.2 isoforms, which have alternative first exons and promoters. Stimulation of TG-elicited peritoneal M ϕ and RAW 264.7 cells with TLR4 and A_{2A}R agonists synergistically increased the expression of both isoforms with different kinetics. However, only HIF-1- α I.1 was involved in cytokine production and down-regulated the LPS-induced production of pro-inflammatory cytokines (e.g., TNF α , IL-1 β , IL-6, IL-12p40, MIP-1 α , MCP-1, and MIP-2), but not IL-1

or VEGF, as was demonstrated in HIF-1 α I.1^{-/-} cells. Hypoxia had no effect on the expression of both isoforms (Ramanathan et al., 2009).

The only case that we are aware of hypoxia-induced alternative splicing is of CD80, where hypoxia reduced its surface expression on RAW 264.7 cells while increasing the concentrations of the soluble protein in the supernatants. As amplification of the total mRNA coding for CD80 remained unchanged, but the mRNA coding for the transmembranal region was reduced, it was concluded that hypoxia triggers alternative splicing to generate soluble CD80 (Lahat et al., 2003).

Stability of mRNAs is mediated through the AU-rich elements (AREs) located on the 3'-UTRs of many labile mRNAs and their interaction with different RBP. Presence of AREs is often correlated with rapid mRNA degradation, as occurs with many pro-inflammatory genes such as cytokines (e.g., TNF α , GM-CSF, IL-8), growth factors (e.g., VEGF), transcription factors (e.g., HIF-1 α), and other genes (e.g., iNOS, COX-2, uPA, MMPs; Khabar, 2010). Efficient AREs consist of overlapping repetitions of the pentamer AUUUU, giving rise to one or several UUAUUUUUU nonamers, and can assume either a linear or a stem-and-loop conformation. The length of the 3'-UTR and of the ARE itself affects mRNA stability, and longer AREs are associated with shorter half-lives of the mRNAs.

Several RBPs bind to 3'-UTR AREs and target the mRNAs for degradation, including tristetraprolin (TTP) and K-homology splicing-regulatory protein (KSRP), while the HuR promote their stabilization. HuR is mostly a nuclear protein, which upon stimulation translocates to the cytoplasm where it binds to selected mRNAs and stabilizes them. Alternatively, phosphorylation of TTP and KSRP reduce their affinity to AREs and thus stabilize the mRNA. In its unphosphorylated form, TTP recruits factors that mediate decapping, deadenylation, and mRNA destruction. Thus, there is a complex interplay between RBPs that bind to the AREs and determine the mRNA half-life (Khabar, 2010).

The 3'-UTR of the HIF-1 α mRNA contains six scattered pentamer AREs and one nonamer ARE, suggesting that TTP could bind to the HIF-1 α 3'-UTR and regulate its stability. In several cancer cell lines, hypoxia increased TTP expression, and the protein directly bound to the HIF-1 α mRNA AREs. Consistently, over-expression of TTP resulted in decreased HIF-1 α mRNA, suggesting that hypoxic cells use TTP to modulate HIF-1 α expression (Shin et al., 2010). Although this was not tested in Mo/M ϕ , another study demonstrated that prolonged hypoxia and LPS increased the expression of the dephosphorylated form of TTP in RAW 264.7 cells, and reduced the stability of TNF α , IL-6, MIP-2, and GM-CSF. Knock-down of TTP by siRNA abolished this destabilization (Werno et al., 2010b). On the other hand, the expression of CXCR4 mRNA was stabilized in hypoxia, although the mechanism was not studied (Schioppa et al., 2003).

TRANSLATIONAL REGULATION: RIBOSWITCHES AND microRNAs

Hypoxia leads to immediate shut-down of general protein translation. However, selected proteins, including the HIF proteins and their targets, are nonetheless translated. How these proteins escape global inhibition of translation remains unclear.

Translational regulation is achieved by several mechanisms, and structural elements in the non-coding regions of messenger RNA were shown to modulate gene expression. An example of such an element is the riboswitch, a regulatory element that binds small molecules or ribonucleoprotein (RNP) complexes that induce its conformational change. Riboswitches are usually identified in bacteria, but recently a similar element was identified in the VEGF-A mRNA. This transcript is equally transcribed in IFN γ -induced U937 cells in normoxia and hypoxia, but the protein translation is inhibited after 24 h in normoxia, whereas hypoxia abolishes this effect (Ray et al., 2009). The VEGF-A transcript contains a 126-nt ARE called hypoxia stability region (HSR) that mediates the mRNA stability in hypoxia. It includes two cis-elements, one is a 21-nt CA-rich element (CARE) that binds with high affinity to the heterogeneous nuclear ribonucleoprotein L (hnRNP L), and the other is a 29-nt IFN γ -activated inhibitor of translation (GAIT) element that binds the GAIT complex and silences inflammatory gene expression. The binding to these two elements is mutually exclusive. Thus, in normoxia IFN γ stimulates the binding of GAIT complex to its element, thereby silencing VEGF-A gene expression, whereas in hypoxia the HSR undergoes a conformational change, which favors binding of hnRNP L and overrides the suppressive effects of IFN γ and the GAIT complex (Ray et al., 2009).

Additional translational regulation is exerted by microRNAs (miRNAs), small non-coding RNA molecules that bind with

imperfect complementarity to specific mRNA target sequences, usually located in the 3'-UTR of target mRNA, and rapidly and reversibly inhibit their translation. As miRNA are usually independently transcribed, and some contain HRE in their promoter, hypoxic stress can stimulate their transcription, as was shown in different cell types (Pocock, 2011). For example, miR-210 whose promoter contains an HRE is induced in hypoxic tumor and endothelial cells, and can bind to target genes that are crucial in the mitochondria electron transport, thus diverting the cells away from oxidative phosphorylation and toward glycolysis. Alternatively, miR-210 can repress normoxic target genes that are not required for the hypoxic response (Pocock, 2011).

In Mo/M ϕ several miRNAs were identified as regulators of the inflammatory response, in particular miR-155 (O'Connell et al., 2007), miR-146a, and miR-132 (Taganov et al., 2006). LPS markedly induced miR-146a expression in Mo/M ϕ in a prolonged kinetics, and negatively regulated IRAK-1 and TRAF-6 expression leading to down-regulation of the NF- κ B pathway, as well as to LPS tolerance and cross tolerance (activation by other TLR ligands) in LPS-primed Mo/M ϕ (Nahid et al., 2009, 2011).

H₂O₂-induced oxidative stress in RAW 264.7 M ϕ activated the NF- κ B pathway and down-regulated the expression of a unique set of miRNA*s (miRNA expressed at low levels relative to the miRNA in the opposite arm of a hairpin; i.e., miR-27a*, miR-27b*, miR-29b*, miR-24-2*, and miR-21*), implicating them, and miR-27b* in particular, in NF- κ B regulation (Thulasigam et al., 2011). Regulation by both miRNA and RBPs is primarily executed on the 3'-UTR region in overlapping sequences, indicating possible crosstalk. Indeed, the translation of VEGF-A mRNA is inhibited by the binding of several miRNAs to the CARE, particularly miR-297 and miR-299 in normoxic monocytic cells (even without additional stimulation with IFN γ). However, hypoxia induces translocation of hnRNP L to the cytoplasm, where it binds the VEGF-A mRNA and prevents miRNA silencing (Jafarifar et al., 2011).

Regulation of protein translation by miRNAs is even further complicated by the finding that miRNA can oscillate between translational repression and activation (which is distinct from alleviating translational repression), depending on their binding location and/or on the conditions in the microenvironment. For example, in proliferating cells TNF α translation is inhibited by the binding of miRNAs to its ARE located in the 3'-UTR. However, upon serum-starvation and cell cycle arrest, HEK293 cells up-regulated TNF α -ARE expression in a manner that depended on miR-369-3. This was mediated by the increased expression of miR-369-3, which recruited the fragile X mental retardation-related protein 1 (FXR1), found exclusively in the activating complex, and Ago2, found in both repressing and activating complexes, into a complex that executed translation activation (Vasudevan et al., 2007). However, as TNF α is mainly produced in Mo/M ϕ , and its 3'-UTR ARE is representative of other pro-inflammatory cytokines with short half-life, it is very important that these results be confirmed in Mo/M ϕ cells, and the precise microenvironmental conditions that activate this switch be defined. These results suggest a new multi-input signal integration mechanism, which could be advantageous particularly in Mo/M ϕ that must adapt to the changing microenvironment.

INTRACELLULAR TRAFFICKING AND PROTEIN–PROTEIN INTERACTIONS

The ability of Mo/M ϕ to migrate through the ECM is mediated by the balanced secretion of different MMPs, particularly MMP-9, and their endogenous inhibitors TIMPs. Hypoxia inhibits migration of human monocyte-derived dendritic cells by disrupting this balance, as TIMP-1 was increased whereas MMP-9 and MT1-MMP were decreased (Qu et al., 2005). These inhibitory effects of hypoxia may have been mediated by the adenosine receptor A_{2B} in a cAMP and PKC dependent manner (Zhao et al., 2008). Hypoxia also inhibited the TNF α -induced secretion of MMP-9 from U937 cells and primary Mo, thus markedly inhibiting their migration (Rahat et al., 2006). This was not regulated at the transcriptional level, as MMP-9 mRNA was unchanged, or at the translational levels, as the amounts of the intracellular enzyme were increased in hypoxia. Instead, confocal microscopy revealed that in hypoxia MMP-9 was attenuated in secretory vesicles due to the effects of hypoxia on the cytoskeleton. Indeed, secretion of proMMP-9 was reduced by the addition of cytochalasin B or nocodazole, which inhibits the polymerization of actin and tubulin fibers, or by the addition of the Rho kinase inhibitor Y27632, suggesting the involvement of the cytoskeleton and the Rho GTPases in the process of enzyme secretion (Rahat et al., 2006).

Reduced MMP-9 in hypoxia could explain the immobilization of Mo/M ϕ in hypoxic regions, in addition to other mechanisms that were reviewed elsewhere (Murdoch et al., 2004; Bosco et al., 2008). Another example of intracellular trafficking is mentioned later (see TNF α Trafficking).

The hallmark of M1-activated M ϕ is iNOS expression and high NO production, which is crucial for the killing abilities of the M ϕ , whereas low levels are considered pro-angiogenic (Weigert and Brune, 2008). The main regulatory checkpoint for iNOS is transcriptional, and NF- κ B, IRF-1, Stat1, HIF-1 α , and C/EBP β are implicated in its regulation (Pautz et al., 2010). In hypoxia, induction of iNOS transcription and protein expression is demonstrated in differently activated M ϕ (e.g., peritoneal TG-elicited M ϕ , wound M ϕ , spleen M ϕ). However, the enzyme is inactivated and may produce only very low amounts of NO (Melillo et al., 1996; Daniliuc et al., 2003). This inactivation was attributed to the lack of the oxygen substrate during hypoxic stress, however, hypoxic M ϕ lysates failed to produce NO in an a-cellular system in normoxia, where all cofactors and substrates were abundantly present, suggesting a more intricate regulation. Further investigation revealed that in normoxia iNOS is associated with α -actinin-4, a protein responsible for the cross-linking of actin fibers. This interaction recruits iNOS to the sub-membranal area and ensures its activity. However, hypoxia disrupts these interactions, interferes with iNOS localization and causes its inactivation (Daniliuc et al., 2003).

These two examples highlight a special role for the cytoskeleton in regulating enzyme activity and M ϕ phenotype. As cytoskeleton fibers are responsible for the cellular protein trafficking, their disruption may attenuate vesicles within the M ϕ and prevent secretion of their content, or inhibit delivery of proteins to their destination. For example, hypoxia increased tubulin stabilization and changed vesicle trafficking in breast carcinoma cells (Yoon et al., 2005). This is further supported by recent findings linking actin cytoskeleton to HIF-1 α stability (Shin et al., 2010).

THE EFFECTS OF PREVIOUS M ϕ ACTIVATION ON THE HYPOXIC STIMULATION – THE TNF α EXAMPLE

TNF α TRAFFICKING

In addition to the MMP-9 example, where hypoxia attenuated secretory vesicles, hypoxia also affects TNF α transport. In TG-elicited peritoneal M ϕ and in RAW 264.7 cells, hypoxia did not change the LPS-induced TNF α mRNA levels relative to normoxia, whereas the membranal and intracellular amounts of the protein were reduced, suggesting degradation of TNF α . The proteosomal inhibitor MG132 did not change the hypoxic down-regulation of TNF α , whereas the lysosome inhibitor bafilomycin A1 dose-dependently inhibited degradation of intracellular TNF α , suggesting that TNF α is degraded at the lysosome. Using several Rab proteins as markers for different vesicles, TNF α trafficking was mapped to the endosomal pathway, and its presence was demonstrated in recycling endosomes, early endosomes, lysosome, and secretory lysosomes. The increased hypoxic co-localization of TNF α and LAMP-1, the lysosome marker, and the ability of bafilomycin A1 to increase intracellular TNF α to normoxic values, suggested that TNF α is directed to the lysosome and that hypoxia enhances its degradation (Lahat et al., 2008).

EFFECTS OF PREVIOUS M ϕ ACTIVATION ON THE HYPOXIC STIMULATION

The overall effects of hypoxia on the expression of TNF α are controversial. It is widely accepted that hypoxia and LPS increase M ϕ TNF α secretion (Table 2). However, in some cases hypoxia can inhibit TNF α secretion. In our own hands hypoxia exerted opposing effects on LPS-induced secretion of TNF α , as it enhanced it from human monocytes (Lahat et al., 2003), but down-regulated its secretion from peritoneal TG-elicited M ϕ and RAW 264.7 cells (Lahat et al., 2008). We have demonstrated that hypoxia enhances the lysosomal degradation of TNF α , thus causing its reduction in pro-inflammatory induced RAW 264.7 cells (Lahat et al., 2008). Werno et al. (2010b) have also shown that hypoxia reduced TNF α secretion from pro-inflammatory RAW 264.7 cells, although they suggested a mechanism of reduced mRNA stability, which also affected other pro-inflammatory mediators such as MIP-2 and IL-6, and is not mutually exclusive with lysosomal degradation.

This apparent contradiction between the enhancing and inhibiting effects of hypoxia on TNF α secretion may be resolved by taking into account the priming state of the M ϕ . Priming signals may by themselves be insufficient to stimulate M ϕ , but they sensitize it for further triggering. Traditionally, IFN γ is used to prime M ϕ that consequently encounter TLR ligands (such as LPS) and become fully M1 activated, although the sequence of these events is insignificant (Schroder et al., 2006). We suggest that “naïve” M ϕ that were not previously primed respond to a combination of hypoxia and LPS by elevating TNF α secretion, whereas M ϕ that are already primed or M1-activated react by down-regulating TNF α secretion, relative to activation with LPS alone. In this sense, RAW 264.7 cells behaved like primed cells, and we speculate that as the cell line was obtained from a tumor induced by the Abelson murine leukemia virus, the viral transformation primes the macrophage.

To further make the point, we compared resident peritoneal M ϕ with TG-elicited M ϕ , by exposing them for 48 h to hypoxia,

Table 2 | Controversial effects of hypoxia on TNF α secretion from M ϕ .

M ϕ type	Priming	Hyp. (%)	Time (h)	Stim.	Mechanism	Effect	Reference
NAiVE MO/Mϕ							
Human Mo	None	<0.3	24	LPS	Transcription	Up-regulation	Lahat et al. (2003)
	None	3	16	LPS	Transcription	Up-regulation	Guida and Stewart (1998)
	None	1		None	Transcription	Up-regulation	Demasi et al. (2003)
Resident peritoneal M ϕ	None	<0.3	24	LPS	Transcription	Up-regulation	Lahat et al. (2003)
	None	0	18	LPS	Transcription	Up-regulation	Meng et al. (2001)
	None	0	24	LPS + IFN	Transcription	Up-regulation	Albina et al. (1995)
Alveolar M ϕ	None	2	2	LPS	Transcription	Up-regulation	Leeper-Woodford and Detmer (1999)
Wound M ϕ	None	0	24	LPS + IFN	Transcription	Up-regulation	Albina et al. (1995)
BV-2 microglial cells	None	3	12	None	Transcription	Up-regulation	Li et al. (2009)
THP-1	None	1	18	None	Transcription	Up-regulation	Scannell et al. (1993)
PRIMED MO/Mϕ							
RAW 264.7	(Viral) trans-formation?		16	LPS	mRNA stability	Down-regulation	Werno et al. (2010b)
			24	None	Not specified	Down-regulation	Yun et al. (1997)
		<0.3	24	LPS	Enhanced lysosomal degradation	Down-regulation	Lahat et al. (2008)
TG-elicited peritoneal M ϕ	TG	<0.3	24	LPS	Enhanced lysosomal degradation	Down-regulation	Lahat et al. (2008)

Stim., stimulus; Hyp., hypoxia.

with or without addition of LPS. **Figure 1** shows the opposing effects of hypoxia on these M ϕ , although they originate from the same organ. Given the wide use of TG-elicited M ϕ , it is important to note that these cells are derived from a site of ongoing inflammation, and are therefore at least partially activated and should be considered primed in comparison to the “naïve” resident peritoneal M ϕ (as stated in Takeda et al., 2010).

Likewise, when we primed U937 or THP-1 cells by incubating them for 48 h with LPS (simulating gram negative bacterial activation) or with IFN β (simulating viral activation), and then exposed them for additional 24 h to LPS (at a higher concentration) and hypoxia, we again observed the inhibitory effects of hypoxia on IFN β -primed cells, but not on “naïve” cells (**Figure 1**). Note that the lack of response when cells were primed with LPS suggests the involvement of LPS tolerance, which is mediated by the NF- κ B p50:p50 homodimers (Frede et al., 2009). It may also imply that IFN β -priming is mediated by a different mechanism. Thus, the inhibited secretion of TNF α , a typical pro-inflammatory marker, from primed M1-activated M ϕ is inhibited by hypoxia, suggesting that hypoxia may act to restrain the pro-inflammatory M ϕ .

CONCLUDING REMARKS AND PERSPECTIVES

Generally, the hypoxic response depends on cell type, duration of exposure, severity of the hypoxic insult, and as demonstrated for TNF α , previous priming of the M ϕ . Thus, hypoxia can activate M ϕ in a pro-inflammatory way, or skew the pro-inflammatory M ϕ toward a phenotype approximating the M2 or resolution phenotypes. Since hypoxic M ϕ display a mixture of markers of both

M1 and M2 activation, classifying them is not straightforward, and they may represent a unique mode of activation which has several common markers with both M1 and M2 activation. In this respect, hypoxia may serve to restrain the pro-inflammatory M ϕ and prevent exacerbation of tissue damage, or alternatively ensure that M ϕ retained in the hypoxic tissue for long periods of time can gradually become M2 activated and engage in processes such as wound healing and/or resolution. In physiological scenarios (e.g., acute inflammation) this has a favorable outcome, whereas in chronic inflammation (e.g., in autoimmune diseases or solid tumors) the inhibitory effects of hypoxia may have devastating results.

Hypoxia profoundly changes Mo/M ϕ activation mode by regulating gene expression. Most of the known regulatory mechanisms are transcriptional, and rely on the cooperation between the master regulator NF- κ B and HIFs to mediate this induction of gene expression. However, in some cases HIF can also act as a transcriptional suppressor. Determination of M ϕ activation mode further rely on the cooperation with additional transcriptional factors that are expressed or modified according to the signals received by the microenvironment. The effects of hypoxia on differently activated M ϕ and the molecular mechanisms it exerts (summarized in **Figure 2**) were not investigated enough. For example, it seems reasonable to assume that differently activated M ϕ located in divergent microenvironments would express different HIF isoforms in different proportions and with different kinetics. However, this assumption merits further study.

Additional levels of post-transcriptional and post-translational regulation are gradually revealed. These include regulation of

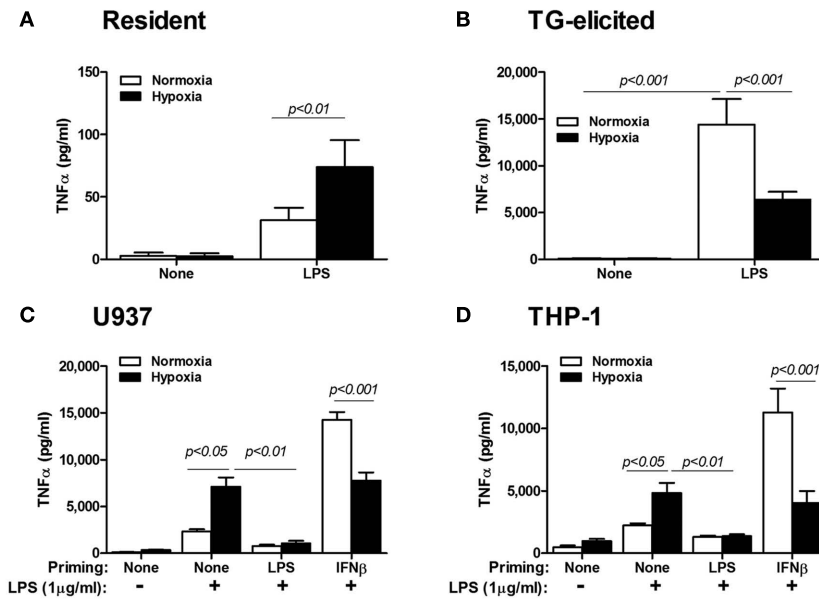


FIGURE 1 | Priming of Mφ affects the hypoxic response. (A) Resident peritoneal Mφ (n = 14) or (B) TG-elicited peritoneal Mφ (n = 16) were subjected to hypoxia (<0.3% O₂) for 24 h, with or without stimulation with LPS (1 μg/ml). Hypoxia inhibited the secretion of high amounts of TNFα that were produced in normoxia in primed TG-elicited Mφ, while an opposite response to hypoxia was observed for the naïve resident Mφ. The

human monocytic cell lines (C) U937 (n = 12) and (D) THP-1 (n = 18) were primed with either LPS (100 ng/ml) or IFNβ (10 U/ml) for 48 h in normoxia, before LPS (1 μg/ml) was added and cells were incubated for additional 24 h in normoxia or hypoxia. Hypoxia increased TNFα secretion in naïve Mo, but inhibited it in IFNβ-primed cells. TNFα was determined in the supernatants using ELISA.

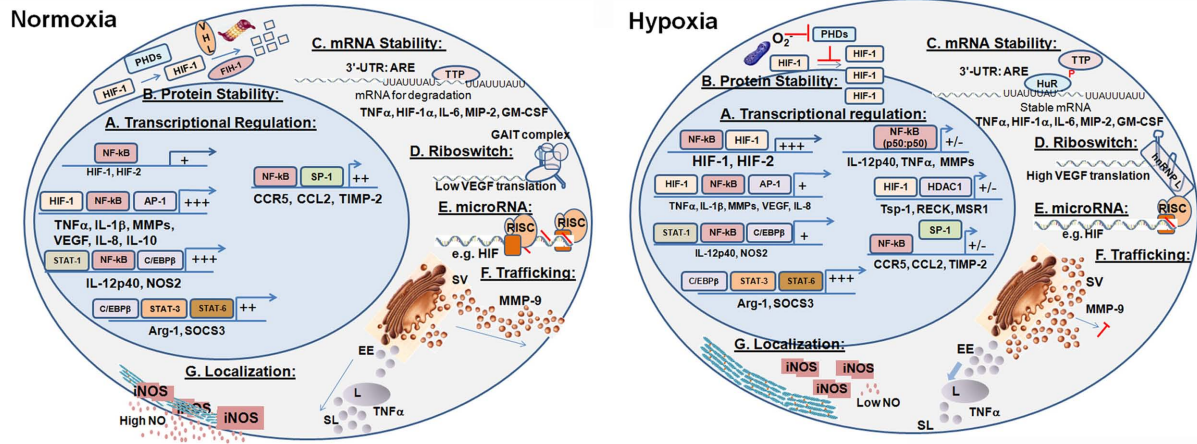


FIGURE 2 | Molecular mechanisms mediating the regulatory effects of hypoxia. Hypoxia affects the production, secretion, and activity of key Mφ proteins in several regulatory checkpoints, generally keeping the pro-inflammatory Mφ in check. (A) Transcriptional regulation in the M1-activated Mφ in normoxia is based on the collaboration between NF-κB, low level of HIF-1, and additional transcription factors. In hypoxia, more HIFs are produced, and genes involved in angiogenesis, metabolism, and survival are triggered. (B) HIFs protein stability in hypoxia is increased, as the prolyl hydroxylases that target them for proteosomal degradation in normoxia are inhibited. (C) Stability of mRNA is regulated by RNA-binding proteins to the AU-rich elements (ARE) located in the 3'-UTR. (D) Post-transcriptional regulation – Riboswitch: The VEGF example illustrates how hypoxia changes secondary structures of 3'-UTR elements, and the

binding of protein complexes to them, thus increasing their stability and translation. (E) Post-transcriptional regulation – microRNAs: Hypoxia modulates the expression of microRNAs that bind selected transcripts, thereby inhibiting or alleviating inhibition of their translation. (F) Post-translational regulation – Trafficking: Hypoxia retains secretory vesicles by inhibiting the actin cytoskeleton, thus attenuating the secretion of proteins such as MMP-9. Hypoxia also enhances the lysosomal degradation of other proteins, such as TNFα, which are secreted via the endosomal pathway. (G) Post-translational regulation – Localization: Hypoxia disrupts the interactions between iNOS and α-actinin-4, which anchors it to the cortical cytoskeleton, resulting in its inactivation. SV, secretory vesicles; EE, early endosomes; L, lysosome; SL, secretory lysosomes. +/– indicated reduced or inhibited transcription.

mRNA stability, regulation of translation via RBP and microRNA, protein trafficking and protein–protein interactions which may be regulated via reorganization of the cytoskeleton. We now begin to understand how some of these mechanisms allow M ϕ to integrate the different microenvironmental signals and to determine the M ϕ activation mode. Therefore, these mechanisms should be further studied in the context of M ϕ activation, to provide better insights and new approaches to manipulate M ϕ in the hypoxic

microenvironment while taking advantage of their selective ability to home to and accumulate in hypoxic regions.

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