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Review

Neutrophils and inflammatory metabolism in antimicrobial functions of the mucosa

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ABSTRACT

In this mini-review, we will discuss recent findings that implicate neutrophil infiltration and function in establishing a metabolic environment to facilitate efficient pathogen clearance. For decades, neutrophils have been regarded as short lived, nonspecific granulocytes, equipped with toxic antimicrobial factors and a respiratory burst generating ROS. Recent findings demonstrate the importance of HIF signaling in leukocytes and surrounding tissues during inflammation. Here, we will review the potential mechanisms and outcomes of HIF stabilization within the intestinal mucosa. *J. Leukoc. Biol.* 98: 517–522; 2015.

OXYGEN METABOLISM IN THE INTESTINE

The intestinal mucosa has a fascinating oxygen profile and has become a model tissue for understanding inflammation-associated changes in metabolism. Even under physiologic conditions, the intestinal mucosa experiences profound fluctuations in blood flow and tissue oxygenation, as well as steep gradients of oxygen across the luminal surface [1]. With the use of oxygen-sensitive tissue stains (i.e., nitroimidazole-derived compounds), we and others have profiled oxygen gradients from the anaerobic lumen of the colon across the epithelium into a highly vascularized and metabolically active serosa [1]. This analysis has revealed that pO_2 levels at the surface of intestinal tissue (1–2 cell layers deep) may be as low as 10 mmHg under normal physiologic conditions [2]. More recent studies that use phosphorescence-quenching methods have revealed that oxygen diffuses radially from the tissue into the lumen and that oxygen-tolerant microbes exist closer to the surface epithelium than in the stool [3].

Given these normal physiologic conditions, it is perhaps not surprising that the epithelium has evolved a number of features to adapt to significant metabolic shifts during normal tissue function. As an example, a comparison of barrier-function responses between epithelial cells from different tissues revealed that IECs appear to be uniquely resistant to low oxygen culture conditions and that the normally low level of oxygenation within the healthy intestinal epithelium may be a regulatory adaptation mechanism to this steep oxygen gradient observed morphologically [2].

Detection of and adaption to diminished oxygen is essential for survival. HIF transcription factors are critical regulators of the cellular response to hypoxia, regulating metabolism, angiogenesis, and inflammatory responses [4]. HIF belongs to the basic loop-helix-loop family of transcription factors and forms a heterodimeric complex, comprised of an α and β subunit. Both subunits are constitutively expressed, but under conditions of normal oxygen tension (i.e., normoxia), the α subunits undergo proline hydroxylation by PHD enzymes. Hydroxylated HIF-α subunits are then polyubiquitinated by the VHL-associated E3 ligase complex, where they are targeted for proteasomal degradation. Under conditions of diminished oxygen (i.e., hypoxia), PHD enzymes lack oxygen substrates, and HIF-α subunits are stabilized, form heterodimeric complexes with HIF-β, and bind to consensus HREs to control the transcription of adaptive HIF target genes. HIF signaling plays key roles in cancer, vascular disease, and inflammation [5]. Three α subunits exist (HIF-1α, HIF-2α, and HIF-3α); however, specific differences between the gene regulation by these transcription factors are still largely unknown and remain a topic of significant interest [6].

MUCOSAL INFLAMMATION AND OXYGEN METABOLISM

Sites of mucosal inflammation that occurs in diseases, such as IBD and infectious colitis, are characterized by profound changes in tissue metabolism, including local depletion of oxygen, nutrient imbalances, and the generation of large quantities of reactive oxygen intermediates [1]. These changes in metabolism are, at least in part, attributable to recruitment of inflammatory cells, including neutrophils (PMN) and monocytes. PMNs are recruited by chemical signals, such as the chemokine IL-8, complement factor C5a, *N*-formylated peptides, platelet-activating factor, and leukotriene B4, which are generated at sites

Abbreviations: A1AT = α-1 antitrypsin, AMP = antimicrobial peptide, CD = Crohn's disease, CGD = chronic granulomatous disease, CYBA/B = cytochrome b α/β, *DEFB* = defensin β, DMOG = dimethyloxalylglycine, H_2O_2 = hydrogen peroxide, hBD1 = human β-defensin-1, HIF = hypoxia-inducible factor, HRE = hypoxia-response element, IBD = inflammatory bowel disease, IEC = intestinal epithelial cell, LL-37 = cathelicidin, NCF = neutrophil cytosolic factor,

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of active inflammation as part of the innate host immune response to microorganisms. In transit, PMN cells expend tremendous amounts of energy (i.e., large amounts of ATP are needed for the high actin turnover required for cell migration [7]). Once at the sites of inflammation, the nutrient, energy and oxygen demands of the PMN increase to accomplish the processes of phagocytosis and microbial killing [8]. It is known that PMNs are primarily glycolytic cells, with few mitochondria and little energy produced from respiration [9]. A predominantly glycolytic metabolism ensures that PMNs can function at the low oxygen concentrations (even anoxia) associated with inflammatory lesions.

Once at the inflammatory site, PMNs recognize and engulf pathogens and activate the release of antibacterial peptides, proteases, and ROS ($O_2^{\cdot -}$, H_2O_2, hydroxyl radical, and hypochlorous acid) into the vacuole, which together, kill the invading microbes [10]. $O_2^{\cdot -}$ is produced by phagocytes in a powerful oxidative burst, driven by a rapid increase in oxygen uptake and glucose consumption, which in turn, triggers further generation of ROS; H_2O_2 is generated via SOD, which can be converted to hypochlorous acid via myeloperoxidase enzymes or form the hydroxyl radical via the Fenton reaction [11]. When activated, it is estimated that PMNs can consume up to 10 times more O_2 than any other cell in the body. Notably, the PMN oxidative burst is not hindered by even relatively low O_2 (as low as 4.5% O_2) [12], which is important, as it means that ROS can be generated in the relatively low O_2 environments of inflamed intestinal mucosa [1].

It was demonstrated recently that during acute inflammatory disease, infiltrating neutrophils "mold" the tissue microenvironment in ways that significantly promote the stabilization of HIF and HIF-dependent transcriptional responses [13]. Microarray analysis of epithelial cells following PMN transmigration identified the induction of a prominent cohort of HIF target genes. With the use of HIF reporter mice, $Gp91^{phox-/-}$ mice (lack a respiratory burst), and PMN depletion strategies in acute colitis models, these studies revealed that transmigrating neutrophils rapidly deplete the microenvironment of molecular oxygen in a NOX-dependent manner and "transcriptionally imprint" a molecular fingerprint that significantly reflects PMN induction of HIF target genes onto the surrounding tissue (**Fig. 1**). Importantly, this molecular signature promotes effective HIF-dependent inflammatory resolution. Indeed, $Gp91^{phox-/-}$ mice developed highly accentuated colitis relative to controls with exaggerated PMN infiltration, diminished inflammatory hypoxia, and increased microbial invasion. In this regard, a clinical corollary to these findings has indicated that patients who lack a functional NOX (i.e., CGD) often present with an IBD-like syndrome [14]. At this point, it is unclear to what extent mucosal hypoxia is dependent on PMN NOX, relative to other ROS-generating leukocytes (e.g., monocytes or eosinophils). It is likely that in other disease models with different leukocyte recruitment dynamics, NOX-expressing cells, other than PMN, may elicit tissue hypoxia.

FUNCTIONAL HIF TARGETS IN MUCOSAL INFLAMMATION

Intestinal epithelia form a selective barrier to permit absorption of nutrients, regulate water transport, and prevent translocation of commensal microbes. Epithelial junctional complexes (i.e., adherens and tight junctions) maintain cell–cell contact in juxtaposed epithelia, whereas factors that regulate cytoskeletal dynamics modulate paracellular flux [15]. A number of studies have shown that HIF triggers the expression of genes that enable IECs to function as an effective barrier [16–19]. Originally shown by microarray analysis of hypoxic IECs [18], these studies have been validated in animal models of intestinal inflammation [2, 20–24] and in inflamed human intestinal tissues [25–27]. The functional proteins encoded by hypoxia-induced, HIF-dependent mRNAs localize primarily to the most luminal aspect of polarized epithelia. Molecular studies of these hypoxia-elicited pathway(s) have shown a dependence on HIF-mediated transcriptional responses. Notably, epithelial barrier-protective pathways driven by HIF tend not to be the classic regulators of epithelial permeability, such as modulation of adherens or tight-junction protein organization or regulation of actin dynamics. Rather, the HIF-regulated pathways are more to do with overall tissue integrity, ranging from increased mucin production [28], including molecules that modify mucins, such as intestinal trefoil factor [16], to xenobiotic clearance by P-glycoprotein [17], to nucleotide metabolism (by ecto-5′-nucleotidase and CD73) [18, 19]. and nucleotide signaling through the adenosine the A2BR [19].

As an extension of the original studies identifying HIF induction within the intestinal mucosa, Karhausen et al. [2] generated mice lacking expression of intestinal epithelial Hif-1α (causing constitutive repression of *Hif1a*) or constitutive expression of HIF-1 in intestinal epithelia (via targeting of the VHL gene). Loss of epithelial HIF-1α resulted in a more severe colitic phenotype than wild-type animals, with increased weight loss, decreased colon length, and increased intestinal permeability, whereas constitutively active intestinal epithelial HIF was protective for each of these parameters. These findings may well be somewhat model dependent, as epithelial HIF-based signaling has also been shown to promote inflammation in other studies [24, 29]. However, the findings confirmed that IECs can adapt to hypoxia and that HIF may contribute to such adaptation.

Our studies, addressing the role of PMN in promoting HIF-dependent protection during colitis, revealed that pharmacological HIF stabilization within the mucosa significantly protected $Gp91^{phox-/-}$ mice [13]. These findings confirm a number of ongoing studies that have provided efficacy for pharmacologic compounds, which function to stabilize HIF and provide protection in intestinal inflammation models. In most instances, the pharmacologic approach to achieve HIF stabilization involves the inhibition of PHDs. The targeting of the catalytic domain of PHDs can be achieved by the generation of molecules that interfere with critical cofactors, such as the 2-oxoglutarate

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NOX = NADPH oxidase complex, $O_2^{\cdot -}$ = superoxide anion, PHD = prolyl hydroxylase, RAC1/2 = Ras-related C3 botulinum toxin substrate 1/2, ROS = reactive oxygen species, SOD = superoxide dismutase, TNBS = 2,4,6-trinitrobenzenesulfonic acid, UC = ulcerative colitis, VHL = von Hippel-Lindau

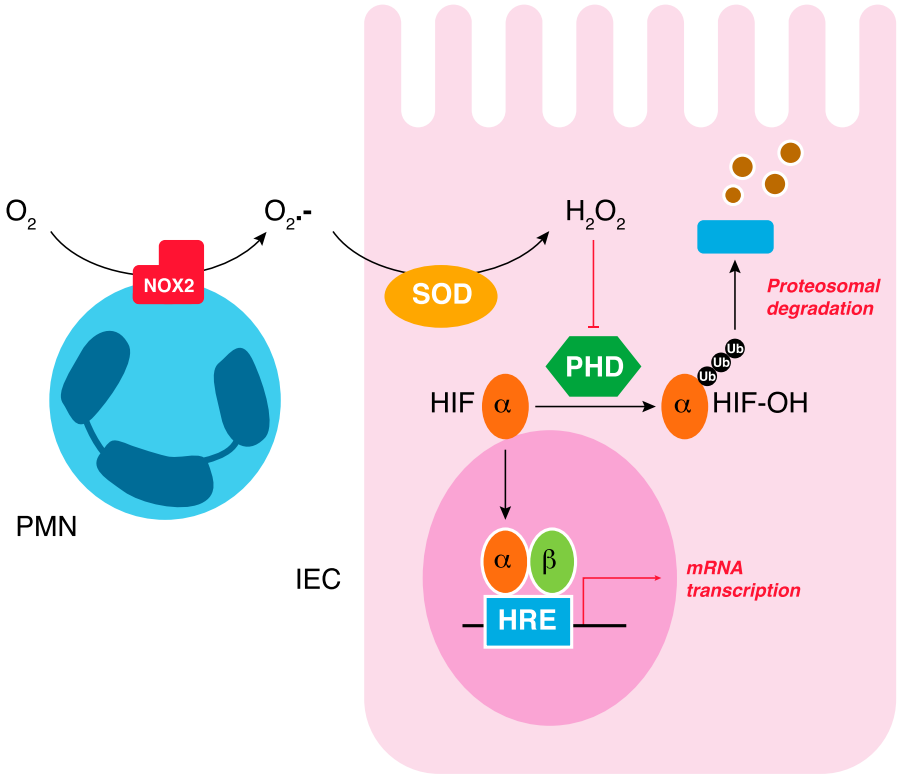

Figure 1. Proposed mechanism for IEC stabilization of HIF by activated neutrophil NOX2 activity. Neutrophils (PMNs) use microenvironmental oxygen in the respiratory burst via the NOX2, with resultant generation of $O_2^{\cdot -}$. SODs in IECs catalyze the reduction of $O_2^{\cdot -}$ to H_2O_2. PHDs post-translationally modify HIF-α subunits under normal oxygen tensions, hydroxylating proline residues. Hydroxylated HIF-α (HIF-OH) is targeted for ubiquitination (Ub) and subsequent proteosomal degradation. PHD enzymes are inhibited by H_2O_2 and result in HIF-α stabilization, nuclear translocation, interaction with the HIF-β subunit, and binding to HREs, regulating gene expression and adaptive responses to hypoxia. Adapted from ref. [13].

molecule, by structural mimicry, as for example, in the case of DMOG [30]. Indeed, 2 studies simultaneously demonstrated a protective role for HIF activators in different models of intestinal inflammation. The first study used the PHD inhibitor DMOG for the treatment of intestinal inflammation during chemically induced colitis [20]. A second study used the HIF activator FG-4497 during TNBS-induced intestinal inflammation. Similar to DMOG, FG-4497 blocks the active site of PHDs [30]. In both studies, HIF activator treatment was associated with profound improvements of multiple disease parameters, including weight loss, intestinal inflammation, and histologic tissue injury [1, 31]. Indeed, the use of more HIF-1 selective stabilizers, including AKB-4924 [32], holds promise in such models and suggests that IBD may be one of the more promising potential indications for PHD-inhibitor treatment.

HIF AND ANTIMICROBIAL ACTIVITY IN THE MUCOSA

The hypoxic microenvironment of the inflammatory lesion and associated HIF activity has been implicated in the function of myeloid cells for the clearance of infections. Not only is HIF-1 essential for support of glycolytic metabolism of phagocytes, but furthermore, it regulates key functions, such as bacterial uptake and production of antimicrobial effector molecules [e.g., LL-37-related AMP and serine proteases], and enhances the longevity of neutrophils [33]. In this regard, a fundamental difference between innate and adaptive immunity is the means by which individual leukocyte populations obtain energy. Cells of myeloid lineages derive their energy almost exclusively from glycolysis, whereas lymphocytes use predominantly oxidative phosphorylation [34]. Original studies by Cramer et al. [35] and by Peyssonnaux et al. [36] revealed an important role for HIF-1 in innate immune function of myeloid phagocytes. These studies used conditional deletion of Hif-1α in myeloid populations and showed a decreased bactericidal capacity of myeloid phagocytes lacking functional Hif-1α. Conversely, these same studies also revealed that genetic loss of myeloid VHL (i.e., stabilization of HIF) results in enhanced acute inflammatory responses. Given that VHL has a number of substrates, it remains to be determined to what extent HIF contributes to this hyperinflammatory response. With regard to adaptive immunity, parallel studies that use recombination activation gene 2-deficient blastocyst complementation to bypass embryonic lethality revealed that Hif-1α deficiency in T and B lymphocytes resulted in major defects in the development of B cells and significant autoimmunity reflected as IgG and IgM deposits in the kidney and increased anti-dsDNA antibodies [37].

The structure of AKB-4924 reveals an α-hydroxy carbonyl group similar to other iron chelators (e.g., L-mimosine) [38], suggesting that AKB-4924 inhibits PHDs through a chelation-dependent mechanism. Recent extensions of these studies by the Johnson group [39], using the HIF-1-predominant PHD inhibitor AKB-4924, revealed potent antimicrobial activity of this compound in stimulating the killing of the pathogens *Pseudomonas aeruginosa* and *Acinobacter baumanii*. With the consideration that AKB-4924 functions as an iron chelator, the authors tested direct influences of AKB-4924 on bacterial growth and demonstrated a bacteriostatic influence on log-phase growth; however, it was not directly bactericidal. Conversely, treatment of keratinocytes with AKB-4924 enhanced their antimicrobial defense, likely via

enhanced HIF-regulated antimicrobial factor (e.g., LL-37) expression [40]. Others have shown that pulmonary infections with *P. aeruginosa* are significantly attenuated with the PHD inhibitor DMOG in a HIF-2- and Rho kinase-dependent manner [41].

It was shown recently that HIF contributes significantly to the homeostatic regulation of epithelial AMPs, particularly hBD1 [42]. AMPs represent a substantial part of the infectious protection along the length of the intestinal mucosa, and β-defensins are the dominant class of AMP secreted by the epithelium. The 4 characterized hBD1–4, encoded by *DEFB1*, *DEFB4*, *DEFB103*, and *DEFB104*) are small (30–47 aa), cationic, cysteine-rich peptides that possess broad antimicrobial activity [43, 44]. In the gut, hBD1 has 2 characteristics that confer prominence. First, its antimicrobial activity is potentiated under reducing conditions that exist in the hypoxic gut lumen, whereas other AMPs, such as hBD3 are diminished in the reduced state [45]. Second, expression of hBD1 is constitutive, whereas other defensins are expressed in response to inflammatory stimuli or microbial invasion [46–48]. Given these properties, it is not surprising that defective expression of hBD1 is associated with mucosal disease, such as IBD [49–51], *Candidia* infection [52], and dental infections [53]. It is likely that this homeostatic role of HIF in epithelial AMP expression compliments other aspects of HIF-dependent mucosal barrier function [31]. Likewise, HIF-1 is strongly expressed in the skin and regulates AMP production by keratinocytes, (e.g., LL-37) [40]. Keratinocyte-specific deletion of HIF-1 enhanced susceptibility to group A streptococcal infection, whereas treatment with AKB-492 enhanced keratinocyte bactericidal activity in vitro and in vivo [39, 40]. LL-37 exerts weaker antimicrobial activities than most AMPs; however, it exerts pleiotropic effects by inhibiting biofilm [54], and as a neutrophil chemoattractant, finally, it also regulates synthesis of IL-8 [55], a potent neutrophil/monocyte chemoattractant.

DISEQUILIBRIUM OF NEUTROPHIL FUNCTION AND IBD

IBDs broadly describe chronic inflammatory conditions of the gastrointestinal tract, including UC and CD. Innate immune deficiencies, in particular, relating to neutrophils, have been implicated in both conditions; however, they differ in their involvement—in UC, excessive or uncontrolled PMN activity is detrimental. Contrastingly, bacterial DNA has been reported to be present in granulomas [56]; thus, it would appear that inadequate PMN activity or insufficient accumulation may contribute to pathogenesis. Indeed, neutrophil density is important for retarding bacterial growth within tissues [57]. Clearly, excessive neutrophil recruitment or prolonged activity is deleterious for a tissue, whereas insufficient recruitment is equally undesirable. Aside from absolute numbers of neutrophils recruited during inflammation, strict regulation of their function also contributes to tissue homeostasis. Neutrophil azurophilic granules contain the potent antimicrobial serine protease elastase. Elevated fecal elastase levels have been detected in UC patients [58]. Mucosal tissues respond by releasing inhibitors of secreted elastase (e.g., A1AT) to limit nonessential tissue damage. Fecal levels of A1AT correlate negatively with disease severity in IBD [59, 60]. Experimentally, A1AT administration has improved outcomes in acute and chronic models of murine colitis and ileitis, respectively [61].

CGD patients exhibit congenital defects in genes coding the subunits comprising the neutrophil NOX (i.e., mutations in CYBA, CYBB, NCF1, NCF2, NCF4, RAC1, and RAC2). As mentioned previously, NOX is responsible for the generation of ROS and used by innate immune cells to kill invading pathogens. Interestingly, ~40% of CGD patients develop IBD-like symptoms [62]; however, the presentation is distinct from CD or UC [63, 64]. Most recently, variants in the genes coding NOX can predict very early onset IBD [65].

In UC patients, excessive PMN accumulation has been argued to be a causative factor in tissue damage through generation of excessive levels of ROS. Mechanisms exist to protect tissues from ROS-induced damage; for example, SOD is widely expressed in the mucosa and converts $O_2^{\cdot -}$ to H_2O_2. H_2O_2 has been demonstrated to inhibit the function of the PHD-2 enzyme, resulting in HIF stabilization [66]. Unsurprisingly, disturbances in SOD expression have been identified in IBD [67], and experimentally exogenous SOD ameliorates TNBS colitis [68].

NEUTROPHILS AND TRAINED IMMUNITY

The concept of trained immunity is relatively recent and challenges the established dogma that innate immune cells, such as neutrophils, monocytes, and NK, elicit rapid, nonspecific responses and lack immune "memory" [69]. Trained immunity has been demonstrated in NK cells and monocytes; however, neutrophils have been dismissed as a result of their high turnover. However, in chronic inflammatory conditions and cancer, neutrophils can reside for much longer at target sites than their unstimulated circulating counterparts. The mere act of neutrophil recruitment and engagement by endothelial cells results in signaling cascades that extend the PMN half-life [70]. Elegant findings by the Whyte and Walmsley groups [71, 72] indicate that HIFs, in particular, HIF-2α, function to promote PMN longevity by delaying apoptosis. Aside from contributing to the generation of a hypoxic milieu, it is possible that this HIF stabilization in neutrophils is necessary to survive severe acute inflammatory episodes within a tissue long enough to kill invading pathogens. Mechanistically, the Netea group [73] has elucidated that trained immunity appears to require HIF stabilization, where pre-exposure of monocytes to β-glucan from *Candida albicans* results in HIF stabilization and aerobic glycolysis, protecting mice from subsequent infection-induced lethality. In this seminal paper, myeloid-specific HIF-1α conditional knockout mice succumb to *Staphylococcus aureus*-induced sepsis. The authors propose a mechanism whereby β-glucan stimulates monocyte Dectin-1, initiating an Akt → mammalian target of rapamycin → Hif-1α signaling cascade, leading to aerobic glycolysis in monocytes. However, it is unclear if the protection afforded by pre-exposure to β-glucan is necessarily specific to that stimulus or if merely HIF stabilization alone could be sufficient to facilitate this protection. Whereas PMN may not experience trained immunity themselves, their contribution to

establishing a hypoxic milieu may consequently stabilize HIF in other innate immune cells to elicit trained immunity.

CONCLUSION

Taken together, it is evident that neutrophils are not merely short-lived cells destined to kill invading microbes nonspecifically. Emerging literature suggests that neutrophils can shape a metabolic environment to facilitate not only host defense but also via HIF-mediated pathways to coordinate other innate and adaptive immune cells in orchestrating clearance of pathogens and protection of host tissue from metabolic stress during inflammation and potentially establishing an environment for resolution to occur. Most importantly, dysregulation of such pathways can be compensated for by use of pharmacological inhibition of PHD enzymes.

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DISCLOSURES

The authors declare no financial interests in any of the work submitted here.

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