The role of HIF in immunity and inflammation.

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ABSTRACT

Uncontrolled or non-resolving inflammation underpins a range of disease states including rheumatoid arthritis, inflammatory bowel disease and atherosclerosis. Hypoxia is a prominent feature of chronically inflamed tissues. This is due to elevated oxygen consumption by highly metabolically active inflamed resident cells and activated infiltrating immunocytes, as well as diminished oxygen supply due to vascular dysfunction. Tissue hypoxia can have a significant impact upon inflammatory signalling pathways in immune and non-immune cells and this can impact upon disease progression. In this review, we will discuss the relationship between tissue hypoxia and inflammation and identify how hypoxia-sensitive signalling pathways are potential therapeutic targets in chronic inflammatory disease.

Hypoxia in inflammation.

Metazoans have evolved a highly efficient bio-energetic strategy which involves the oxidative metabolism of carbohydrates and fatty acids to produce biochemical energy equivalents in the form of adenosine triphosphate (ATP). This process occurs in mitochondria in the presence of sufficient levels of molecular oxygen (O_2). Under normal circumstances, the majority of oxygen available in a tissue is consumed during oxidative metabolism, however, a reserve of non-mitochondrial oxygen is also normally available [1, 2]. This non-mitochondrial oxygen acts both as an oxygen reserve and a sensed signal which provides information to a cell that the tissue is receiving sufficient oxygen to meet metabolic requirements. Tissue hypoxia occurs when this state of oxygen homeostasis is disrupted and a condition where oxygen demand exceeds supply ensues. Under hypoxic-conditions, the reserve of non-mitochondrial oxygen is consumed to maximize ATP production and adaptive pathways are activated. Tissue hypoxia may occur as a result of diminished oxygen supply or increased oxygen demand.

It has recently become clear that tissue hypoxia is a prominent feature in a range of disorders where inflammation plays a prominent causative role including atherosclerosis, arthritis, inflammatory bowel disease (IBD), infection, obesity and cancer [3]. Taking the intestinal mucosa as an example, a number of studies have now demonstrated that this tissue becomes profoundly hypoxic under conditions of inflammation [4, 5]. In the normal physiologic state, the intestinal mucosa has a steep oxygen gradient from crypt to villus tip [6]. This is likely a consequence of a countercurrent exchange [7] of oxygen flow in the villus, and the fact that the intestinal epithelium is in direct contact with the anoxic lumen of the gut. Under conditions where the mucosa becomes inflamed such as inflammatory bowel disease (IBD), a profound degree of hypoxia occurs (e.g. surface oxygen tension measured in rabbits ranged from 36+/-5 torr in control animals, 11+/-5 torr in mild colitis and 4+/-1torr in severe

colitis [8]). The reasons for the occurrence of hypoxia at sites of inflammation are manifold and will be outlined below (Figure 1).

A key reason for the occurrence of tissue hypoxia in inflammation is the increased demand for oxygen which occurs when a tissue becomes inflamed. Following an immunogenic insult, there is an increase in the metabolic activity in the inflamed tissue. Inflammation is a metabolically costly process which requires increased synthesis of high levels of inflammatory enzymes and cytokines by inflamed resident cells leading to an increase in oxygen demand. Furthermore, sites of inflammation are characterized by the influx of inflammatory cells which add to the metabolic requirements / oxygen demand at the inflamed site. For example, it has recently been demonstrated that infiltrating neutrophils consume sufficient amounts of oxygen during the oxidative burst to elicit a state of hypoxia in neighboring resident cells [9, 10].

Another cause of hypoxia during inflammation is disrupted oxygen delivery. This is particularly the case in chronic inflammation where the combination of prolonged inflammatory activity and associated fibrosis and thrombosis results in diminished blood (and consequently oxygen) supply to the site of inflammation [11]. Therefore, a combination of increased oxygen consumption by inflamed resident cells and infiltrating immune cells along with a disrupted blood supply due to vascular dysfunction contributes to tissue hypoxia during chronic inflammation (Figure 1). Hypoxia in turn regulates a number of key signaling pathways which facilitate adaptation as outlined below.

The hypoxia-inducible factor (HIF):

Because a continuous supply of molecular oxygen is essential for cell, tissue and organism survival, it is perhaps not surprising that metazoans have evolved multiple mechanisms by which to adapt to and survive periods of hypoxia [12]. The primary signaling pathway activated by hypoxia involves the stabilization of the hypoxia-inducible factor (HIF) [13]. Because the HIF pathway has been extensively reviewed elsewhere in this series, it will be only briefly described here. Under conditions of

normoxia where oxygen is abundant, a family of hydroxylases (termed HIF-hydroxylases) hydroxylate HIF in an oxygen-dependent manner [12]. The hydroxylation of HIFα (HIF-1α / HIF-2α) subunits results in a reduction in both its stability and transcriptional activity, thus rendering HIF inactive in normoxia. In hypoxia, HIF is rapidly stabilized and is responsible for the activation of an adaptive transcriptional response which involves the up-regulation of survival factors such as metabolic enzymes, angiogenic factors and vasoactive substances which co-ordinate an increase in oxygen supply and metabolic activity in the hypoxic tissue [12, 13].

While HIF (the focus of this review) is a key regulator of the transcriptional response to hypoxia, a number of other transcription factors are also responsive to hypoxia and contribute to the overall cellular response [14]. Primary amongst these is the key regulator of immunity and apoptosis, NF-kB. We and others have shown that NF-kB, like HIF is regulated under hypoxic-conditions via alterations in the activity of the oxygen-dependent hydroxylases.

Nuclear Factor kappaB (NF-kB):

NF-kB is a master regulator of genes involved in innate immunity, inflammation and apoptosis. The NF-kB family is composed of five related proteins p65 (RelA, NFκB3), p50 (NFκB1), p52 (NFκB2), c-Rel and RelB (13). NF-kB is activated through a series of phosphorylation events usually initiated following activation of cell surface receptors which recognise specific inflammatory stimuli (e.g. toll-like receptors and cytokine receptors). Diverse stimuli such as interleukin 1β (IL-1β), lipopolysaccharide (LPS) and tumor necrosis factor (TNF) activate distinct cell surface receptors and associated signaling pathways, which converge on activation of the IkappaB kinase (IKK) complex. This heterotrimeric complex is composed of three proteins termed IKKα, IKKβ and NEMO (NF-kB essential modulator), which phosphorylates IκBα on defined serine residues and targets it for ubiquitylation-dependent degradation. IκBα

degradation releases NF-kB dimers allowing them to translocate to the nucleus where they control gene transcription [15]. Signaling by the heterotrimeric IKK complex is known as ‘canonical’ NF-kB signaling. An ‘alternative’ or ‘non-canonical” pathway also exists which is mediated by IKKα homodimers [16]. This pathway is more selectively activated by ligands such as lymphotoxin and CD40L (Figure 2.). NF-kB is responsible for the expression of many genes implicated in immune, inflammatory and apoptotic pathways such as TNFα, ICAM, and iNOS. Activation of the NF-kB pathway in response to inflammatory stimuli has been expertly reviewed elsewhere [17, 18]. Here we will focus on the relationship between hypoxia and NF-kB.

Koong et al. initially observed that exposure to hypoxia caused IκBα degradation in turn leading to increased NF-kB DNA binding [19]. Chandel et al. proposed that mitochondrial derived ROS generation in hypoxia may cause NF-kB activation [20]. Alternatively, a direct link between hypoxia and NF-kB activity was provided by the demonstration that the NF-kB pathway, like HIF, was regulated by prolyl hydroxylases (19). Intriguingly, both IKKα and IKKβ contain motifs similar to those known to be hydroxylated in HIF (LxxLAP) in their kinase domains, suggesting that hydroxylation of these proteins might be responsible for the oxygen-dependent regulation of NF-kB [21]. This hypothesis is supported by the recent observation of PHD-1 / EGLN2-dependent hydroxylation of an IKKβ protein in a de-carboxylation assay [22], and the observation that the canonical NF-kB pathway (and not the non-canonical pathway) is sensitive to hypoxia [23]

Recently, hydroxylase inhibitors have been employed in a number of models of inflammatory disease (discussed later). Interestingly, while hypoxia or hydroxylase inhibition in isolation are modest activators of NF-kB, the net effect of hydroxylase inhibition in the context of inflammation is to decrease NF-kB signaling [24, 25].

Recent evidence has shown that the prolyl hydroxylases can interact with members of the NF-kB family independently of their hydroxylase activity. Fu et al. demonstrated

that PHD3 overexpression can prevent NF-kB activation through physical interaction with IKKγ (NEMO), thus preventing IKKγ ubiquitylation and subsequent NF-kB activation [26]. Although this provides further insight into the interactions of NF-kB with the prolyl hydroxylases, this regulation of NF-kB by the PHDs is likely unaffected by hypoxia.

Of note, several NFkB family members have been proposed as substrates for the asparaginyl hydroxylase FIH. Cockman et al. first identified FIH-dependent hydroxylation of the NFkB subunits p105 and IκBα in their ankyrin repeat domains [27]. However, the functional significance of asparaginyl hydroxylation of these residues is not clear given the authors did not observe major effects on p65/p50 interactions or NF-kB-dependent transcriptional activity. More recently, Scholz et al. observed protein-protein interactions between several NFkB family members and FIH. Interestingly, many of these interactions were enhanced with the addition of DMOG (which is postulated to stabilize interactions between hydroxylases and their substrates) [25]. The authors subsequently went on to demonstrate in detail, the consequences for FIH-dependent hydroxylation on OTUB1 [28] (a de-ubiquitase important in linking IL-1β dependent signaling to the NFkB pathway). Interestingly, Scholz et al. note that pan-hydroxylase inhibition with DMOG significantly suppresses IL-1β-dependent NFkB activity, while a compound that selectively inhibits prolyl hydroxylases do not. Thus, there is strong evidence in support of a relationship between FIH and the NFkB pathway, but the functional/ signaling consequences for this are not yet full elucidated. These points are summarized in Figure 2.

In summary, cells are equipped with an array of factors which co-ordinate an adaptive response to hypoxia including HIF and NF-kB. Because hypoxia and inflammation are coincidental events, the possibility for significant cross- talk between these two pathways exists. The evidence for this is discussed below.

NF-kB and HIF cross-talk:

NF-kB and HIF signalling are interdependent. NF-kB has been shown to play a role in basal and stimulated HIF-1α mRNA expression. The, p50 and p65 NF-kB subunits can bind to a κB binding site located in the HIF-1α promoter in response to hypoxia. When these subunits are overexpressed, an increase in HIF-1α mRNA levels and promoter activity is observed. Subsequent mutation of this κB site in the HIF-1α promoter prevented hypoxic induction of HIF-1α promoter activity. Furthermore, a dominant negative IκBα mutant was shown to affect HIF-1α mRNA and protein levels in both hypoxic and normoxic conditions [29] [30]. Other studies have demonstrated that siRNA-mediated knockdown of NF-kB subunits showed a decrease in basal HIF-1α mRNA in normoxia. In agreement, overexpression of NF-kB promotes HIF-1α protein expression in normoxia [31]. Frede et al. showed that bacterial LPS can cause NF-kB dependent up-regulation of HIF-1α mRNA and protein [32]. Using an *in vivo* system Rius et al. demonstrated that IKKβ deficient mice have an absolute requirement of a functioning NF-kB pathway for HIF-1α mRNA. Knockout of IKKβ resulted in down-regulated HIF-1α mRNA, HIF protein and HIF-dependent gene expression *in vivo* [33]. Taken together there is significant evidence for the requirement of an intact NF-kB pathway for proper oxygen and ligand-induced HIF activity.

In addition to the requirement for a functional NF-kB pathway for HIF activity, NF-kB-dependent activity is also subject to regulation by HIF is some systems [34]. Together these studies highlight the complex and inter-related inflammatory signaling cascades that are initiated via the HIF and NF-kB pathways in hypoxia.

While the central role of NF-kB in immunity and inflammation has been well described previously, the role of HIF in the regulation of inflammation is less clearly understood. For the remainder of this review, we will focus on recent genetic and pharmacologic

studies which have identified the potential role of HIF in immune and non-immune cells as a key regulator and therapeutic target in inflammatory disease.

Regulation of immune effector pathways by HIF:

In this section, we will focus on the function(s) of the HIF and the HIF-hydroxylases in specific immune cell sub-types of both the innate and adaptive immune systems. This has been an area of intense research over the last decade with the use of genetically modified organisms in multiple models of infection/inflammation being investigated. We will attempt to reconcile the data emerging from the different experimental approaches to give an overview of the importance of the HIF pathway and hydroxylase enzymes in specific immune cell types.

HIF and Innate Immunity

1) Myeloid Cells

HIF-1α and HIF-2α levels are increased in primary human macrophages exposed to hypoxia [35]. Indications as to the importance of a functional oxygen-sensing pathway in immune cells was first provided by Cramer et al. [36]. Using a targeted deletion of HIF-1α in the myeloid cell lineage (granulocytes and monocytes/ macrophages) achieved by cre expression driven by the lysozyme M promoter, they observed a profound effect of HIF-1α loss on myeloid cell metabolism. It is known that classically activated macrophages and neutrophils produce much of their ATP via glycolysis, a pathway under heavy regulatory control by HIF-1α [36]. Glycolysis and energy generation were severely impaired by loss of HIF-1α and consequently many of the key innate immune functions of the myeloid cells were diminished (e.g. killing of bacterial pathogens, invasion, aggregation and motility). Interestingly, these effects were observed without a large change in the numbers of circulating monocytes or neutrophils. Taken together these data highlight the importance of a functional HIF-1α

pathway in myeloid cells that is central to the basal metabolic programing of the immune cell and facilitates innate immune function.

Specifically looking at neutrophil function in these animals, Walmsley et al. demonstrated that hypoxia-induced HIF-1α inhibits neutrophil apoptosis [34] while Peyssonnaux et al. demonstrated that HIF-1α played a role in the regulation of neutrophil bactericidal activity [37].

Elevated expression of HIF2α mRNA and protein are a particular feature of tumor associated macrophages [38] . Using a LysM-cre strategy, a conditional deletion of HIF-2α in the myeloid lineage was generated. HIF-2α deficient macrophages, similar to HIF-1α deficient macrophages demonstrated impaired immune function as indicated by altered cytokine production upon ligand activation, as well as suppressed migration and invasion [39]. Interestingly, there was no difference in ATP production in HIF-2α deficient macrophages (which is in marked contrast to the HIF-1α deficient phenotype reported by Cramer et al. [36]). Taken together these data suggest distinct roles for the two different HIF isoforms in the cellular response to low oxygen and that macrophage HIF-2α appears to be of particular importance in governing the pro-tumorigenic functions of tumor associated macrophages (TAM). Myeloid HIF-2α deficiency also results in decreased neutrophilic inflammation in a model of lung injury, likely due to a phenotype of increased neutrophil-apoptosis. Interestingly, HIF-2α is up-regulated in neutrophils taken from patients with inflammatory disease, an observation that the authors propose might indicate selectively targeting HIF-2α in conditions of neutrophilic inflammation [40].

2) Epithelial cells.

While epithelial cells may not be classically thought of as cells of the immune system they play a crucial role in lining the internal and external surfaces of the body and

acting as physical barrier between the internal environment of the body and the external environment and therefore perform a vital innate immune function. Epithelial cells are found in a number of tissues and experience significantly different pO_2 levels. For example, lung epithelial cells are exposed to relatively high levels of oxygen, while at the apical surface of gut, epithelial cells are exposed to the anoxic environment of the intestinal lumen [41]. Maintaining an effective barrier between the internal and external environment however is a key common function of the epithelium, and when this barrier becomes compromised, inflammation occurs [42]. The epithelium must then play a key role in restoring tissue integrity and barrier function.

The intestinal epithelium is known to exist in a state of relative 'physiological hypoxia' with a sharply decreasing oxygen gradient between the well vascularised intestinal mucosa, and the apical epithelial membrane which is juxtaposed with the anoxic gut lumen [41]. Furthermore, the nature of the villus microcirculation permits a countercurrent shunt of oxygen, which effectively reduces the oxygenation at the villus tip [6]. In conditions of chronic inflammation (e.g. IBD) the colonic mucosa can become even further depleted of oxygen as neutrophils infiltrate the tissue consuming large amounts of oxygen and creating a hypoxic inflammatory microenvironment [10]. The role of HIF-1α in the intestinal epithelium was initially investigated by Karhausen et al. Using a fatty acid binding protein-cre they conditionally depleted HIF-1α in the intestinal epithelium [41]. Mice lacking HIF-1α in the epithelium were more susceptible to experimental colitis. Furthermore, mice lacking VHL in the epithelium were protected in TNBS colitis. Thus, this study concluded that HIF-1α was protective in the context of colitis likely due to the beneficial effect of HIF-1α in transcribing genes associated with barrier protection (e.g. Intestinal trefoil factor and CD73). Interestingly, a different conditional knockout of HIF-1α in the intestinal epithelium (this time cre-recombinase was driven by a villin promoter) displayed the opposite phenotype in a Dextran sodium sulphate (DSS)- induced model of experimental colitis [43].. In this instance mice

lacking HIF-1α or HIF-1β (ARNT) in the intestinal epithelium were not significantly different from control littermates. VHL loss however, resulted in an exacerbated colitis phenotype that was dependent on HIF signaling. Interestingly, VHL loss in the intestinal epithelium resulted in accumulation of HIF-2α. HIF-1α was not detected in the colon epithelium. Thus, the authors ascribe the exacerbation of colitis seen in the VHL deficient mice to HIF-2α- dependent regulation of macrophage inhibitory factor (MIF) [43]. The reasons for the discrepancy in these phenotypes are not fully understood and are likely a consequence of different genetic models employed (fabp-cre Vs villin-cre) and models of colitis used (TNBS Vs DSS). Regardless, the fact that opposing phenotypes can be observed in such seemingly similar model systems encourages caution and circumspection in our interpretation of data from conditional knock-out models alone. Nevertheless, the studies reviewed above highlight the importance of the oxygen-sensing pathway in the normal regulation of epithelial cell function. Furthermore, there is significant evidence to support the concept that promoting activation of the HIF pathway with pharmacological hydroxylase inhibitors is of benefit in colitis [44].

The skin and lung epithelium interface directly with the atmosphere. The skin, like the intestinal epithelium has a sharp oxygen gradient outwards from the dermis to the epidermis and exterior hair follicles and sebaceous glands [45]. HIF-1α and HIF-2α are both expressed in keratinocytes and their function investigated.

HIF-1α was conditionally deleted in keratinocytes using a K16-cre. These mice displayed an abnormal skin phenotype in the aging mouse and had delayed wound healing [46]. Interestingly, using a K14-cre approach to knock out HIF-2α in keratinocytes, Cowburn et al. demonstrated that these transgenic mice show faster wound closure and keratinocyte migration speed [47]. Importantly; in the context of infection and inflammation; fewer bacteria were recovered in from the keratinocyte specific HIF-2α knock out mice. Thus, there appears to be conflicting roles for HIF-1α

and HIF-2α in the context of skin wound healing. The authors [47] propose that this might be explained by the known differential roles for HIF-1α and HIF-2α with respect to c-Myc [48].

3) Dendritic cells

Dendritic cells (DCs) are key immune cells involved in antigen presentation to T-cells acting at the interface between innate and adaptive immunity. Similar to other immune cell types the local environment surrounding a dendritic cell can experience drastically different oxygen tensions between conditions of health and disease. The role of hypoxia and HIF has been studied in DCs. The combination of hypoxia and a pro-inflammatory stimulus resulted in decreased DC expression of co-stimulatory markers [49]. Mancino et al. also demonstrated impairment of specialised DC functions in DCs exposed to hypoxia while in contrast reporting higher expression of pro-inflammatory cytokines including TNFα and IL-1β. The authors postulate that the hypoxic microenvironment can uncouple or dissociate two important roles of the DC in (i) tissue repair and (ii) promotion of inflammation [50]. The role of HIF-1α in DCs has been addressed using an siRNA approach in BMDM derived DCs. Naldini et al. demonstrated that hypoxia induces cell death in immature DCs and that is attenuated with siRNA directed to HIF-1α [51]. Together this supports the concept of HIF-1α playing a key role in cell fate/survival in immature DCs exposed to hypoxia. Using myeloid deficient HIF-1α knock out mice Wobben et al. observed that DCs lacking HIF-1α have impaired interferon α and β production [52]. Using a different genetic approach to deplete HIF-1α in DCs Köhler et al. used CCL17-cre and CD11c-cre mice in combination with $HIF1\alpha^{flox}$ mice. This study demonstrated hypoxia-dependent changes in DC migration and IL-22 production, which were dependent on HIF-1α as well as alterations in the expression of other cytokines independent of HIF-1α [53]. Thus, taken together there is clearly a role for hypoxia-dependent signaling and HIF-1α in

shaping DC cell signaling with other hypoxia-sensitive transcription factors likely also playing a role.

4) Natural Killer T-cells

NKT cells are early response immune cells that play a role in several pathologies including ischemia-reperfusion injury (IRI). They are the only lymphocyte sub-type involved primarily in innate immunity. Zhang et al. investigated the role of HIF- 2α in NKT cells using an Lck-cre – dependent genetic approach in a model of renal IRI. Mice with HIF-2α deficient NKT cells exhibited a more severe form of renal injury suggestive of HIF-2α normally playing a role in restricting NKT cell activity in IRI [54].

HIF and adaptive immunity

1) Lymphocytes

T-lymphocytes (T-cells) are key immune cell-types of the adaptive immune response. Lymphoid progenitors originating in the bone marrow migrate to the thymus for maturation and they can differentiate into a variety of subtypes including T helper (Th) (CD4+), cytotoxic T-cell (CD8+) and T regulatory (T-Reg) cells [55]. These cells can then reside in primary or secondary lymphoid tissue. This migratory and differentiation life-cycle ensures that these lymphocytes encounter different oxygen gradients during their development [56] which alters the proportion of T-cell sub-types present. Furthermore, the energetic/ metabolic profile of the distinct T-cell sub-types is different e.g. Treg cells use lipid oxidation and oxidative phosphorylation to a greater extent than pro-inflammatory Th cells which have a greater reliance on glycolysis [57, 58]. Thus, interpreting the contribution of HIF-1α which is oxygen, pathogen and metabolism sensitive is highly complex in the context of multiple T-cell sub-types. The role of HIF-1α in T-cell differentiation and function has been expertly reviewed previously [59].

Helper T-cells (Th) and regulatory T-cells (Treg).

Makino et al. reported a key role for HIF-1α in regulating survival in human T cells (along with TCR activation). This concept was further investigated by Biju et al. using mice that conditionally lacked VHL and HIF [60]. Thymocytes that lacked VHL (and consequently had elevated HIF-1α levels) had decreased survival levels as a consequence of HIF-1α dependent increase in caspase 8 enzymatic activity, at least in part. HIF-1α expression however is thought to favor Th17 differentiation via increased IL-17 production (downstream of elevated RORγT) [61] and the subsequent HIF-dependent increase in glycolysis facilitates Th17 differentiation [58]. Thus, pro-inflammatory Th-17 differentiation is intrinsically linked to the expression of HIF-1α. The role of HIF-1α in Treg differentiation appears more complex with HIF-1α reported to attenuate Treg development by targeting FOXP3 for degradation [61]. However, hypoxia can promote FOXP3 expression (and consequently Treg differentiaton) and HIF-1α is required for optimal Treg function in models of disease [62]. These divergent responses with respect to the role of HIF-1α in Treg cells are not straight-forward to reconcile. Clambey et al. propose that the ultimate fate of Treg cells in hypoxia is likely due to the integrated action of HIF-1α, TGFβ (a growth factor that can regulate and be regulated by HIF, and is involved in promotion of both Treg and Th17 cells) and the cytokines present in the local microenvironment [62].

Cytotoxic CD8+ T cells.

The role of HIF-1α has also been studied in cytotoxic T-lymphocytes (CTLs). Finlay et al. used a conditional knockout of HIF-1β in CTLs [63]. HIF-1β is the constitutive nuclear binding partner of both HIF-1α and HIF-2α. Thus, through deletion of the transcriptional co-activator, HIFα –dependent signaling would be ablated in CTLs (as well as other HIF1β-dependent signaling pathways (e.g. the dioxin signaling pathway). mTORC1 is a key transcriptional regulator in CTLs and its control of glucose uptake and glycolysis in CTLs was found to be dependent on HIF-1. Of note, HIF-1 was not

responsible for proliferation in CTLs, but HIF-1β deficient CTLs expressed diminished levels of cytolytic effector molecules such as perforin and granzyme [63]. Using a different conditional knockout model where VHL is depleted in CTLs (in order to amplify HIFα-dependent signaling) Doedens et al. observed augmented CTL glycolytic metabolism and effector capacity. The role of HIF-1α vs HIF-2α was addressed with respect to HIF-dependent effector molecule expression (e.g. Granzyme B). Interestingly, the cytokine milieu played a key role in determining whether the augmented effector molecule expression was exclusively HIF-1α dependent or due to a dual role for HIF-1α and HIF-2α. Taken together these studies highlight an important role for HIFα in host protection [63]. In the context of infection or cancer HIF plays a central role in the dynamic interplay between the different immune cell types present. For example macrophages (via a HIF-1α -dependent mechanism) can suppress localized T-cell responses [64]. This has the potential to limit immunopathology or inhibit tumor cell killing depending on the context.

B-lymphocytes.

The role of the oxygen-sensing pathway has also been studied in B lymphocytes. . Kojima et al. used a genetic strategy which involved generating a chimeric HIF-1α-/- → Rag2-/- mouse. This mouse overcame the embryonic lethality of the HIF-1α knockout phenotype and generated lymphocytes which were derived from the injected embryonic stem cells (i.e. HIF-1α +/+ or HIF-1α -/-). The HIF-1α-/- peritoneal B1-like cells were abnormal and distortions in B-2 lymphocyte maturation was evident in bone marrow [65]. Thus, HIF-1α was deemed essential for normal B-cell development and self-tolerance. The mechanisms downstream of HIF in this phenotype were further explored. Kojima et al. identified that B-cells have a 'stage-specific' glucose dependency that might explain the central role of HIF-1α in B-cell development [66].

In summary there is significant evidence that the HIF pathway is of central importance to several aspects of immune cell function e.g. metabolism, survival/ apoptosis, differentiation and cytokine expression. It is clear that the roles of HIF-1α and HIF-2α are not redundant in this context with distinct and overlapping targets. With the advent of conditional knockout-deletion strategies our ability to study the role of the HIFs in specific cell types has expanded enormously. However, the apparent conflicts that can exist in genetically similar models should sound a word of caution regarding over-reliance on these models in isolation. This is compounded by the fact that immune cells by their very nature migrate to areas that vary greatly in their microenvironment (e.g. oxygen levels, pH, CO_2 levels, presence of other cells, cytokines, bacteria, virus etc.). Thus, some of the effects detailed above become more or less obvious depending on factors such as the differentiation stage of the immune cell or the presence or absence of a specific cytokine. It will be interesting to evaluate the contribution of the next generation of HIF-modulating- pharmacological agents [44] in models of inflammation and immunity.

PHDs and innate immunity

1) Myeloid cells

The genetic approaches detailed above have been very informative in defining the role of the HIFα subunits in the myeloid cell response to low oxygen. The contribution of specific prolyl and asparagynl hydroxylases to these cellular effects has been further investigated using whole animal or conditional knock out of PHDs1-3.

PHD-2 is the hydroxylase which is most associated with the oxygen-dependent degradation of HIFα [67]. Whole animal PHD2 deletion is embryonic lethal [68]. Haploinsufficiency for PHD2 promotes macrophage skewing towards an increase in M2 marker expression e.g TGFβ in a model of hindlimb ischemia. Haplodeficiency for PHD2 was thus deemed to be pro-arteriogenic and mechanistically dependent on

activation of canonical NF-kB [69]. Myeloid specific deletion of PHD2 resulted in macrophages with generally suppressed M1 macrophage markers e.g. TNF, IL-6, IL1β. Interestingly, the authors suggest that this decreased pro-inflammatory cytokine production occurs independently of the HIF pathway and propose altered NF-kB activity as a mechanism [70].

PHD-3 knockout mice are viable and the role of PHD3 in myeloid cells has been studied in whole animal and conditional knockout mice. Swain et al. investigated the impact of selective deletion of PHD3 (EGLN3) in the myeloid lineage (LysM cre-driven). BMDMs from these mice did not demonstrate altered HIF-1α or HIF-2α expression nor did they exhibit aberrant M1/M2 polarisation [71]. These PHD3 depleted macrophages did however show an anti-apoptotic phenotype suggesting a role for PHD3 in controlling macrophage apoptosis and life expectancy within hypoxic and inflamed niches.

Kiss et al. studied the role of PHD3 in macrophages derived from a whole animal PHD3 knockout mouse. These PHD3-/- mice were more susceptible to sepsis, which was associated with enhanced pro-inflammatory macrophage activity, altered macrophage maturation and polarization. This phenotype was associated with enhanced NF-kB activity [72]. In the same PHD3-/- mouse model, Walmsley et al. studied the role of PHD3 on neutrophil function. Similar to Swain et al. [71] these PHD3 deficient neutrophils also demonstrated an apoptosis/ survival associated phenotype. The previously reported hypoxia-induced neutrophil survival phenotype is lost in PHD3 deficient neutrophils with the consequence that there is reduced neutrophilic inflammation in PHD3-/- animal exposed to colitis [73]. Interestingly, there is a distinct transcriptional profile in PHD3-/- neutrophils compared to HIF-1α -/- neutrophils, with the hydroxylase deficient neutrophils maintaining normal metabolic and antimicrobial function. Thus, the PHD3-/- pro-apoptotic neutrophil phenotype is more complicated than a simple feedback regulation via HIF. In fact, the phenotype seen in the HIF-1α

conditional knockout neutrophils may in part be attributable to loss of PHD3 (which is evident in HIF-1α-/- neutrophils exposed to hypoxia). Also notable, is the fact that there was no evidence of up-regulation of NF-kB targets in PHD3 deficient neutrophils [73] (as was seen in the PHD3 deficient macrophages[72]).

The role of PHD1 in myeloid cells is less well studied compared to PHD2 and PHD3. The likely reason for this is that PHD1-/- mice did not demonstrate exacerbation of lethality in a model of abdominal sepsis whereas PHD3-/- mice did [72]. In addition PHD1 expression levels were relatively insensitive to hypoxia and peptidoglycan stimulation [73].

In summary there is significant evidence linking PHD enzyme expression and myeloid cell function. The relative hierarchy of importance for distinct hydroxylase isoforms in myeloid cells is difficult to assess.. PHD2 is the main hydroxylase responsible for the regulation of HIF-1α and we have already discussed the major importance of HIF-1α in myeloid function. However, the embryonic lethality of the whole animal PHD2 knockout has left us with fewer tools to test myeloid PHD2 function, compared to PHD1 and PHD3 function. However, myeloid phenotypes from haplo-insuffient PHD2 mice and myeloid deficient PHD2 mice point more to signaling roles for NFkB than HIF. Furthermore the phenotype of PHD3-/- and HIF-/- neutrophils are distinct. Perhaps the main effects of PHD loss in the context of myeloid cells are primarily on pathways other than HIF.

2) Epithelial cells

Intestinal epithelium

Several studies using pharmacological inhibition of the hydroxylase enzyme family demonstrated improvements in a number of inflammatory conditions including models of IBD (discussed in more detail later). For this reason, the role of the PHDs in the intestinal epithelium was investigated. Using whole animal knock outs of PHD1, PHD3

and a heterozygous depletion of PHD2, Tambuwala et al demonstrated that loss of PHD-1 (and not PHD-3 or PHD2+/-) was protective in a DSS model of colitis. This protection was a consequence of increased barrier protection downstream of an inhibition of epithelial cell apoptosis [74]. Furthermore, Tambuwala et al. demonstrated that PHD1 expression correlated with disease severity in patients with IBD [74]. In a more expansive clinical analysis of PHD expression in IBD, PHD1 was again found to be overexpressed in IBD patients at the mRNA and protein level. PHD-2 expression was unchanged and PHD3 was altered at the mRNA level only [75]. Together these studies illustrate the importance of PHD1 expression in maintenance of intestinal barrier function.

Chen et al. examined the role of PHD-3 in the intestinal epithelium using a Villin-cre strategy [76]. Interestingly, these PHD-3 deficient mice developed spontaneous colitis which was not evident in the whole animal knockout [74]. The authors propose a mechanism whereby PHD3 stabilises the junctional protein occludin in a manner independent of catalytic hydroxylase activity [77]. Furthermore the protein level of PHD3 was inversely correlated with the severity of UC in this study. Given that the functional role for PHD3 in this model is as a structural protein (as opposed to an oxygen sensing enzyme), the significance for this finding in the context of inflammatory hypoxia is unclear. Pan-hydroxylase inhibition has consistently been shown to be anti-inflammatory in the context of inflammation, whereas PHD3 deletion in this study leads to spontaneous colitis. These findings can however be reconciled due to the fact that one study is modulating hydroxylase activity whereas the other is modulating hydroxylase expression. This is an important point to note as we continue to develop our arguments for using pharmacological inhibitors of hydroxylases, informed by phenotypes derived from animals that are genetically deficient in hydroxylase enzymes..

Skin epithelium

Examining the effect of PHD-2 specifically in keratinocytes Kalucka et al. employed a K14-cre deletion strategy. Similar to the studies above, a wound healing phenotype was observed. Deletion of PHD2 in keratinocytes resulted in accelerated wound healing and keratinocyte migration in these mice that was downstream of HIF-1α dependent regulation of β3 integrin [78].

PHDs and adaptive immunity

1) Lymphocytes

As discussed above the role of HIF in the context of tumor biology is complex. Mamlouk et al. investigated the role of PHD2 in conditional knockout mouse under the control of CD68-cre. This mouse lacked PHD2 in a number of cell types [79] which was associated with a suppression of tumor growth. Using further genetic tools Mamlouk et al were able to implicate loss of PHD2 in myeloid cells and T-lymphocytes together as key cells involved in conferring the tumor suppression phenotype [80]. This is thought to be at least in part as a consequence of an altered cytokine profile in PHD2 deficient myeloid and T-cells.

In summary there is significant evidence for the prolyl hydryoxylase enzymes playing key roles in immune cell function including macrophage polarisation, neutrophil survival, epithelial barrier function and integrin expression. The consequences of PHD loss in animal models cannot be fully explained by effects on the HIF pathway alone and other related pathways are likely involved including NF-kB. Similarly, there is emerging evidence for non-enzymatic functions for these proteins in the regulation of processes including barrier function. A careful dissection of phenotypes exhibited by hypoxia/pharmacological hydroxylase inhibition compared to genetic loss of a PHD enzyme will be helpful in dissecting these two distinct (enzymatic and non-enzymatic) regulatory functions of the PHDs in immune cells.

Pharmacological PHD inhibition.

The discussion above of the complex roles of oxygen-sensing hydroxylases and the HIF pathway in inflammation give rise to the question: what is the net effect of hypoxia / hydroxylase inhibition and the activation of the HIF pathway on inflammation? This is probably the most pertinent question from the point of view of potential therapeutic interventions in inflammatory disease. Because HIF performs many pro- and anti-inflammatory functions in different immune and non-immune cell sub-types, it makes it difficult to predict as to what role HIF would play in the complex milieu of a chronically inflamed tissue. In order to address this question, a pharmacological approach was first taken where the impact of systemic HIF hydroxylase inhibitors (which activate the HIF pathway) on *in vivo* models of inflammation were tested. Initial studies into the impact of this intervention in chemical models of colitis indicated a profoundly anti-inflammatory effect. These anti-inflammatory effects were subsequently recapitulated in a diverse range of models of inflammation both of the gastrointestinal tract and of other organs [42, 44]. Therefore while the role of HIF in inflammation and immunity is clearly complex and cell-type specific (Figure 3), the pharmacologic activation of this pathway *in vivo* appears to provoke a largely anti-inflammatory effect. These data provide support for the use of drugs such as hydroxylase inhibitors in the treatment of chronic inflammatory diseases.

A note of caution with respect to the use of systemic administration of hydroxylase inhibitors needs to be sounded. Because the HIF pathway is primarily responsible for the regulation of erythropoiesis [13], systemic exposure to hydroxylase inhibitors may provoke unwanted vascular side effects. In order to overcome this potential limitation, targeted drug delivery approaches have recently been adopted in order to achieve local delivery while minimizing systemic exposure [81]. In summary, targeting the

HIF/PHD pathway in inflammatory disease is an exciting area for potential future therapeutics.

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FIGURE LEGENDS

Figure 1: Causes of tissue hypoxia in the inflamed intestine. In the healthy state (left hand side), the intestinal epithelium serves as an effective barrier separating the intestinal microbiota from the mucosal immune system. Under such conditions, the tissue receives sufficient oxygen supply through the mucosal microvasculature. In the inflamed intestine (right hand side), an increase in intestinal permeability leads to transmigration of intestinal microbiota and luminal antigens to the submucosal compartment resulting in the initiation of mucosal inflammation. The infiltration of activated neutrophils which consume oxygen during the oxidative burst and the induction of microvascular dysfunction combine to render the inflamed mucosa hypoxic.

Figure 2. Evidence for hydroxylation in the NFkB pathway. The canonical NF-kB pathway and non-canonical pathway are outlined in this schematic. FIH-dependent hydroxylation of NF-kB family members are highlighted in red, and a putative PHD-dependent hydroxylation of IKKβ highlighted in green.

Figure 3: Impact of HIF and PHDs in immune cells. The functions of HIF and PHDs in cells of the innate (left hand side) and adaptive (right hand side) immune systems are outlined. Key references which described these roles are included in parentheses and are colour coded (orange for HIF-dependent roles, blue for PHD-dependent roles)