Hypoxic Influences On Myeloid Cells During Inflammation And Inflammation-Associated Cancer

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Abstract
Hypoxia is a prominent characteristic of many acute or chronic inflammatory diseases, and exerts significant influence on their progression. Macrophages and neutrophils are major cellular components of innate immunity and contribute not only to O2 deprivation at the site of inflammation, but also alter many of their functions in response to hypoxia to either facilitate or suppress inflammation. Hypoxia stabilizes HIF-αs in macrophages and neutrophils, and these O2-sensitive transcription factors are key regulators of inflammatory responses in myeloid cells. This body of work investigates the role of myeloid HIF-αs in the settings of several acute and chronic inflammatory diseases.

First, the role of pan-HIF signaling in acute intestinal inflammation is investigated by depleting myeloid ARNT, the obligate heterodimeric binding partner for both HIF-α subunits, in a dextran sodium sulfide-induced murine acute colitis model. Myeloid pan-HIF deficiency exacerbates infiltration of pro-inflammatory neutrophils and monocytes into inflamed colon. Myeloid HIF ablation also hinders macrophage functional conversion to a protective, pro-resolving phenotype, and elevates gut serum amyloid A levels during the resolution phase of colitis. These data suggest that myeloid HIF signaling promotes the effective resolution of acute colitis.

Second, the effect of HIF inhibition in colitis-associated cancer (CAC) and other inflammatory diseases is studied using myeloid-specific depletion of each HIF-α subunit or panHIF. Myeloid HIF deficiency elevates tumor burden and advances disease progression of murine CAC. Acute skin inflammation is slightly mitigated upon loss of myeloid ARNT. However, myeloid HIFs are dispensable for leukocyte influx in a peritonitis model and animal survival in an endotoxemia model. Collectively, these data suggest that HIF-αs adopt diverse roles during inflammation, and their functions highly depend on specific types and stages of disease.

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HYPOXIC INFLUENCES ON MYELOID CELLS DURING INFLAMMATION AND INFLAMMATION-ASSOCIATED CANCER

Nan Lin

A DISSERTATION

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ABSTRACT

HYPOXIC INFLUENCES ON MYELOID CELLS DURING INFLAMMATION AND INFLAMMATION-ASSOCIATED CANCER

Nan Lin
M. Celeste Simon

Hypoxia is a prominent characteristic of many acute or chronic inflammatory diseases, and exerts significant influence on their progression. Macrophages and neutrophils are major cellular components of innate immunity and contribute not only to O₂ deprivation at the site of inflammation, but also alter many of their functions in response to hypoxia to either facilitate or suppress inflammation. Hypoxia stabilizes HIF-αs in macrophages and neutrophils, and these O₂-sensitive transcription factors are key regulators of inflammatory responses in myeloid cells. This body of work investigates the role of myeloid HIF-αs in the settings of several acute and chronic inflammatory diseases.

First, the role of pan-HIF signaling in acute intestinal inflammation is investigated by depleting myeloid ARNT, the obligate heterodimeric binding partner for both HIF-α subunits, in a dextran sodium sulfide-induced murine acute colitis model. Myeloid pan-HIF deficiency exacerbates infiltration of pro-inflammatory neutrophils and monocytes into inflamed colon. Myeloid HIF ablation also hinders macrophage functional conversion to a protective, pro-resolving phenotype, and elevates gut serum amyloid A levels during the resolution phase of colitis. These data suggest that myeloid HIF signaling promotes the effective resolution of acute colitis.

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# TABLE OF CONTENTS

ACKNOWLEDGEMENT.................................................................................................................. III

ABSTRACT ..................................................................................................................................... IV

LIST OF TABLES.......................................................................................................................... VIII

LIST OF FIGURES........................................................................................................................ IX

CHAPTER 1 ..................................................................................................................................... 1

INTRODUCTION........................................................................................................................... 1

INFLAMMATION AND HYPOXIA.............................................................................................. 1

HYPOXIA AND HYPOXIA-INDUCIBLE FACTORS................................................................. 3

MYELOID CELLS IN INFLAMMATION ..................................................................................... 4

HIF IN MYELOID CELLS ........................................................................................................... 7

SUMMARY .............................................................................................................................. 21

CHAPTER 2 ................................................................................................................................... 22

MYELOID CELL HYPOXIA-INDUCIBLE FACTORS PROMOTE RESOLUTION OF
INFLAMMATION IN EXPERIMENTAL COLITIS ................................................................. 22

INTRODUCTION ..................................................................................................................... 22

RESULTS ................................................................................................................................ 24

DISCUSSION .......................................................................................................................... 52

CHAPTER 3 ................................................................................................................................... 57

DIFFERENTIAL ROLES OF MYELOID CELL HIFS IN OTHER INFLAMMATORY DISEASES .. 57

INTRODUCTION ..................................................................................................................... 57

RESULTS ................................................................................................................................ 58

DISCUSSION .......................................................................................................................... 73

CHAPTER 4 ................................................................................................................................... 79
LIST OF TABLES

Chapter 1: Hypoxia-inducible factors: key regulators of myeloid cells during inflammation

Table 1 Summary of myeloid HIF’s role in various inflammatory scenarios

Chapter 4: Concluding Remarks

Table 2 Significantly changed diseases and biological functions in ARNT-deficient lamina propria macrophages
LIST OF FIGURES

Chapter 1: Hypoxia-inducible factors: key regulators of myeloid cells during inflammation

Figure 1 Overview of the known roles of HIF-1α and HIF-2α in myeloid cells
Figure 2 Contact-dependent myeloid HIF-α effector functions

Chapter 2: Myeloid cell hypoxia-inducible factors promote resolution of inflammation in experimental colitis

Figure 3 Arnt deletion in macrophages disrupts both HIF-1α and HIF-2α transcriptional activity
Figure 4 Myeloid ARNT depletion does not affect phagocytosis, ATP generation in BMDMs, and peripheral lymphocytes composition
Figure 5 Myeloid HIF-α/ARNT heterodimer deficiency impairs resolution of DSS-induced acute colitis
Figure 6 Myeloid HIF-α/ARNT heterodimer deficiency promotes secretion of pro-inflammatory cytokines in the colon
Figure 7 ARNT deficiency suppresses AhR ligand-induced AhR target gene expression, but does not affect their expression under hypoxia
Figure 8 Myeloid HIF-1α and HIF-2α are required for proper resolution of DSS-induced acute colitis
Figure 9 Characterization of myeloid cells in LysMCre and LysMCre;Arntfl/fl lamina propria
Figure 10 Myeloid HIF deficiency does not alter composition of T cells and B cells in lamina propria
Figure 11 Arnt is partially deleted in neutrophils
Figure 12 Increased neutrophil numbers in lamina propria of LysMCre;Arntfl/fl mice is due to elevated CXCL1, not enhanced survival
Figure 13 HIF deficiency renders macrophages less pro-resolving
Figure 14 Myeloid HIF deficiency favors M1 polarization of macrophages in vitro
Figure 15 Myeloid HIF deficiency does not favor macrophage M1 polarization in vivo
Figure 16 Myeloid HIF deficiency elevates serum amyloid As gene expression in lamina propria macrophages
Figure 17 Myeloid HIF deficiency results in increased serum amyloid As gene expression and production in colon
Figure 18 Myeloid HIF deficiency alters PGE2 production in macrophages and in colon
Chapter 3: Differential roles of myeloid cell HIFs in other inflammatory diseases

Figure 20  Deficiency of both HIF-αs in myeloid cells worsens tumor burden of murine colitis-associated colorectal cancer

Figure 21  Deficiency of both HIF-αs in myeloid cells results in larger and more advanced tumors in murine CAC model

Figure 22  Macrophage infiltration in murine CAC model is not altered upon loss of both HIF-αs in myeloid cells

Figure 23  Mice with myeloid HIF-2α deficiency are more susceptible to DSS-induced inflammation in AOM/DSS CAC model, displaying a survival disadvantage

Figure 24  Myeloid HIF-2α deficiency does not significantly affect disease onset and resolution in a DSS-induced chronic colitis model

Figure 25  Myeloid HIF-2α deficiency does not change the composition of lamina propria immune cell populations in a DSS-induced chronic colitis model

Figure 26  Myeloid HIF deficiency mildly alleviates TPA-induced cutaneous inflammation

Figure 27  Myeloid HIF deficiency increases myeloid cell numbers at the site of TPA-induced cutaneous inflammation

Figure 28  Myeloid HIF deficiency significantly increases CD11c⁺, F4/80⁺ neutrophils during TPA-induced cutaneous inflammation

Figure 29  Myeloid HIF deficiency does not affect recovery in the chronic cutaneous inflammation model induced by TPA

Figure 30  Myeloid HIF deficiency does not affect immune cell infiltration in the thioglycollate-induced peritonitis model

Figure 31  Survival in a LPS-induced endotoxemia model is not dependent on myeloid HIF signaling
CHAPTER 1

Introduction

This chapter has been adapted from the following review article: Lin, N. and Simon, M.C. (2016). Hypoxia-inducible factors: key regulators of myeloid cells during inflammation. J Clin Invest. 2016;126(10):3661-3671. https://doi.org/10.1172/JCI84426.

INFLAMMATION AND HYPOXIA

Inflammation constitutes the body’s defensive response to injury and/or infection in order to eliminate pathogens and damaged tissue, while initiating tissue repair and healing. Upon tissue injury or pathogen invasion, local sentinel cells such as resident macrophages and mast cells respond to dilate blood vessels, increase vascular permeability, and recruit a variety of leukocytes to the site of inflammation. During the acute phase of inflammatory responses, a major task of such recruited cells is the clearance of damaged tissue or pathogens. Upon transition into the resolution phase, tissue homeostasis is gradually restored. If acute inflammation fails to subside, it progresses into chronic inflammation with potentially serious consequences for the afflicted patient (Nathan and Ding 2010, Fullerton and Gilroy 2016). One feature of inflammation sites is low oxygen (O2) tension, termed “hypoxia.” Oxygen tension ranges between 2.5% and 9% in most healthy tissues. However, poor O2 availability resulting from damaged vasculature, high metabolic rates of bacteria and other pathogens, and numerous infiltrating immune cells deprive inflamed tissue of O2, frequently leading to partial O2 pressures (pO2) of less than 1% (Imtiyaz and Simon 2010, Lewis et al. 1999).

The interdependence between inflammation and hypoxia has been evident for many years. Hypoxia is prevalent in multiple inflammatory scenarios, such as inflammatory bowel diseases (IBDs) and rheumatoid arthritis (RA) (Sivakumar et al.
2008, Muz et al. 2009, Colgan and Taylor 2010, Taylor and Colgan 2007). The intestinal mucosa exhibits an O2 gradient from crypt to villus, wherein O2 is highest in the crypts and lowest in the villus tips, which are closest to the anoxic gut lumen (Zheng, Kelly, and Colgan 2015). This “physiological” hypoxia is largely extended with intestinal inflammation (Colgan and Taylor 2010, Taylor and Colgan 2007). Hypoxia is also a characteristic of inflamed joints in patients with RA. Using a highly sensitive gold microelectrode, investigators accurately measured synovial O2 tension in RA patients (Sivakumar et al. 2008), demonstrating that RA median O2 tension in synovial tissue (2%–4%) was much lower than that in the noninflamed synovium (9%–12%) (Sivakumar et al. 2008, Muz et al. 2009).

Low O2 tension can also directly contribute to inflammation. In the setting of obesity, hypoxia develops as adipose tissue mass expands, initiating inflammatory responses. Secretion of inflammation-related adipokines (e.g., TNF-α and leptin) increases in hypoxic adipose tissue. Together with additional disruptions in glucose and lipid metabolism, this inflammation can become chronic and systemic, eventually leading to insulin resistance (Ye 2009, Trayhurn 2013). In the lung, alveolar hypoxia can be induced by exposing rats to 10% O2 for up to 8 hours, which triggers macrophage recruitment, enhances expression of HIF-1α and inflammatory cytokines (e.g., macrophage inflammatory protein 1-α [MIP-1α], monocyte chemoattractant protein-1 [MCP-1], and TNF-α), promotes NF-κB activity, and elevates albumin leakage (Madjdpour et al. 2003). Similar observations were made in mice exposed to 5% O2 for 60 minutes, where levels of IL-6, TNF-α, and IL-1α were elevated in serum and isolated peritoneal macrophages and Kupffer cells (liver macrophages) (Ertel et al. 1995). In humans, hypoxia-induced inflammation is evident in individuals with high-altitude illness. Those who ascend rapidly are at risk of developing high-altitude pulmonary and cerebral
edema, caused by hypoxic pulmonary vasoconstriction, high arterial and capillary pressure, and elevated levels of circulating IL-6, IL-1 receptor antagonist (IL-1RA), and C-reactive protein (CRP) (Bartsch and Swenson 2013, Hartmann et al. 2000). All of these examples indicate that the relationship between inflammation and hypoxia exists in many pathological settings, and is a potentially attractive therapeutic target.

HYPOXIA AND HYPOXIA-INDUCIBLE FACTORS

In many of the pathological situations described above (He et al. 2011, Lee et al. 2011, Madjdpour et al. 2003, Colgan and Taylor 2010, Sivakumar et al. 2008, Taylor and Colgan 2007), HIFs are activated in response to the hypoxic and inflammatory microenvironment. HIFs represent the primary O₂-sensing transcription factors (Majmundar, Wong, and Simon 2010, Mucaj, Shay, and Simon 2012, Keith, Johnson, and Simon 2012, Lee and Simon 2015), as heterodimers comprised of an O₂-sensitive α subunit (HIF-α) and a constitutively expressed β subunit (HIF-1β or aryl hydrocarbon receptor nuclear translocator [ARNT]). Three α subunits have been discovered thus far: HIF-1α, HIF-2α, and HIF-3α. While HIF-1α and HIF-2α are well characterized, relatively little is known about HIF-3α (Kaelin and Ratcliffe 2008, Gu et al. 1998). The HIF3A gene encodes multiple HIF-3α variants, which are structurally distinct from HIF-1α and HIF-2α, as they lack a C-terminal transactivation domain. Divergence in structure and variant diversity allow HIF-3α to have numerous modes of action, regulating a transcriptional program that is distinct from that of HIF-1α (Duan 2016). In this chapter, we will focus on HIF-1α and HIF-2α, but additional details about HIF-3α are reviewed elsewhere (Zhang et al. 2014, Duan 2016, Gu et al. 1998). Under normoxia, the O₂-sensitive α subunit is hydroxylated on two conserved proline residues (P402/P405 and P564/P531 for HIF-1α/HIF-2α, respectively) within the O₂-dependent degradation domain (Kuhl et al.) by prolyl hydroxylase domain–containing proteins (PHDs) (Kaelin 2005, Kaelin and Ratcliffe 2008). Hydroxylated HIF-α subunits are then polyubiquitinated by the von Hippel-Lindau (VHL) tumor suppressor E3 ubiquitin ligase complex and subsequently degraded via the 26S proteasome (Maxwell et al. 1999, Cockman et al. 2000,
Jaakkola et al. 2001). Under hypoxia, PHDs cannot hydroxylate key HIF-α proline residues due to limited access to their substrate (O₂) or redox imbalance (Chandel et al. 2000, Bell and Chandel 2007, Lin et al. 2008, Lee and Simon 2015), resulting in HIF-α stabilization. Stabilized HIF-αs translocate into the nucleus, dimerize with their obligate binding partner ARNT, recruit additional coactivators, and bind to hypoxia-response elements (HREs) to enhance transcription of hundreds of genes whose products mediate cellular adaptation to hypoxia. Such pathways include metabolism, angiogenesis, and inflammatory responses.

Other than O₂-dependent HIF posttranslational modifications, HIF-α stabilization can be induced by inflammatory stimuli independently of hypoxia. The pro-inflammatory cytokines TNF-α and IL-1β promote HIF-1α accumulation in an NF-κB–dependent manner (Jung, Isaacs, Lee, Trepel, Liu, et al. 2003, Zhou, Schmid, and Brune 2003, Jung, Isaacs, Lee, Trepel, and Neckers 2003). Bacterial products, such as LPS, can also stabilize HIF-1α under normoxia through multiple pathways, such as NF-κB (Fang et al. 2009, Rius et al. 2008), ROS (Nishi et al. 2008), PHDs (Peyssonnaux et al. 2007), and MAPKs (Frede et al. 2006). On the other hand, hypoxic responses can also be HIF independent. For example, hypoxia suppresses mTOR activity independently of HIF signaling (Arsham, Howell, and Simon 2003), via the mTOR inhibitor REDD1 and the TSC1/TSC2 complex (Brugarolas et al. 2004). Other hypoxia-responsive pathways include endoplasmic reticulum (ER) stress (Koumenis et al. 2002) and NF-κB (Cummins et al. 2006, Rius et al. 2008, Safronova et al. 2009) pathways. One myeloid-specific example is mentioned later in this chapter (Snodgrass et al. 2016).

MYELOID CELLS IN INFLAMMATION

Macrophages are key cellular components of innate immunity and encompass a highly heterogeneous population of cells with a broad array of phenotypes and functions. Some of these cells are distributed over most of the body, residing in many tissues (e.g., Kupffer cells in the liver, osteoclasts in the bone, and microglia in the brain), while others are differentiated monocytes that infiltrate sites of inflammation to promote adaptive responses or facilitate restoration of tissue
homeostasis (Davies et al. 2013, Davies and Taylor 2015). Upon pathogen invasion or injury, tissue-resident macrophages represent the first responders, recruiting neutrophils via secretion of chemokines (e.g., IL-8 in humans and CXCL1 in mice). Once neutrophils arrive at the compromised site, they release monocyte chemoattractants (e.g., MCP-1) so that large numbers of recruited monocytes/macrophages extend the inflammatory response (Wynn, Chawla, and Pollard 2013, Ginhoux and Jung 2014, Fournier and Parkos 2012). These macrophages normally adopt a pro-inflammatory or “classically activated” (M1) phenotype, which is often induced by IFN-γ and Toll-like receptor ligands. M1 macrophages elevate their secretion of reactive oxygen and nitrogen species (ROS and NOS) and pro-inflammatory cytokines, to eliminate pathogens and damaged tissues while recruiting additional immune effector cells. When most of the pathogens or tissue debris are removed, hyperactivation of macrophage bactericidal activity may result in unnecessary destruction of healthy tissue. As highly plastic cells, macrophages then respond to microenvironmental cues (e.g., T\textsubscript{H}2-type cytokines IL-4 and IL-13) and adopt an “alternatively activated” (M2) phenotype, which suppresses host defenses and facilitates wound healing and tissue remodeling to resolve inflammation and restore homeostasis at the inflamed site (Galli, Borregaard, and Wynn 2011, Gordon 2003, Sica and Mantovani 2012, Mantovani et al. 2013). This oversimplified segregation of macrophage phenotypes was originally applied to in vitro systems and has been widely used to provide a conceptual framework for subsequent research. However, given the complexity of in vivo microenvironments, macrophages exhibit phenotypes across a broad spectrum of activation states, and the simple M1/M2 dichotomy is unlikely to reflect physiological macrophage phenotypes (Martinez and Gordon 2014, Mosser and Edwards 2008, Xue et al. 2014).

macrophages are typically observed at atherosclerotic plaques. These maladaptive macrophages can induce a nonresolving inflammatory response leading to robust accumulation of cells, lipid, and matrix at the plaque. Defective macrophage efferocytosis (engulfment of dead cells) and enhanced apoptosis contribute to formation of a necrotic plaque core that might eventually rupture, causing platelet aggregation and thrombus formation (Moore and Tabas 2011, Moore, Sheedy, and Fisher 2013). The functional importance of myeloid cells in atherosclerosis is supported by experimental evidence that interventions to alter monocyte recruitment and/or survival can markedly affect disease progression (Lessner et al. 2002, Swirski et al. 2006, Landsman et al. 2009). Airway inflammation typically accompanies airway allergic asthma, another disease involving macrophages. The microenvironment in asthma is dominated by type 2-associated cytokines (e.g., IL-4 and IL-13), which preferentially polarize macrophages into the M2 state (Wynn, Chawla, and Pollard 2013). Elevated numbers of IL-4R+ macrophages have been reported in asthmatic patients with defective lung function (Melgert et al. 2011). Moreover, the presence of IL-4R+ macrophages exacerbates allergen-induced airway inflammation, whereas reduction of IL-4R+ macrophages alleviates this disease (Ford et al. 2012, Moreira et al. 2010).

Neutrophils, another major component of the innate immune response, are among the first cells recruited to inflammatory sites. These cells possess multiple means of eliminating invading pathogens, i.e., phagocytosis of microorganisms, degranulation to release antibacterial proteins, and emanation of neutrophil extracellular traps (NETs) (Kolaczkowska and Kubes 2013, Wright et al. 2010). Recently, many properties of neutrophils favoring the resolution of inflammation have been revealed (Jones et al. 2016), including production of Annexin A1 (Scannell and Maderna 2006) and lipid (e.g., LXA₄ and 13-series resolvins) pro-resolution mediators (Fierro et al. 2003, Dalli, Chiang, and Serhan 2015), chemokine/cytokine scavenging (e.g., CCL3 and CCL5) (Ariel et al. 2006), and apoptosis-induced macrophage efferocytosis (Leitch et al. 2008, Hallett et al. 2008). Similarly to macrophages, neutrophils are associated with multiple inflammatory syndromes, such as RA (Wipke and Allen 2001, Wright, Moots, and Edwards 2014, Wittkowski et al. 2007), chronic obstructive pulmonary disease (Hoenderdos and Condliffe 2013, Pesci et al. 1998), and IBDs (Fournier and Parkos 2012, Campbell et al. 2014). In
IBDs, for example, neutrophils contribute to elimination of pathogens and immune cell (e.g., macrophages) recruitment and activation, as well as mucosal wound healing and resolution of inflammation. Of note, precise roles of neutrophils during intestinal inflammation are currently under investigation and are highly debated (Campbell and Colgan 2015, Fournier and Parkos 2012). Some studies using colitis models, either chemically induced (dextran sulfate sodium [DSS] or dinitrobenzene sulfonic acid/trinitrobenzene sulfonic acid [DNBS/TNBS]) or immune system dysregulation–induced (CD4⁺CD45RBʰⁱ T cell transfer), showed that neutrophil depletion exacerbates colitis, suggesting a beneficial role of neutrophils in this setting (Kuhl et al. 2007, Zhang et al. 2011); however, other studies showed a completely opposite phenotype in which neutrophil depletion ameliorates colitis (Natsui et al. 1997, Kankuri et al. 2001). Additionally, the role of neutrophils could also depend on the concomitant presence of monocytes and macrophages (Qualls et al. 2006). Therefore, while neutrophils are clearly associated with intestinal inflammation, whether they exert beneficial or detrimental effects appears to be model dependent and condition dependent.

**HIF IN MYELOID CELLS**

It is noteworthy that myeloid cells localize predominantly within hypoxic subdomains of tumors and sites of inflammation, and multiple mechanisms have been proposed to explain how hypoxia promotes recruitment and retention of myeloid cells (Murdoch, Giannoudis, and Lewis 2004). Both HIF-1α and HIF-2α regulate myeloid migratory activity: HIF-1α is recruited to the CXCR4 promoter, stimulating CXCR4 transcription in human monocytes experiencing hypoxia (Schioppa et al. 2003). Moreover, CXCR4 is a key chemokine receptor mediating chemotactic responses to CXCL12 ligand, which is upregulated in ischemic tissues such as arthritic joints (Hitchon et al. 2002). The HIF-1α/PDK1 axis has been recently shown to contribute to macrophage migratory activity via induction of active glycolysis (Semba et al. 2016). For HIF-2α, Casazza and colleagues demonstrated a semaphorin 3A/neuropilin1–dependent (SEMA3A/NRP1-dependent) means of macrophage positioning within the tumor. Here, NRP1
Figure 1. Overview of the known roles of HIF-1α and HIF-2α in myeloid cells. Both HIF-1α and HIF-2α are required for key macrophage functions, such as cytokine production and the ability to migrate and invade. However, macrophage glycolysis, ATP generation, and bactericidal activity have been related to HIF-1α exclusively. Nevertheless, both isoforms contribute to pathogenesis of various acute inflammatory syndromes. Additionally, the roles of myeloid HIF-αs in the setting of tumor inflammation are currently being investigated. As compared with macrophages, less is known about HIF-αs in neutrophils. However, it is very clear that both isoforms are required to inhibit neutrophil apoptosis and elongate their lifespan. While HIF-1α facilitates bacterial killing by neutrophils, many neutrophil functions seem less dependent on HIF-2α, including respiratory burst, chemotaxis, and phagocytosis. Nevertheless, increased neutrophil HIF-2α accumulation correlates with increased neutrophilic inflammation and lung injury in an LPS-induced acute lung injury murine model.
repression, which triggers macrophage retention in hypoxic regions, is mediated by HIF-2α–dependent NF-κB activity (Casazza et al. 2013).

Given the preferential localization of myeloid cells in hypoxic regions, significant efforts have defined how HIF-1α and/or HIF-2α promote myeloid cell adaptation to hypoxic environments and mediate inflammation (Figure 1). Cramer and colleagues were the first to demonstrate the importance of HIF-1α in macrophage and neutrophil function in the setting of inflammation (Cramer et al. 2003). HIF-1α was ablated in myeloid cells using Lysozyme M (LysM) promoter–driven Cre recombinase, which is specific for the myeloid lineages, i.e., monocytes, macrophages, neutrophils, etc. Myeloid-specific Hif1a deletion results in defective glycolysis and ATP generation, leading to impairment of myeloid cell motility, invasiveness, aggregation, and bacterial killing. Moreover, mice with myeloid-specific HIF-1α deficiency are protected against acute and chronic cutaneous inflammation and arthritis. Subsequent studies investigating HIF-1α specifically in neutrophils demonstrated that hypoxia-induced inhibition of neutrophil apoptosis is dependent on HIF-1α (Walmsley et al. 2005), and that HIF-1α is required for phagocytes to fully exert their bactericidal activity (Peyssonnaux et al. 2005). More recent attention has focused on myeloid cell immunometabolism (Kelly and O'Neill 2015, O'Neill and Pearce 2016). Myeloid cells can undergo metabolic reprogramming to adapt to critical changes in the microenvironment. Tannahill and colleagues demonstrated that LPS exposure can alter glutamine-dependent anaplerosis (replenishment of TCA cycle intermediates) to elevate succinate levels, which further stabilize HIF-1α in macrophages, resulting in increased production of IL-1β (Tannahill et al. 2013). These findings serve as an excellent example of how HIF-1α–dependent immunometabolism can directly affect cytokine production by macrophages.

The role of HIF-2α in myeloid cells has also been investigated using the LysM-Cre–mediated deletion strategy. Macrophages lacking HIF-2α exhibit defects in the production of inflammatory cytokines/chemokines in response to hypoxia, migration, and invasion. Myeloid HIF-2α deficiency also protects mice in models of sepsis, cutaneous inflammation, peritonitis, hepatocellular carcinoma, and colitis-associated colorectal cancer (Pesci et al.) (Imtiyaz et al.
Like HIF-1α, neutrophil HIF-2α contributes to hypoxia-induced inhibition of apoptosis. HIF-2α deficiency increases neutrophil apoptosis in vivo and ex vivo, leading to suppression of neutrophilic inflammation and inflammatory responses during acute lung injury (Thompson et al. 2014).

One interesting observation from the work of Imtiyaz and colleagues (Imtiyaz et al. 2010) is that, unlike myeloid HIF-1α, HIF-2α deficiency does not alter cellular ATP production. The notion that HIF-1α and HIF-2α exert non-redundant or even opposing functions in macrophages is further supported by a study showing that HIF-1α and HIF-2α differentially regulate NO production by controlling expression of iNOS and arginase 1, respectively (Takeda et al. 2010).

Given the complex roles of HIF-1α and HIF-2α in macrophages, pan-HIF inhibition via pharmacological or genetic methods (i.e., Arnt deletion) is warranted. In a murine CAC model, treatment with the HIF inhibitor acriflavine reduces both tumor burden and macrophage infiltration (Shay et al. 2014). Additionally, myeloid cell–specific ARNT deficiency reduces macrophage pro-inflammatory cytokine production, and mice lacking myeloid ARNT are protected from cutaneous inflammation and exhibit delayed wound healing (Scott et al. 2014).

In the following sections, we will discuss the roles of myeloid HIF-αs in the settings of specific inflammatory diseases, as summarized in Table 1.

**Atherosclerosis**

Atherosclerosis is a chronic inflammatory disease of the arterial vasculature. Retention of apolipoprotein B–containing lipoproteins and accumulation of cholesterol-laden macrophages in the artery wall contribute to this syndrome. Monocytes are first recruited to differentiate into mononuclear phagocytes and ingest lipoproteins; however, lipid buildup in these cells transforms them into foam cells that exhibit dysregulated lipid metabolism and elevated secretion of pro-inflammatory cytokines (e.g., IL-6 and TNF-α) and macrophage retention factors (i.e., netrin 1 and semaphorin 3E). Foam cells promote the further progression of atherosclerosis (Moore and Tabas 2011, Moore, Sheedy, and Fisher 2013).
Table 1. Summary of myeloid HIF’s role in various inflammatory scenarios

<table>
<thead>
<tr>
<th>Inflammatory disease</th>
<th>HIF subunit</th>
<th>Overall effect</th>
<th>Proposed mechanisms</th>
<th>References</th>
</tr>
</thead>
</table>
| Atherosclerosis      | HIF-1α      | Promotes inflammation | 1. Enhances lipid uptake  
2. Induces sterol synthesis 
3. Suppresses cholesterol efflux 
4. Elevates proteoglycans secretion 
5. Promotes angiogenesis 
6. Increases glycolytic flux 
7. Sustains viability 
|                      | HIF-2α      | Promotes inflammation | Elevates proteoglycans secretion | Asplund et al. 2010 |
|                      | HIF-1α      | Promotes inflammation | Enhances macrophage M1 polarization | Fujisaka et al. 2013 |
|                      | HIF-2α      | Suppresses inflammation | 1. Represses NO and pro-inflammatory cytokines production from macrophages 
2. Improves insulin resistance in adipocytes | Choe et al. 2014 |
|                      |             | No effect |                     | Snodgrass et al. 2016 |
| Sepsis               | HIF-1α      | Promotes inflammation | Pro-inflammatory cytokine production | Peyssonnaux et al. 2007, Mahabeleshwar et al. 2012 |
|                      | HIF-2α      | Promotes inflammation | 1. Maintains serum levels of pro-inflammatory cytokines 
2. Lowers IL-10 level | Imtiyaz et al. 2010 |
|                      | HIF-1α      | Promotes inflammation | 1. Promotes VEGF and CXCL1 expression 
2. Enhances eosinophil infiltration | Byrne et al. 2013, Crotty Alexander et al. 2013 |
|                      | HIF-2α      | Suppresses inflammation | 1. Elevates IL-10 level to block dendritic cell and T helper cell response 
2. Inhibits neutrophil apoptosis | Toussaint et al. 2013, Shepardson et al. 2014 |
| Airway allergy and asthma | HIF-1α | Promotes inflammation |                     |                |
|                      | HIF-1α      | Suppresses inflammation |                     |                |
|                      | HIF-2α      | Suppresses inflammation |                     |                |
| Gastritis            | HIF-1α      | Suppresses inflammation |                     | Malak et al. 2015 |
| Renal fibrosis and inflammation | HIF-1α | Suppresses inflammation | 1. Represses CCR2 and CCL2 expression to inhibit macrophage infiltration 
|                      | HIF-2α      | Suppresses inflammation | Represses CCR2 and CCL2 expression to inhibit macrophage infiltration | Kobayashi et al. 2012 |
| Arthritis            | HIF-1α      | Promotes inflammation |                     | Cramer et al. 2003 |
|                      | HIF-2α      | Promotes inflammation |                     | Cramer et al. 2003 |
| Cutaneous inflammation | HIF-1α  | Promotes inflammation |                     |                |
|                      | HIF-2α      | Promotes inflammation | Increases neutrophil infiltration | Imtiyaz et al. 2010 |
Elevated levels of HIF-1α and HIF-2α are detected in human atherosclerotic carotid plaques compared with normal arteries, where HIF-1α colocalizes with CD68, a macrophage marker (Pedersen et al. 2013). Hypoxia has been implicated as a pathogenic factor in atherosclerosis and contributes to the pro-atherosclerotic functions of macrophages (Figure 2A) (Marsch, Sluimer, and Daemen 2013). Several reports showed that lipid uptake and foam cell formation are dependent on hypoxia and HIF-1α (Jiang et al. 2007, Crucet et al. 2013, Parathath et al. 2011). Both murine and human macrophage cell lines increase cellular neutral lipid content when cultured under hypoxic conditions; however, this effect is reversed upon HIF-1α depletion. Multiple HIF-1α–dependent mechanisms have been proposed for this phenotype. For example, hypoxia enhances expression of lectin-like oxidized low-density lipoprotein (oxLDL) receptor-1 (LOX-1), which promotes oxLDL uptake in the murine macrophage cell line RAW264.7; silencing of HIF-1α diminishes the upregulation of LOX-1 (Crucet et al. 2013). Hypoxic J774 murine macrophages exhibit elevated sterol accumulation due to (a) enhanced sterol synthesis via increased 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase activity and (b) suppressed cholesterol efflux due to altered subcellular localization of ATP-binding cassette subfamily A member 1 (ABCA1) (Parathath et al. 2011). These phenotypic changes are also HIF-1α-dependent. In U937 human monocytes, oxLDL treatment increases the expression of 70 out of 96 key genes that are known to be involved in atherosclerosis, while 57 of these genes (e.g., cyclooxygenase-2 [COX-2], vascular cell adhesion molecule [VCAM-1], and IL-1β) are downregulated with HIF-1α siRNA pretreatment (Jiang et al. 2007). Other pro-atherosclerotic functions of macrophages, such as promotion of angiogenesis and proteoglycan synthesis, are also dependent on HIF-αs (Hutter et al. 2013, Asplund et al. 2010). In a coculture system of human monocytes/macrophages and endothelial cells, oxLDL strongly induces HIF1A and VEGFA expression in macrophages, while increasing endothelial cell tube formation. Of note, oxLDL pro-angiogenic effects are partially lost upon HIF-1α inhibition (Hutter et al. 2013). The notion that myeloid HIF-1α can promote angiogenesis through VEGF upregulation is demonstrated in other studies as well (Ahn et al. 2014). Macrophages can also contribute to pathogenesis by secreting proteoglycans (PGs) such as versican, which modulate lipoprotein
retention and the activity of enzymes, cytokines, and other growth factors in atherosclerotic lesions. Increased versican and perlecan expression is detected in macrophages under hypoxia; versican is coregulated by HIF-1α and HIF-2α, while perlecan is only dependent on HIF-1α (Asplund et al. 2010). Myeloid HIF-1α is also a critical regulator of both glycolytic metabolism and pro-inflammatory activation of macrophages, and is stabilized by cues in the atherosclerotic microenvironment, such as hypoxia and cytokines. HIF-1α increases transcription of the gene encoding 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3), a key enzyme in the glycolytic pathway, leading to (a) increased glycolytic flux, (b) increased pro-inflammatory cytokine production (e.g., TNF-α), and (c) maintenance of macrophage viability (Tawakol et al. 2015). Together, these studies reveal that HIF-αs are crucial components in determining macrophage pro-atherosclerotic functions.

Unfortunately, in vivo studies do not always provide consistent findings. In a wire-induced vascular injury model, myeloid HIF-1α promotes vascular inflammation and remodeling manifested by increases in TNF-α and IL-6 levels proximal to the injury site and neointimal thickening of injured arteries (Nakayama et al. 2013). However, a recent in vivo genetic and drug-based approach suggested the opposite effect in a different mouse model, indicating that HIF-1α and HIF-2α accumulation correlates with reduced atherosclerosis development. The authors inhibited PHD2, resulting in HIF-1α and HIF-2α stabilization, by administering a pharmacological inhibitor (FG-4497) in an LDL receptor–deficient model of atherosclerosis or by crossing Hif-p4h-2 hypomorphic (Hif-p4h-2<sup>−/−</sup>) mice with LDL receptor–deficient mice. PHD2 inhibition led to reductions in levels of atherosclerotic plaque formation, weight gain, insulin resistance, liver and white adipose tissue (WAT) mass, adipocyte size, number of inflammation-associated WAT macrophage aggregates, and high-fat diet–induced increases in serum cholesterol levels (Rahtu-Korpela et al. 2016). The discrepancy with previous findings could be due to non–myeloid-specific inhibition of PHD2 in vivo. As such, the in vivo role of myeloid HIF signaling in atherosclerosis requires further investigation.
Figure 2. Context-dependent myeloid HIF-α effector functions. Myeloid HIF-1α and HIF-2α exhibit diverse functions that differ in distinct pathological settings. The two isoforms sometimes work in a similar fashion, but can also oppose each other. (A) In the setting of atherosclerosis, both myeloid HIF-1α and HIF-2α contribute to pathogenesis. HIF-1α promotes lipid uptake in macrophages through induction of LOX-1. Elevation in HMG-CoA reductase activity and surface ABCA1 perinuclear relocation downstream of HIF-1α increases cholesterol synthesis while simultaneously blocking cholesterol efflux. Through VEGF production, myeloid HIF-1α also facilitates oxLDL's proangiogenic effects. Regulation of PFKFB3 by HIF-1α enhances glycolytic flux and is crucial for both viability and proinflammatory activation of macrophages. Both isoforms contribute to proteoglycan secretion. (B) In adipose tissue of obese subjects, ATM HIF-1α enhances inflammation via induction of hypoxic and proinflammatory genes, while ATM HIF-2α alleviates insulin resistance and adipose tissue inflammation. ATM HIF-2α not only suppresses proinflammatory responses in ATM via induction of arginase 1 (ARG1) expression, but also sensitizes adipocytes to insulin signaling while inhibiting proinflammatory gene transcription. Abbreviations: oxLDL, oxidized low-density lipoprotein; LOX-1, lectin-like oxLDL receptor-1; ABCA1, ATP-binding cassette subfamily A member 1; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3; Glut1, glucose transporter 1; Saa, serum amyloid A.
Adipose tissue inflammation and obesity

Adipose tissue hypoxia, chronic inflammation, and macrophage infiltration are key characteristics of obesity (Xu et al. 2003, Weisberg et al. 2003, Wellen and Hotamisligil 2005, Olefsky and Glass 2010). In lean mice, a majority of the adipose tissue macrophages (ATMs) are alternatively activated M2 macrophages, which suppress pro-inflammatory responses and maintain adipocyte insulin sensitivity by elevated expression of arginase 1 and IL-10, among other factors. In the setting of obesity, the number of macrophages is increased and their phenotype altered. Many macrophages are in a classically activated (M1) state, which produces NO and secretes pro-inflammatory cytokines, such as IL-1β, TNF-α, and IL-6. These cytokines potentiate inflammatory responses in adipose tissue that eventually result in insulin resistance (Wellen and Hotamisligil 2005, Odegaard and Chawla 2011, Chawla, Nguyen, and Goh 2011, Lumeng, Bodzin, and Saltiel 2007). However, a shift in macrophage polarization in lean versus obese humans is debated. Aron-Wisnewsky and colleagues reported a more M1 than M2 polarization of macrophages (defined by CD40 and CD206 expression, respectively) in obese patients, which shifts to a less pro-inflammatory profile after weight loss (Aron-Wisnewsky et al. 2009). A more recent study, however, demonstrated that even though macrophage numbers increase in adipose tissue of obese patients, most of these ATMs are predominantly M2 macrophages (defined by CD163 and IL-10 expression) (Fjeldborg et al. 2014). These contrasting observations may be a consequence of the oversimplified dichotomy of macrophage polarization (see above). In the work of Wentworth and colleagues, ATMs were found to be positive for both M1 (CD11c) and M2 (CD206) markers, exhibiting a pro-inflammatory status associated with insulin resistance in obese humans (Wentworth et al. 2010).

In obese patients, higher HIF-1α levels are evident in adipose tissue (Cancello et al. 2005), and hypoxia and HIF signaling regulate ATM functions in the setting of obesity (Figure 2B). Fujisaka and colleagues showed that adipose tissue hypoxia induces pro-inflammatory phenotypes of M1 ATMs, with elevated expression levels of pro-inflammatory cytokines and hypoxia-related genes (Fujisaka et al. 2013). In contrast with HIF-1α’s pro-inflammatory roles in
ATMs, macrophage HIF-2α has been suggested to ameliorate adipose tissue inflammation and insulin resistance (Choe et al. 2014). Choe and colleagues demonstrated that HIF-2α overexpression in macrophages represses NO production and expression of pro-inflammatory cytokine genes. On the other hand, silencing HIF-2α in palmitate-treated macrophages increases NO production, indicating that HIF-2α is required to downregulate palmitate-induced NO production. Macrophage HIF-2α also regulates the crosstalk between macrophages and adipocytes. Adipocytes cocultured with wild-type macrophages exhibit decreased insulin signaling, while coculture with HIF-2α–deficient macrophages not only reverses the decrease in insulin signaling, but also stimulates adipocyte pro-inflammatory responses. In a murine model of high-fat diet–induced obesity, HIF-2α haplodeficient (Epas1+/−, “Hif2a+/−” herein) mice were more susceptible to adipose tissue inflammation and became insulin resistant. Upon macrophage depletion, both insulin resistance and adipose tissue inflammation improved in this model (Choe et al. 2014). In summary, myeloid HIF-1α promotes adipose tissue inflammation by aiding macrophage M1 polarization, while myeloid HIF-2α constrains the inflammatory response and insulin resistance in adipose tissue. These conclusions are consistent with the understanding that different polarization states of macrophages exert opposite effects on adipose tissue inflammation, and are also consistent with the notion that HIF-1α is required for M1 polarization of macrophages, and HIF-2α for M2 polarization (Takeda et al. 2010). However, another study suggests that hypoxia potentiates palmitate-induced expression of the pro-inflammatory genes IL-6 and IL-1β independently of HIF-1α and HIF-2α in human macrophages. Instead, their induction occurs via activation of JNK and p38 MAPK signaling (Snodgrass et al. 2016). Another group proposed that insulin resistance and metabolic dysregulation in obese mice are mainly regulated by adipocyte HIF-2α, but not myeloid HIF-2α (Garcia-Martin et al. 2015). Clearly, additional effort is needed to determine the extent of HIF-α–mediated regulation of ATM phenotypes and whether HIF-α–dependent ATM phenotypic changes are sufficient to alter adipose tissue inflammation and obesity.
**Sepsis**

Sepsis is a life-threatening systemic illness that is normally induced by microbial infection and may result in fatal multi-organ failure in patients. Hyperactivation of the innate immune system is believed to be a key component of this pathophysiology. Macrophages and neutrophils release cytokines, chemokines, and complement-activation mediators soon after the initial microbial stimuli (Rittirsch, Flierl, and Ward 2008, Fink and Warren 2014, Zinkernagel, Johnson, and Nizet 2007). LPS, a lipoglycan found in the outer membrane of gram-negative bacteria and often used to induce murine sepsis or endotoxemia, has been shown to stabilize macrophage HIF-1α via p42/44 MAPK and NF-κB signaling pathways (Frede et al. 2006). HIF-1α subsequently promotes macrophage *in vitro* production of pro-inflammatory cytokines such as TNF-α, IL-6, IL-1β, IL-1α, IL-4, and IL-12. When mice with conditional *Hif1a* deletion in the myeloid lineage are challenged with LPS, they exhibit reduced hypothermia and hypotension, along with enhanced survival compared with mice that express myeloid HIF-1α (Peyssonnaux et al. 2007). Myeloid HIF-1α deficiency is also protective in a gram-positive endotoxin-induced murine sepsis model (Mahabeleshwar et al. 2012). Similar to HIF-1α, deletion of myeloid HIF-2α is also protective against sepsis. Cultured bone marrow-derived macrophages (BMDMs) isolated from mice with myeloid HIF-2α deficiency also exhibit decreased pro-inflammatory cytokine and increased antiinflammatory cytokine production in response to LPS stimulation. Additionally, myeloid-specific HIF-2α deficiency promotes survival in LPS-challenged mice (Imtiyaz et al. 2010). Collectively, these data show that both HIF-1α and HIF-2α contribute to macrophages’ pathogenic roles in septic pathology. This conclusion is further supported by a more recent study in which 2-methoxyestradiol (2-ME2), a HIF-1α inhibitor (Mooberry 2003), protected mice from both LPS- and cecal ligation and puncture–induced (CLP-induced) sepsis. Suppression of cytokines by 2-ME2 was observed in LPS-stimulated peritoneal macrophages, indicating that macrophage phenotypic alterations also contributed to the survival phenotype (Yeh et al. 2011)
**Airway allergy and asthma**

Although airway allergy is a chronic inflammatory disease primarily driven by DCs and Th2 T lymphocytes, lung macrophages have also been implicated in airway inflammation and asthma (Careau and Bissonnette 2004, Careau et al. 2010, Bang et al. 2011). In a house dust mite (HDM) antigen–induced experimental model of airway allergy, myeloid HIF-1α deficiency renders mice more susceptible to these stimuli (Toussaint et al. 2013). Toussaint and colleagues found that lung macrophage HIF-1α drives expression of immunosuppressive IL-10 to impair DC activation and Th responses (Toussaint et al. 2013). A similar protective effect of myeloid HIF-1α is also evident in the setting of pulmonary fungal infections. Shepardson and colleagues found that mice with myeloid HIF-1α deficiency are more susceptible to pulmonary challenge with *Aspergillus fumigatus*, are defective in fungal clearance, and exhibit decreased lung neutrophil numbers. These phenotypes can be partly attributed to decreased production of CXCL1 and increased neutrophil apoptosis (Shepardson et al. 2014). Contradictory to the finding that macrophage HIF-1α prevents airway allergy, a study by Byrne and colleagues suggests that development of airway allergy is dependent on macrophage HIF-1α. They demonstrated that HDM increases HIF-1α abundance in the lung, inducing VEGF and CXCL1 production in primary lung macrophages in a HIF-1α–dependent manner. Pharmacological HIF-1α inhibition in this model suppresses pulmonary allergic inflammation and VEGF and CXCL1 secretion (Byrne et al. 2013). Using an ovalbumin-induced (OVA-induced) asthma model, others have shown that myeloid HIF-1α deficiency reduces airway hyperresponsiveness and eosinophil infiltration. Furthermore, HIF-1α and HIF-2α directly regulate eosinophil chemotaxis in opposing ways (Crotty Alexander et al. 2013). Therefore, the role of myeloid HIF-1α in airway diseases remains very complex, and varies in different experimental models.

**Gastritis**

Inflammation in the gastric mucosa is most commonly induced by *Helicobacter pylori* infection in humans. Chronic gastritis may progress to gastrointestinal ulcers or gastric cancer (Fox and Wang 2007). Like other inflammatory diseases, recruitment of immune cells is also
evident during gastric inflammation, and macrophage depletion using drug-loaded liposomes has been shown to ameliorate the pathology of *H. pylori*–induced gastritis (Kaparakis et al. 2008). A recent report (Matak et al. 2015) specifically examined the role of myeloid HIF-1α in gastritis. The authors found that HIF-1α levels are positively correlated with the severity of gastritis in patients with *H. pylori* infections, and HIF-1α is readily observed in macrophages from patient biopsies. *In vitro*, *H. pylori* preferentially upregulates Hif1a and downregulates Hif2a transcription in BMDMs, while expression of pro-inflammatory cytokines is dependent on HIF-1α. Elevated HIF-1α levels also contribute to bactericidal activity of both neutrophils and macrophages. Interestingly, in a murine model of *H. pylori*–induced gastritis, mice with myeloid-specific HIF-1α deletion failed to exhibit changes in bacterial loads as compared with wild-type animals. Even though myeloid-specific HIF-1α deficiency blocks the induction of pro-inflammatory gene expression upon *H. pylori* infection, more severe gastritis is observed in these animals, characterized by worsened histopathological grading, greater immune cell infiltration, and a higher cellular proliferation index compared with infected wild-type animals (Matak et al. 2015). Overall, myeloid HIF-1α appears to be protective in *H. pylori*–mediated gastritis; however, additional work is needed to fully explain these counterintuitive phenotypes.

**Renal fibrosis and inflammation**

Macrophages represent the dominant infiltrating cell type during progression of chronic kidney disease (CKD), driven partially by low O₂ availability in the kidney (Fu, Colgan, and Shelley 2016, Erwig 2008, Duffield 2010). Kobayashi and colleagues addressed the role of myeloid HIF-αs using the typical *LysM-Cre* strategy in a murine unilateral ureteral obstruction–induced (UUO-induced) kidney injury model. Activation of myeloid HIF via *LysM-Cre*–driven *Vhl* deletion attenuates renal inflammation, while deletion of both myeloid *Hif1a* and *Hif2a* enhances inflammation, as indicated by increased F4/80⁺ cell numbers in the kidney. However, the presence of myeloid HIF-αs does not alter renal fibrosis. The authors suggest that hypoxia and/or myeloid HIF-α activation alleviates renal inflammation via suppression of *Ccr2* and *Ccl2*, which are crucial for monocyte recruitment (Kobayashi et al. 2012). The notion that myeloid HIF-1α
regulates UUO-induced nephropathy is further supported by another study using the same \textit{LysM-Cre} model; however, Tateishi and colleagues reported that myeloid HIF-1α deletion promoted renal fibrosis but did not alter macrophage accumulation in the UUO model. They suggested a different mechanism for the protective role of myeloid HIF-1α in renal fibrosis: suppression of renal connective tissue growth factor (CTGF) within renal cells (Tateishi et al. 2015). The discrepancy between the two reports could be due to deletion of two isoforms of HIF-α versus deletion of HIF-1α alone. Nevertheless, both studies suggest a protective role for myeloid HIF-αs in CKD, which partly supports the observation in patients with CDK that elevated renal HIF-1α expression correlates with less severe disease (Hung et al. 2013).

\textbf{Cancer-associated inflammation}

A strong link between chronic inflammation and tumor progression has been clearly evident for some time. For example, patients with IBDs are at increased risk of developing colorectal cancer (Eaden, Abrams, and Mayberry 2001, Jess, Rungoe, and Peyrin-Biroulet 2012, Danese and Mantovani 2010). Similar to sites of inflammation, the tumor microenvironment is also highly hypoxic. Macrophages predominantly accumulate in hypoxic regions, change their gene expression profiles in response to low O\textsubscript{2}, and function in response to limited O\textsubscript{2} availability (Murdoch, Giannoudis, and Lewis 2004). Significant effort has delineated the respective roles of myeloid HIF-1α and HIF-2α in the tumor setting, beyond the two examples we will summarize here. In a PyMT model of breast cancer, loss of myeloid HIF-1α significantly decreases tumor mass and inhibits tumor progression, likely through suppression of cytotoxic T cell response to the tumors (Doedens et al. 2010). As for HIF-2α, Imtiyaz and colleagues demonstrated that myeloid HIF-2α deficiency leads to reduced tumor burden and progression in a murine CAC model, while ablating macrophage infiltration of murine hepatocellular carcinoma. The authors suggest that these results could partly be due to defective migration and invasion of macrophages with HIF-2α loss (Imtiyaz et al. 2010). For a more comprehensive discussion of myeloid HIF-αs in cancer, please refer to these reviews (Henze and Mazzone 2016, Triner and Shah 2016).
Overall, hypoxia and inflammation are clearly inextricably linked. Hypoxia can be a strong contributory factor in certain inflammatory diseases; in turn, inflammation sites often exhibit low O$_2$ tension. Myeloid cells are major components of innate immunity that are tightly associated with inflammation in different tissues and found predominantly localized within the hypoxic regions of inflamed tissues. Myeloid cell infiltration on its own can contribute to O$_2$ deprivation at these sites. In response to hypoxia, myeloid cells stabilize HIF-αs, which facilitates their metabolic reprogramming and other adaptations, allowing myeloid cells to take on transient roles in different stages of disease progression. In many types of inflammation described in this chapter, the roles of myeloid HIF-αs remain incompletely described. In many cases, myeloid HIF-1α and HIF-2α have nonredundant or even opposing effects on myeloid cell functions (Keith, Johnson, and Simon 2012). Therefore, many questions concerning the role of HIF-αs in myeloid cells require further investigation. For example, in specific inflammatory diseases, it is unclear if it would be beneficial or detrimental to target HIF-αs. If targeting of HIF-αs is beneficial, then should a specific HIF-α isoform or both isoforms be targeted? Even if all mechanisms mediated by HIF in inflammatory myeloid cells are elucidated, clinical translation will still be challenging. For example, how can HIF be specifically targeted in myeloid cells? How efficient will these therapies be? Nevertheless, oxygen-sensing pathways in myeloid cells are clearly key determinants of their physiological and pathological functions and these pathways remain attractive therapeutic targets.
CHAPTER 2

Myeloid cell hypoxia-inducible factors promote resolution of inflammation in experimental colitis

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INTRODUCTION

Hypoxia (low oxygen tension) is evident in many pathological contexts, including chronic intestinal inflammation, commonly known as inflammatory bowel diseases (IBD) (Colgan and Taylor 2010, Taylor and Colgan 2007). Healthy colon tissue exhibits O₂ partial pressures ranging from ~85 mmHg in intestinal crypts to <10 mmHg in villus tips (Zheng, Kelly, and Colgan 2015). This “physiological” hypoxia is profoundly exacerbated in the context of active inflammation, as revealed in murine models of IBD (Colgan and Taylor 2010, Taylor and Colgan 2007, Karhausen et al. 2004) . A well characterized cellular response to O₂ deprivation is activation of hypoxia-inducible factor (HIF) transcriptional regulators, comprised of an O₂-sensitive α-subunit (HIF-1α or HIF-2α) and a constitutively expressed β subunit (HIF-1β, or aryl hydrocarbon receptor nuclear translocator [ARNT]). In the presence of O₂, HIF-α is hydroxylated by prolyl hydroxylase domain-containing proteins (PHDs), and then poly-ubiquitinated by the von Hippel-Lindau (pVHL) tumor suppressor E3 ubiquitin ligase complex, leading to its degradation by the 26S proteasome (Maxwell et al. 1999, Cockman et al. 2000, Jaakkola et al. 2001). Hypoxic conditions inhibit PHD enzymes, allowing HIF-α subunits to accumulate, dimerize with their obligate binding partner ARNT, and bind to hypoxia-response elements (HREs) to enhance transcription of hundreds of genes whose products mediate cellular adaptation to hypoxia, including glycolysis, angiogenesis, and inflammatory responses (Majmundar, Wong, and Simon 2010, Lee and Simon 2015, Lin and Simon 2016). As hypoxia is a prominent characteristic of inflamed colon tissue, activation of both
HIF-1α and HIF-2α, the two best-characterized HIF-α subunits, is also frequently detected in the colon of IBD patients (Giatromanolaki et al. 2003, Xue et al. 2013, Ortiz-Masia et al. 2012).

Inflamed tissues can become hypoxic due to abnormal vascular function (Hatoum, Binion, and Gutterman 2005) and enhanced metabolic activities of bacteria and infiltrating immune cells, such as myeloid cells, which include granulocytes (i.e. neutrophils, basophils, eosinophils and mast cells) and monocytes that differentiate into macrophages and dendritic cells (Lin and Simon 2016, Soehnlein and Lindbom 2010). When recruited to sites of inflammation, these cells eliminate invading pathogens by driving innate immune responses, e.g. phagocytosis or inflammatory cytokine secretion. Macrophages and neutrophils accumulate in the mucosa of IBD patients (Rugtveit et al. 1997, Thiesen et al. 2014, Kamada et al. 2008, Bressenot et al. 2015, Demir et al. 2015) and play critical roles in modulating and resolving inflammation (Qualls et al. 2006, Zigmond et al. 2012, Platt et al. 2010, Kuhl et al. 2007, Zhang et al. 2011, Natsui et al. 1997, Kankuri et al. 2001). Moreover, their preferential localization within hypoxic regions suggests a potential role of O₂ limitation in dictating myeloid cell inflammatory responses. Significant effort has elucidated that HIF-1α and HIF-2α in myeloid cells (Lin and Simon 2016) have common (Cramer et al. 2003, Imtiyaz et al. 2010, Walmsley et al. 2005, Thompson et al. 2014), non-redundant (Cramer et al. 2003, Imtiyaz et al. 2010), and opposing functions (Takeda et al. 2010, Keith, Johnson, and Simon 2011), reflecting the complexity of HIF function in these cells.

ARNT depletion represents an attractive approach to study pan-HIF inhibition in multiple contexts (Wong et al. 2015, Majmundar et al. 2015, Krock et al. 2015). In a murine model of colitis, pharmacological HIF stabilization using PHD inhibitors proved to be protective, partly through anti-apoptotic effects on epithelial cells (Robinson et al. 2008, Marks et al. 2015, Cummins et al. 2008). Notably, HIF-1α and HIF-2α can oppose each other during intestinal inflammation: for example, epithelial cell HIF-1α helps maintain intestinal barrier functions during colitis (Karhausen et al. 2004, Furuta et al. 2001, Hirota et al. 2010), whereas HIF-2α worsens colitis by promoting tumor necrosis factor alpha (TNFα) production in epithelial cells (Xue et al. 2013, Ortiz-Masia et al. 2012).
HIF functions can also differ drastically depending on cell type. For example, dendritic cell-specific HIF-1α suppresses intestinal inflammation via activation of regulatory T cells (Fluck et al. 2016), whereas macrophage-specific HIF-1α has been implicated as pathogenic (Backer et al. 2017, Kim et al. 2018). However, the role(s) of pan-HIF activation specifically in myeloid cells during colitis are yet to be fully investigated.

In this chapter, we show that myeloid HIF-α/ARNT heterodimers are required for efficient resolution of inflammation in a dextran sulfate sodium (DSS)-induced acute colitis murine model, and confirm that these effects are due to disruption of HIF-1α and HIF-2α signaling. Lamina propria neutrophil and monocyte numbers are elevated in mice with myeloid HIF deficiency during the resolution phase of acute colitis. Microarray analysis of colonic macrophages indicates that their conversion to a “pro-resolving” profile requires a full complement of HIF activities. We also identify serum amyloid A s (SAAs) as a likely mechanism through which HIF-deficient macrophages contributes to aberrant disease resolution. Our findings are the first to connect HIFs to SAAs in colitis, and highlight potential clinical benefits of activating myeloid HIF signaling as a way to resolve intestinal inflammation.

RESULTS

ARNT depletion in myeloid cells disrupts both HIF-1α and HIF-2α transcriptional activity

To study HIF function in myeloid cells, we generated LysMCre;Arnt<sup>fl/fl</sup> mice to achieve myeloid cell-specific Arnt deletion, whereas LysMCre;Arnt<sup>+/+</sup> (henceforth LysMCre) mice were used as controls. The LysMCre transgene drives efficient deletion of conditional “floxed” alleles in neutrophils and macrophages, which increases as monocytes mature into macrophages (Clausen et al. 1999). Highly specific Arnt recombination was confirmed using polymerase chain reaction (PCR) assays to detect the deleted (1 lox) Arnt allele in DNA isolated from bone marrow-derived macrophages (BMDMs) (lane 3-4), but not tail tissues (lane 7-8), in LysMCre;Arnt<sup>fl/fl</sup> mice (Figure 3A). ARNT protein abundance was assessed by western blot analysis of lysates from BMDMs cultured under either normoxic (21% O₂) or hypoxic (0.5% O₂) conditions for 24 hours, at which
Figure 3. *Arnt* deletion in macrophages disrupts both HIF-1α and HIF-2α transcriptional activity. (A) PCR analysis of genomic DNA prepared from bone marrow-derived macrophages (BMDMs) and tail of LysMCre and LysMCre;Arnt6/6 mice. The 2 lox band is the floxed allele of Arnt, and the 1 lox band indicates successful Cre-mediated recombination. (B) Western blotting of murine ARNT, HIF-1α, and HIF-2α in protein lysates prepared from LysMCre and LysMCre;Arnt6/6 BMDMs cultured under 21% O₂ (normoxia) or 0.5% O₂ (hypoxia) for 24 hours. Beta-actin was used as loading control. Densitometry was applied for quantification of HIF-1α and HIF-2α normalized to β-actin. The numbers indicate average values of duplicated samples. (C-E) Real-time quantitative PCR (RT-qPCR) analysis of HIF target genes in LysMCre (n=3; mean ± s.e.m.) and LysMCre;Arnt6/6 (n=3; mean ± s.e.m.) BMDMs cultured under 21% O₂ or 0.5% O₂ for 24 hours. Two-way ANOVA, *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.
Figure 4. Myeloid ARNT depletion does not affect phagocytosis, ATP generation in BMDMs, and peripheral lymphocytes composition. (A-B) Phagocytosis response (A) and ATP generation normalized to total protein (B) of BMDMs cultured under normoxia or hypoxia for 24 hours (n=3; mean ± s.e.m.). Phagocytosis response was measured using Vybrant Phagocytosis Assay Kit (Thermo Fisher Scientific). ATP levels were determined using ATPLite Luminescence ATP Detection Assay System (PerkinElmer). (C) Percentage in CD45+ cells (left panel) and absolute cell counts of major immune cell populations normalized to weight of spleen (right panel) from LysMCre (n=4; mean ± s.e.m.) and LysMCre;Armtn (n=4; mean ± s.e.m.) mice without DSS challenge. Two-way ANOVA, *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.
time ARNT protein was significantly depleted in LysMCre;Arntf/fl BMDMs (Figure 3B). Both HIF-1α and HIF-2α were stabilized under hypoxia, irrespective of the presence or absence of ARNT protein (Figure 3B). While not as dramatic as HIF-1α protein stabilization (>8-fold increase), HIF-2α exhibited perceptible accumulation (>3-fold increase) under hypoxic conditions (Figure 3B). The same cells were examined to test whether ARNT depletion was sufficient to abrogate both HIF-1α and HIF-2α transcriptional activity. HIF-1α-specific (Ldha, Pkg1), HIF-2α-specific (Arg1, Serpine1), and common target genes of both HIF-α subunits (Adm, Vegfa) displayed increased transcription in LysMCre BMDMs challenged with hypoxia; however, this induction was greatly diminished in LysMCre;Arntf/fl BMDMs (Figure 3C-E). Notably, neither phagocytosis nor intracellular ATP levels were significantly affected by ARNT loss in BMDMs (Figure 4A-B), and age-matched LysMCre and LysMCre;Arntf/fl mice exhibited comparable body weight and fertility (data not shown). Furthermore, no obvious differences were observed in peripheral lymphocyte composition of mouse spleen, including B cells, T cells, macrophages, neutrophils, monocytes, and dendritic cells (Figure 4C). Overall, these data indicate that ARNT depletion disrupts both HIF-1α and HIF-2α transcriptional activity in myeloid cells, offering an excellent opportunity to study myeloid pan-HIF inhibition in multiple disease models, including colitis.

Myeloid deficiency of HIF-α/ARNT heterodimers hinders resolution of DSS-induced acute colitis

To determine the effects of myeloid ARNT deficiency in a model of acute colitis, LysMCre and LysMCre;Arntf/fl animals were administered drinking water containing 3% DSS for 5 days, followed by 3 days of regular water. Importantly, mice of both genotypes were housed in the same cages to minimize potential confounding influences from differing microbiomes. Mice were sacrificed on Day 5 ([+DSS, Day5] and Day 8([+DSS, Day8) to compare effects on colitis “induction” and “resolution” phases, which are crucial for pathogen elimination and homeostatic tissue restoration, respectively (Soehnlein and Lindbom 2010, Prame Kumar, Nicholls, and Wong 2018). As expected, LysMCre and LysMCre;Arntf/fl mice imbibing regular drinking water for 8 days ([-DSS, Day8) showed no body weight loss, diarrhea, or fecal blood (collectively
Figure 5. Myeloid HIF-α/ARNT heterodimer deficiency impairs resolution of DSS-induced acute colitis. (A) Graphical summary of body weight changes (left panel) and Disease Activity Index (right panel) of LysMCre and LysMCre;Arntfl/fl mice. See Methods for scoring system of Disease Activity Index. Experimental groups (n=24 for each genotype; mean ± s.e.m.) received 3% DSS in drinking water for 5 days, followed by 3 days on regular drinking water. Control groups (n=5 for each genotype; mean ± s.e.m.) received regular drinking water for 8 days. (B-C) Colon length (B) and H&E images of colon about 1 cm from rectum (C) from untreated groups sacrificed on day 8 and DSS-treated groups sacrificed on day 5 and day 8. Scale bars, 100 μm. (D) Histopathological scoring of colon from mice challenged with DSS for 5 days and sacrificed on day 8. Student’s t-test. Two-way ANOVA was applied for (A-B), *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.
Figure 6. Myeloid HIF-α/ARNT heterodimer deficiency promotes secretion of pro-inflammatory cytokines in the colon. (A-B) Following analyses include untreated group ([-]DSS) sacrificed on day 8 (n=4; mean ± s.e.m.) and DSS-treated groups ( [+ ]DSS) sacrificed on day 5 (n=4; mean ± s.e.m.) and day 8 (n=6; mean ± s.e.m.). (A) RT-qPCR analysis of genes encoding pro-inflammatory cytokines from colon tissue. (B) ELISA analysis of IL-1β and IL-6 from colonic explant supernatants. Two way ANOVA was applied for (A-B), *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001. (C) Cytokine array using pooled colonic explant supernatants from DSS-treated LysMcCre and LysMcCre;Amtn mice sacrificed on Day 8. (D) Quantification of cytokine array based on density. N=6 for each genotype; mean ± s.e.m., Student’s t-test, *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.
represented by “Disease Activity Index” [DAI], see Methods for scoring system) over the course of this experiment (Figure 5A). In contrast, DSS treatment induced colonic inflammation in both LysMCre and LysMCre;Arntfl/fl animals, manifested by decreased body weight, increased DAI, and shortened colon length (Figure 5A-B). Body weight loss and DAI on Day 5 were largely identical in LysMCre and LysMCre;Arntfl/fl mice. Interestingly, as body weight and DAI gradually improved in LysMCre mice starting at Day 6, LysMCre;Arntfl/fl mice continued to exhibit weight loss and an elevated DAI through Day 8 (Figure 5A). Similarly, Day 5 colon lengths were comparable between the two groups; however, LysMCre;Arntfl/fl mice displayed significantly shorter colons compared to LysMCre mice on Day 8 (Figure 5B).

Histological evaluation revealed immune cell infiltration and disrupted epithelium on Day 5 in both cohorts (Figure 5C). On Day 8, LysMCre mice displayed a colonic histology similar to untreated colons, whereas colons from LysMCre;Arntfl/fl mice had elevated immune cell filtration and relatively few normal crypt structures (Figure 5C). This difference in histology on Day 8 was confirmed and quantified by histopathological scoring (see Methods) (Figure 5D). RNA expression and secretion of key pro-inflammatory cytokines (IL1β, IL6, IL12α, and TNFα) were also substantially increased in the colon of LysMCre;Arntfl/fl mice, compared to controls, especially on Day 8 (Figure 6A-B). Further unbiased cytokine array analysis revealed a more pro-inflammatory microenvironment in LysMCre;Arntfl/fl colons (Figure 6C-D). Collectively, these data suggest that myeloid ARNT is required for proper resolution of acute colitis.

Loss of either HIF-1α or HIF-2α in myeloid cells phenocopies LysMCre;Arntfl/fl mice in DSS-induced colitis model

It is well established that ARNT can heterodimerize with the bHLH/PAS transcription factor aryl hydrocarbon receptor (AhR), which also regulates immune responses during intestinal inflammation (Mulero-Navarro and Fernandez-Salguero 2016, Lamas, Natividad, and Sokol 2018). As expected, increased expression of two AhR target genes was observed in LysMCre BMDMs exposed to the AhR agonist (6-formylindolo[3,2-b]carbazole, aka FICZ), but not in LysMCre;Arntfl/fl BMDMs (Figure 7A). However, ARNT loss had no effect on AhR target gene
Figure 7. ARNT deficiency suppresses AhR ligand-induced AhR target gene expression, but does not affect their expression under hypoxia. (A-B) RT-qPCR analysis of Cyp1a1 and Ugt1a1 in BMDMs treated with FICZ (100 nM) under normoxia (A) and BMDMs cultured under normoxia or hypoxia (B) for 24 hours (n=3 for each genotype under each condition; mean ± s.e.m.) Two-way ANOVA; *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.
Figure 8. Myeloid HIF-1α and HIF-2α are required for proper resolution of DSS-induced acute colitis. Graphical summary of body weight changes (A, F), Disease Activity Index (B, G), colon lengths (C, H), expression of pro-inflammatory cytokine genes (D, I), and H&E images of colon about 1 cm from rectum (E, J) of LysMCre (n=4; mean ± s.e.m.) and LysMCre;Hif1αCre mice (n=4; mean ± s.e.m.) or LysMCre;Hif2αCre mice (n=4; mean ± s.e.m.). All mice received 3% DSS in drinking water for 5 days, followed by 3 days on regular drinking water, and sacrificed on day 8. Analyses in (C-E, H-J) were performed using colonic tissues from mice sacrificed on Day 8. Student’s t-test, *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.
expression under hypoxic conditions (Figure 7B), suggesting that AhR contributes little to ARNT-dependent responses in hypoxic inflamed colon tissue. Moreover, RNA expression assessment of sorted colonic macrophages failed to reveal changes in AhR target gene expression between the two cohorts (see Figure 13).

To determine whether myeloid HIF-1α and HIF-2α both contribute to the resolution phase of intestinal inflammation, we induced acute colitis in LysMCre;Hif1α\(^{fl/fl}\) and LysMCre;Hif2α\(^{fl/fl}\) mice using an identical treatment regimen as that described above (Figure 5). As before, control LysMCre mice regained normal body weight after removal of DSS water, whereas LysMCre;Hif1α\(^{fl/fl}\) and LysMCre;Hif2α\(^{fl/fl}\) animals did not (Figure 8A and 8F). DAI also remained high following DSS treatment in these mice, compared to LysMCre controls (Figure 8B and 8G). Colon length showed considerable shortening in both HIF-deficient cohorts on Day 8, although the difference between LysMCre and LysMCre;Hif2α\(^{fl/fl}\) mice failed to reach statistical significance (Figure 8C and 8H). Elevated gene expression of key pro-inflammatory cytokines (Figure 8D and 8I) and exacerbated histopathology on Day 8 (Figure 8E and 8J) in LysMCre;Hif1α\(^{fl/fl}\) and LysMCre;Hif2α\(^{fl/fl}\) mice further confirmed unresolved inflammation in mice with myeloid HIF-α depletion. We conclude that expression of both HIF-1α and HIF-2α in myeloid cells is critical for resolving acute colonic inflammation. Interestingly, the hypoxic induction of HIF-2α (see Figure 3) and impact of HIF-2α on colitis are less dramatic than HIF-1α (see below for further discussion).

Myeloid HIF deficiency leads to more neutrophils and monocytes in the inflamed colons

Elevated and persistent immune responses frequently contribute to IBD pathogenesis. To monitor changes in colonic immune cell populations in the context of myeloid HIF disruption, we performed a comprehensive FACS analysis of immune cells recovered from the lamina propria of LysMCre and LysMCre;Arnt\(^{fl/fl}\) mice treated with DSS. Compared to control LysMCre mice, increased numbers of neutrophils (CD45\(^{+}\), CD11c\(^{-}\), CD11b\(^{+}\), Ly6G\(^{+}\)) and monocytes (CD45\(^{+}\), CD11c\(^{+}\), CD11b\(^{+}\), Ly6C\(^{hi}\), Ly6G\(^{-}\)) (Figure 9A) were observed in LysMCre;Arnt\(^{fl/fl}\) mice on Day 8, expressed either as a percentage of CD45\(^{+}\) cells (Figure 9B) or as the number of cells per mg of colon tissue (Figure 9C). DSS treatment elevated neutrophil and monocyte numbers (Figure 9B)
Figure 9. Characterization of myeloid cells in LysMCre and LysMCre;Arnt
tm lamina propria. Following analyses include untreated group ([−]DSS) sacrificed on day 8 (n=4) and DSS-treated groups ([+]DSS) sacrificed on day 5 (n=4) and day 8 (n=10). (A) Representative gating of neutrophils (Ly6G⁺) and monocytes (Ly6G⁻, Ly6C⁺) in live CD45⁺, CD11b⁺, CD11c⁻ cells. (B) Percentages of neutrophils (top panel) and monocytes (bottom panel) in live CD45⁺ cells. (C) Absolute counts normalized to colon weight of neutrophils (left panel) and monocytes (right panel). (D) Representative gating of macrophages (CD11b⁺, F4/80⁺) in live CD45⁺ cells. (E) Percentages in live CD45⁺ cells (top panel) and absolute counts normalized to colon weight (bottom panel) of macrophages in lamina propria. Data presented as mean ± s.e.m. Two-way ANOVA, *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.
Figure 10. Myeloid HIF deficiency does not alter composition of T cells and B cells in lamina propria. (A-B) Percentage in CD45+ cells (A) and absolute cell counts normalized to colon weight (B) of B cells and T cells from LysMCre and LysMCre;Armt/m mice. Data presented as mean ± s.e.m. Two-way ANOVA, *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.
in both LysMCre and LysMCre;Arntfl/fl mice on Day 5; however, no significant difference between genotypes was detected. We hypothesized that increased numbers of neutrophils and monocytes in colonic tissue of LysMCre;Arntfl/fl mice could contribute to more severe inflammation, particularly during the resolution phase of acute colitis.

Interestingly, the percentage of macrophages (CD45+, CD11b+, F4/80+) (Figure 9D) in CD45+ cells was significantly higher in LysMCre;Arntfl/fl mice than in LysMCre mice at Day 5 (Figure 9E). However, by Day 8, the percentage of macrophages between the two groups was comparable, suggesting that HIF deficiency in macrophages could be of particular importance for the shift from the induction phase to the resolution phase of colitis. As expected, both B and T cells showed no significant differences in the lamina propria of LysMCre and LysMCre;Arntfl/fl mice (Figure 10), consistent with previous data indicating that the DSS-induced colitis model is primarily driven by innate immune cells (Kiesler, Fuss, and Strober 2015).

Myeloid HIF deficiency contributes to increased neutrophil numbers primarily through elevated infiltration

Neutrophils often exert pro-inflammatory functions at sites of inflammation (Kolaczkowska and Kubes 2013, Wright et al. 2010), and a neutrophil-derived protein, myeloperoxidase, is widely used as a marker of colitis severity in IBD (Kim et al. 2012, Garrity-Park et al. 2012, Hansberry et al. 2017). Therefore, we investigated the cellular mechanisms contributing to elevated numbers of neutrophils in DSS-treated LysMCre;Arntfl/fl mice. Given that both neutrophils and macrophages are targeted by LysMCre recombination strategies, we first enriched neutrophils from bone marrow (Figure 11A-B) and compared the efficiency of Arnt deletion in these two cell types by PCR (Figure 11C). Neutrophils exhibited relatively lower deletion efficiency (≥60%) compared to macrophages (≥80%) (Figure 11D), which correlated to decreases in Arnt mRNA levels (Figure 11E). Nevertheless, Arnt deletion in neutrophils was sufficient to disable hypoxic induction of Vegfa gene expression in LysMCre;Arntfl/fl BMDNs (Figure 11F). We next determined if neutrophils were affected by cell-intrinsic factors, cell-extrinsic factors, or a combination of the two.
Timely neutrophil apoptosis is a key event that initiates the resolution of acute inflammation (Soehnlein and Lindbom 2010, Ortega-Gomez, Perretti, and Soehnlein 2013). It was therefore plausible that the increase in neutrophil numbers during the resolution phase was due to delayed apoptosis; however, given previously described pro-survival functions of both HIF-1α and HIF-2α in neutrophils (Thompson et al. 2014, Walmsley et al. 2005), this seemed unlikely. Consistent with these observations, untreated LysMCre;Arntf/l/fl mice exhibited decreased neutrophil viability (Figure 12A). Furthermore, the percentage of dead neutrophils in colon tissue from LysMCre and LysMCre;Arntf/l/fl mice was indistinguishable at Day 5 and Day 8 (Figure 12A). In vitro, the percentage of viable BMDNs after 24-hour culture under normoxia or hypoxia were also comparable between LysMCre and LysMCre;Arntf/l/fl cohorts (Figure 12B). Caspase 3/7 activity assessment further supported the notion that ARNT deficiency can promote, as opposed to delay, neutrophil apoptosis under hypoxia (Figure 12C), consistent with previous findings (Thompson et al. 2014, Walmsley et al. 2005).

Another critical step in resolving inflammation is the prevention of further neutrophil recruitment (Soehnlein and Lindbom 2010, Ortega-Gomez, Perretti, and Soehnlein 2013). We therefore tested whether myeloid HIF deficiency enhanced neutrophil infiltration by altering either the microenvironment or neutrophil chemotaxis. CXCL1 has long been recognized as a major neutrophil chemoattractant, and CXCL1 secretion in the supernatant of colonic explants was increased by almost three-fold in LysMCre;Arntf/l/fl mice on Day 8, compared to LysMCre mice (Figure 12D). In contrast, the levels of CXCR2, the CXCL1 receptor expressed by neutrophils (Kolaczkowska and Kubes 2013), were not affected by ARNT status (Figure 12E), suggesting that the increased number of neutrophils may reflect the elevated secretion of chemoattractant in the gut, rather than enhanced neutrophil migratory ability. Given that CXCL1 production by macrophages has been shown to promote neutrophil infiltration in a peritonitis model (De Filippo et al. 2013), we next asked if HIF deficiency promoted CXCL1 production by macrophages. Expression of Cxcl1 was indeed higher in macrophages sorted from the lamina propria of LysMCre;Arntf/l/fl mice, suggesting that macrophages could be a major source of CXCL1 in the intestine (Figure 12F).
**Figure 11. Arnt is partially deleted in neutrophils.** (A) Example of neutrophils from bone marrow after enrichment. (B) Flow cytometry confirmation of high neutrophil percentage after enrichment from bone marrow of LysMCre and LysMCre;Arntfloxed mice. (C) PCR analysis of genomic DNA prepared from BMDNs and BMDMs of LysMCre and LysMCre;Arntfloxed mice. The Cre recombinase-mediated recombination of the conditional allele (2 lox) leaves behind a single LoxP (1 lox) site. (D) Quantification of deletion efficiency based on (C) using density. Deletion efficiency = (1 lox density)/(1 lox density + 2 lox density). (E) RT-qPCR analysis of Arnt in BMDNs (left panel) (n=3 for each genotype; mean ± s.e.m.) and BMDMs (right panel) (n=3 for each genotype; mean ± s.e.m.) Student’s t-test, **p<0.001 (F) RT-qPCR analysis of Vegfa in BMDNs cultured under normoxia or hypoxia for 24 hours (n=3 for each genotype; mean ± s.e.m.). Two-way ANOVA, *p<0.05.
Figure 12. Increased neutrophil numbers in lamina propria of LysMCre;Armtm/m mice is due to elevated CXCL1, not enhanced survival. (A) Percentage of dead neutrophils normalized to total neutrophils in lamina propria. Data represent untreated mice ([-]DSS) sacrificed on day 8 (n=4) and DSS-treated mice ([+DSS) sacrificed on day 5 (n=4) and day 8 (n=10). (B) Viability of bone marrow-derived neutrophils (BMDNs) from LysMCre (n=3) and LysMCre;Armtm/m (n=3) mice cultured under normoxia or hypoxia for 24 hours. Viability was determined by Trypan Blue exclusion. (C) Caspase3/7 activity assay performed with BMDNs from LysMCre (n=3) and LysMCre;Armtm/m (n=3) mice. Baseline measurement was conducted using BMDNs immediately after isolation from bone marrow. (D) ELISA analysis of CXCL1 in colonic explant supernatants. (E) RT-qPCR analysis of Cxcr2 expression in LysMCre and LysMCre;Armtm/m BMDNs. (F) RT-qPCR analysis of Cxcl1 expression in sorted lamina propria macrophages from LysMCre and LysMCre;Armtm/m mice. Data presented as mean ± s.e.m. Two-way ANOVA for (A-D) and Student's t-test for (E-F); *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.
HIF-deficient colonic macrophages have a diminished pro-resolving profile

To overcome inflammation, macrophages must activate “pro-resolving” functions to ensure reconstitution of tissue homeostasis (Soehnlein and Lindbom 2010, Ortega-Gomez, Perretti, and Soehnlein 2013). To elucidate the exact contribution of HIF-deficient macrophages to unresolved colitis, we sorted these cells in colitic tissues from LysMCre (n=5) and LysMCre;Arntfl/fl (n=4) mice on Day 8, and conducted RNA microarray analysis. Unsupervised clustering clearly distinguished the two cohorts, indicating similar gene expression patterns among mice with the same genotype (Figure 13A). Together, 115 upregulated and 138 downregulated genes were identified in LysMCre;Arntfl/fl compared to LysMCre macrophages, based on a minimum 1.5X absolute change cutoff and a false discovery rate (FDR) adjusted p-value (stated as q-value here) of 0.05 (Figure 13B, yellow dots). Decreased Arnt gene expression was also verified (Figure 13B, black dot). Expression of multiple genes previously associated with adverse effects in IBD, including Lcn2, Il1f9, Lrg1, and Mmp9 (Figure 13B, blue dots), was higher in LysMCre;Arntfl/fl macrophages. Simultaneously, several genes whose products act to limit or resolve colitis (e.g. Areg and Fgl2 [Figure 13B, blue dots]) showed lower expression levels in HIF-deficient macrophages. We compared our microarray results with a previous genome profiling study performed over the time course of DSS-induced colitis (Fang et al. 2011). Twenty-eight genes exhibited overlap between both datasets (see Figure 16B), which included Lcn2 and Lrg1, pro-inflammatory genes previously implicated in colitis. Of note, expression of two key target genes of AhR signaling (Cyp1a1 and Ugt1a1) was not significantly altered in ARNT-deficient intestinal macrophages (Figure 13B, purple dots), further supporting the notion that AhR signaling is not a significant contributing factor to the phenotypes we observed.

Given the defective resolution of colitis observed in LysMCre;Arntfl/fl mice, we next asked whether conversion to a pro-resolving phenotype was impaired in HIF-deficient macrophages. Transcriptomic profile of inflammatory macrophages and resolution phase macrophages has been described in a previous study (Stables et al. 2011). By comparing differentially expressed
Figure 13. HIF deficiency renders macrophages less pro-resolving. (A) Unsupervised hierarchical clustering of microarray samples collected as sorted lamina propria macrophages from LysMCre and LysMCre;Armtlox/lox mice. Each sample was from a single mouse. Clustering was performed on log 2 normalized gene intensities (from robust multi-array average). Average linkage was used with Pearson dissimilarity as the distance measure. (B) Volcano plot of the statistical significance (d score, the T-statistic value used in Significance Analysis of Microarrays) against the log 2 ratio of gene expression between lamina propria macrophages from LysMCre and LysMCre;Armtlox/lox mice, based on the microarray analysis. The magnitude of d score scales with statistical significance. Genes with fold change below or above 1.5 and a false discovery rate (q value) smaller than 5% are in yellow. (C) Heat maps of gene expression using the top 20 upregulated (left) and downregulated (right) genes in resolution phase macrophages (Gene Express, E-MEXP-3189). Gene expression data are from our microarray analysis of LysMCre;Armtlox/lox vs. LysMCre lamina propria macrophages. Each panel in (C) displays 19/20 genes differentially expressed (Stables et al. 2011), as one gene from each list was not detected in our microarray analysis.
Figure 14. Myeloid HIF deficiency favors M1 polarization of macrophages in vitro. (A-B) RT-qPCR analysis of M1 (A) and M2 (B) signature genes in BMDMs (n=3 for each genotype, mean ± s.e.m.) cultured under hypoxia with either M1 stimuli (5 ng/mL LPS+1 ng/mL IFNγ) or M2 (5 ng/mL IL-4+5 ng/mL IL-13) for 24 hours. Two-way ANOVA; *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.
Figure 15. Myeloid HIF deficiency does not favor macrophage M1 polarization in vivo. (A-B) Heatmap of M1 (A) and M2 (B) signature gene expression based on microarray analysis of sorted lamina propria macrophages from LysMCre and LysMCre;Arnt<sup>fl/fl</sup> mice. These mice were treated with DSS for 5 days and sacrificed on Day 8.
genes in LysMCre;Arntfl/fl and LysMCre macrophages to the published differentially expressed genes in inflammatory- and resolution-phase macrophages, we observed that approximately 70% of the genes in our list were consistent with a reduction in a pro-resolving phenotype of macrophages in LysMCre;Arntfl/fl mice. For example, of the 20 most highly upregulated genes in resolution macrophages, 13 (65%) were instead downregulated in sorted LysMCre;Arntfl/fl macrophages (Figure 13C, left panel). Similarly, 13 (65%) of the 20 most downregulated genes in resolution macrophages were instead upregulated in sorted LysMCre;Arntfl/fl macrophages (Figure 13C, right panel). Collectively, these data suggest a critical role of HIF signaling for the functional conversion of macrophages, which might underlie the successful shift from induction phase to resolution phase in acute colitis.

Multiple studies suggest that macrophage “polarization” into M1 or M2 fates can regulate a variety of inflammatory conditions (Huo et al. 2004, Handberg et al. 2008, Khallou-Laschet et al. 2010, Nguyen et al. 2011, Kosteli et al. 2010, Moreira et al. 2010, Naura et al. 2010). To test if macrophage polarization was affected by Arnt deletion, we cultured LysMCre and LysMCre;Arntfl/fl BMDMs under either normoxia or hypoxia, with or without cytokines that induce an M1 (5 ng/mL LPS+1 ng/mL IFNγ) or M2 (5 ng/mL IL-4+5 ng/mL IL13) phenotype. Based on qPCR analysis of several canonical M1- and M2-associated markers, we found that M1 polarization was enhanced (Figure 14A), whereas M2 polarization was suppressed (Figure 14B). In contrast, when microarray data of sorted macrophages recovered from colon tissue were subject to a comprehensive analysis of M1 and M2 markers, we observed a mixed profile of polarization, marked by repression of both M1 and M2 gene signatures (Figure 15). Consequently, it remains unclear from these data whether myeloid HIF deficiency favors one polarization state over the other in vivo.

Myeloid HIF deficiency increases serum amyloid A levels in the colon

Among the differentially regulated genes displayed in Figure 13B, those encoding three members of serum amyloid A (SAA) family were identified among the most upregulated genes in LysMCre;Arntfl/fl macrophages (red dots). Ingenuity Pathway Analysis (IPA) also identified acute
phase response signaling as one of the most activated signaling pathways in LysMCre;Arntfl/fl macrophages, which includes Saa1 and Saa3 (Figure 16A). Comparison of our microarray results with the temporal genome profiling study of colitis (Fang et al. 2011) revealed Saa3 as one of the 6 common genes differentially expressed throughout the time course of disease (Figure 16B), implicating a potential role for Saa3 in this disease model. SAA is an acute phase response protein whose elevated production is often observed in Crohn’s disease and other inflammatory conditions (Niederau et al. 1997, de Villiers et al. 2000, Ye and Sun 2015, Huang and Littman 2015). Moreover, SAA has been implicated in mediating defective resolution by competing with lipoxin A4, a key pro-resolving molecule, for binding to their common receptor, formyl peptide receptor 2 (FPR2) (Bozinovski et al. 2012, El Kebir et al. 2007). Upregulation of these genes were first confirmed using qRT-PCR (Figure 16C). Moreover, supernatants from colonic explants from LysMCre;Arntfl/fl mice, contained higher levels of SAA1/2 and SAA3 proteins, particularly on Day 8, compared to controls (Figure 17A). Expression of two key SAA receptors (Fpr2 and Scarb1) was also elevated in the colon tissue of LysMCre;Arntfl/fl mice (Figure 17C), reinforcing a potential role for SAA in colitis. We also assessed Saa gene expression in colon tissue and found that the trend of Saa expression correlated with disease progression (Figure 17B). After 5-day DSS treatment, both LysMCre and LysMCre;Arntfl/fl mice exhibited increased Saa expression compared to untreated mice. On Day 8, Saa gene expression in LysMCre mice declined to a level similar to untreated mice; however, LysMCre;Arntfl/fl mice maintained a high level of Saa gene expression, and an elevated level of Saa3. These results again implicate SAA in the regulation of colitis resolution.

KEGG pathway analysis of significantly upregulated genes in LysMCre;Arntfl/fl lamina propria macrophages suggested changes in arachidonic acid metabolism (Figure 18A), especially increased Ptges1, Cyp2e1 and Ggt1 expression (Figure 18B). Ptges1 encodes prostaglandin E synthase 1 (PTGES1), which catalyzes the production of prostaglandin E2 (PGE2) (Nakanishi and Rosenberg 2013). Since PGE2 elicits diverse functions during inflammation, we primarily assessed PGE2 production and key enzymes responsible for it. Ptges1 and Ptges2 upregulation were confirmed using RT-qPCR (Figure 18C). Interestingly,
Figure 16. Myeloid HIF deficiency elevates serum amyloid A gene expression in lamina propria macrophages. (A) Ingenuity Pathway Analysis (IPA) of canonical pathways differentially expressed between LysMCre (n=5) and LysMCre;Amf<sup>fl/fl</sup> (n=4) lamina propria macrophages based on microarray. Numbers on the right end of each bar are activation Z-Score of that pathway. A positive Z-Score (colored orange) indicates likely activation of that pathway in LysMCre;Amf<sup>fl/fl</sup> compared to LysMCre lamina propria macrophages, and a negative Z-Score (colored blue) indicates likely inactivation. Z-Score > 2 or < -2 is considered significant. (B) Venn diagram showing overlapping between differentially expressed genes in (red circle) LysMCre vs. LysMCre;Amf<sup>fl/fl</sup> lamina propria macrophages (absolute fold change >1.5 and q value <5%), (purple circle) Day 4 vs. Day 0 and (green circle), and Day 6 vs. Day 0 DSS-treated colon (absolute fold change > 2 and q value<5%) based on GSE22307. (C) RT-qPCR analysis of Saa1, Saa2, and Saa3 in sorted lamina propria macrophages from mice challenged with DSS for 5 days and sacrificed on Day 8. Data presented as mean ± s.e.m. Student’s t-test, *p<0.05.
Figure 17. Myeloid HIF deficiency results in increased serum amyloid A gene expression and production in colon. (A-C) Data represent untreated mice (−DSS) sacrificed on day 8 (n=4) and DSS-treated mice (+DSS) sacrificed on day 5 (n=4) and day 8 (n=6). (A) ELISA analyses of SAA3 (left panel) and SAA1/2 (right panel) in colonic explant supernatants. (B-C) RT-qPCR analysis of (B) Saa1, Saa2, and Saa3 and (C) Fpr2, Scarb1 in colon tissues. Data presented as mean ± s.e.m. Two-way ANOVA; *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.
Figure 18. Myeloid HIF deficiency alters PGE₂ production in macrophages and in the colon.  
**A** KEGG pathway analysis of significantly upregulated genes in LysMCre;Armt²⁺/⁻ lamina propria macrophages.  
**B** Simplified schematic of arachidonic acid metabolism. Green upward arrow indicates upregulation of gene expression, and red downward arrow indicates downregulation.  
**C** RT-qPCR analysis of Ptgs2, Ptges1, and Ptges2 expression in LysMCre and LysMCre;Armt²⁺/⁻ lamina propria macrophages. Student’s t-test, *p<0.05, and **p<0.01.  
**D-E** Liquid chromatography–mass spectrometry analysis of PGE₂ in (D) LysMCre and LysMCre;Armt²⁺/⁻ colonic explant supernatants from Day 8 and in (E) supernatants collected from BMDMs (left, n=3 for each genotype, mean ± s.e.m.) and BMDNs (right, n=3 for each genotype, mean ± s.e.m.) cultured under normoxia or hypoxia, with or without LPS stimuli (100 ng/mL) for 24 hours. Two-way ANOVA; *p<0.01, and ****p<0.0001.
LysMCre;Arntfl/fl mice exhibited lower levels of PGE2 as compared with LysMCre mice in the gut (Figure 18D). Further measurement of PGE2 in BMDM culture supernatants suggested that decreased PGE2 production by HIF-deficient macrophages may partially contribute to these observations in the colon (Figure 18E, left). Of note, PGE2 generation in BMDNs was independent of HIF-α/ARNT heterodimers (Figure 18E, right). Decreased Ptgs2 expression (Figure 18C), encoding cyclooxygenase 2 (COX2), in LysMCre;Arntfl/fl lamina propria macrophages may be responsible for lower PGE2 levels, despite Ptges1 and Ptges2 upregulation which are downstream of COX2 in PGE2 production. Given PGE2 has been shown to facilitate resolution of colonic inflammation (Zhang et al. 2015, Grainger et al. 2013, Montrose et al. 2015), lower levels of PGE2 may partially contribute to defective resolution in LysMCre;Arntfl/fl mice. However, we note differences of colonic PGE2 levels between LysMCre and LysMCre;Arntfl/fl mice are modest, suggesting other factors are important as well.

**DISCUSSION**

In the present chapter, we demonstrate that HIF signaling in myeloid cells is essential for resolution of acute colitis (Figure 19). Myeloid HIF deficiency increases the infiltration of pro-inflammatory neutrophils and monocytes, impedes the functional conversion of macrophages to a pro-resolving phenotype, and promotes SAA production in the colon during the resolution phase. Collectively, these disruptions contribute to defective resolution of colitis in mice with myeloid HIF deficiency.

Previously, pharmacological approaches to stabilize HIFα proteins using PHD inhibitors were shown to suppress intestinal inflammation (Marks et al. 2015, Robinson et al. 2008). In line with these findings, our study demonstrates that myeloid HIF function is necessary for proper and timely resolution of acute colitis, highlighting myeloid cells as active contributors to the anti-inflammatory effects of PHD inhibitors. In contrast, a previous study shows that depletion of myeloid ARNT dampens cutaneous inflammation (Scott et al. 2014). The apparent discrepancy between that study and ours is likely explained by the differences in disease models. Given the
Figure 19. Proposed model illustrating the pro-resolving functions of myeloid HIF-α/ARNT heterodimer during resolution of intestinal inflammation.
plasticity of macrophages, it is not surprising for myeloid HIF to elicit different functions in response to distinct microenvironments in various types of inflammation. For example, we found that myeloid ARNT is dispensable for animal survival in a LPS-induced endotoxemia model and macrophage recruitment in thioglycollate (TG)-induced peritoneal inflammation (see Chapter 3). These results strongly suggest that potential pharmacological approaches to stabilize HIF should be selected carefully to match the cellular hypoxic responses in specific diseases.

The importance of HIF signaling in distinct cell types, including epithelial cells (Karhausen et al. 2004, Furuta et al. 2001, Hirota et al. 2010, Xue et al. 2013), dendritic cells (Fluck et al. 2016) and T cells (Higashiyama et al. 2012), has been studied in the context of intestinal inflammation. We add to these findings by demonstrating that myeloid HIF-1α and HIF-2α are crucial to resolve acute colitis. Although myeloid HIF-1α has been recently implicated in promoting DSS-induced colitis (Backer et al. 2017, Kim et al. 2018), we hypothesize that these disparate results are likely due to different phases of inflammation under examination, and/or different effects of the gene promoters driving Cre-mediated recombination. Specifically, Bäcker and colleagues (Backer et al. 2017) examined mice up to Day 6 of DSS treatment without time for recovery, as opposed to our study where the principal phenotype was captured after removal of DSS H2O. Moreover, we utilized LysMCre which targets mature macrophages and Gr-1+ myeloid cells, while Kim and colleagues (Kim et al. 2018) chose hMRP8Cre for recombination primarily in Gr-1+ granulocytes. These reports underscore the complexity of colitis, and roles of different myeloid populations. Moreover, we note the less impressive contribution of HIF-2α, compared with HIF-1α, to colitis resolution (Figure 8), which may be due to its less dramatic induction under hypoxia in macrophages (Figure 3). We conclude that HIF-1α is the major isoform driving proper resolution of intestinal inflammation, with HIF-2α contributing to a lesser extent.

Previous studies have described a protective role for AhR, another established ARNT binding partner, in colitis (Lamas et al. 2016, Wang et al. 2018, Furumatsu et al. 2011). However, AhR was globally deleted in these mice. Our findings suggest that myeloid AhR is not likely a major contributor to colitis resolution, as disruption of HIF-1α and HIF-2α signaling accounts for
the majority of ARNT-dependent effects in our model. Microarray analysis of sorted lamina propria macrophages supports our contention that myeloid AhR signaling is not a significant factor in this intestinal inflammation model. Furthermore, Chinen and colleagues (Chinen et al. 2015) reported that myeloid AhR is dispensable for inflammatory response in DSS-induced colitis by utilizing the same LysMCre strategy.

Temporally ordered apoptosis and clearance of neutrophils signals the initiation of inflammation resolution (Soehnlein and Lindbom 2010, Ortega-Gomez, Perretti, and Soehnlein 2013), and we observed that unresolved colitis in LysMCre;Arntfl/fl mice was associated with elevated numbers of neutrophils in the lamina propria (Figure 9), correlating with enhanced expression of chemotactic signals, as opposed to increased neutrophil lifespan (Figure 12). Based on previous understanding that either HIF-1α or HIF-2α prevents neutrophil apoptosis under hypoxia (Walmsley et al. 2005, Thompson et al. 2014), we anticipated that neutrophil viability would be impaired upon Arnt deletion; however, this was not observed. We speculate that increased SAA expression in the colonic microenvironment counterbalances the effects of ARNT loss on neutrophil viability, given that SAA has been shown to inhibit neutrophil apoptosis (Christenson et al. 2008, El Kebir, Jozsef, and Filep 2008).

A “partnership” between neutrophils and macrophages is common during inflammation (Prame Kumar, Nicholls, and Wong 2018), particularly in its resolution phase (Soehnlein and Lindbom 2010). Here, we suggest that HIF-deficient macrophages promote neutrophil chemotaxis via elevated CXCL1 secretion. As such, we speculate that HIF signaling plays a more dominant role in macrophage functions compared to neutrophils in this setting. In line with this, production of PGE₂, together with other eicosanoid metabolites (e.g. prostaglandin F2α and thromboxane B2) (data now shown), is dependent on HIF signaling in macrophages, but not in neutrophils. However, we cannot exclude the possibility that other neutrophil-intrinsic properties are altered upon the ARNT loss. This will be investigated in future studies.

Macrophages adopt a pro-resolving phenotype to facilitate recovery from inflammation (Soehnlein and Lindbom 2010, Ortega-Gomez, Perretti, and Soehnlein 2013). Detailed molecular
mechanisms underlying this functional macrophage conversion have yet to be elucidated. Here, we uncover a requirement for intact HIF signaling for macrophages to adopt a pro-resolving phenotype in inflamed colons. Similar to a previous report that resolution phase macrophages display neither canonical M1 nor M2 markers (Stables et al. 2011), our study reveals that disruption of macrophage HIF signaling impedes conversion to a pro-resolving phenotype, without promoting either M1 or M2 identities in vivo. This could be due to the highly complex and dynamic microenvironment of colitis. Our findings are consistent with previous studies demonstrating that M1 and M2 polarization is more readily conferred in cell culture settings, which do not necessarily reflect in vivo conditions (Martinez and Gordon 2014).

Microarray analysis of colonic macrophages revealed a link between HIF signaling and Saa expression in macrophages. An initial attempt to rescue abnormal resolution in \textit{LysMCre;Arnt}^{fl/fl} mice by injecting anti-SAA neutralizing antibodies (Li et al. 2015) was unsuccessful due to technical complications (data not shown). Given the dearth of validated, commercially available neutralizing antibodies for murine SAA, subsequent work will focus on generating a genetic deletion of \textit{Saa1/2/3} in myeloid cells; however this is beyond the scope of the current study. Furthermore, although SAA function has been implicated previously in regulating inflammation, other genes upregulated in both colitic tissue and \textit{LysMCre;Arnt}^{fl/fl} macrophages, including \textit{Stfa3}, \textit{Asprv1}, \textit{Wfdc21}, and \textit{Chil1} (Figure 13B), may also contribute important functions.

In conclusion, we identify myeloid cells as essential contributors to the protective effects of HIF activation during colitis, and describe an important role for myeloid HIF signaling in the efficient resolution of intestinal inflammation. Myeloid cells present as an attractive target cell population for approaches to engage HIF signaling, such as using PHD inhibitors, as a way to more effectively bring IBD under control.
CHAPTER 3

Differential roles of myeloid cell HIFs in other inflammatory diseases

INTRODUCTION

In addition to acute colitis, both hypoxia and myeloid cells have been implicated in playing critical roles in many other inflammatory diseases; for example, colitis-associated colorectal cancer (CAC). Macrophages can be “re-educated” by the tumor microenvironment to facilitate tumor initiation and malignant progression as tumor-associated macrophages (TAMs) in multiple cancer types (Noy and Pollard 2014). Our lab has previously reported that loss of myeloid HIF-2α resulted in reduced tumor burden and disease progression in a murine CAC model, coincident with decreased TAM infiltration (Imtiyaz et al. 2010). Along the same line, inhibition of HIF-α:ARNT dimerization by acriflavine (ACF) limited tumor progression and macrophage infiltration in the same CAC model (Shay et al. 2014). Since ACF treatment can also hinder HIF signaling in other cells found in the colon, it is difficult to tease out the myeloid-specific contribution of HIF inhibition. Therefore, we decided to carefully delineate individual and pan-HIF roles that are specific to myeloid cells in this CAC model using conditional deletion of either Hif1α, Hif2α, or both by LysMCre.

A previous study by Imtiyaz and colleagues demonstrated that HIF-2α is required for murine skin inflammation, peritonitis, and sepsis (Imtiyaz et al. 2010). This finding was consistent with other reports focusing on the role of HIF-1α in skin inflammation and sepsis. Cramer et al. reported that conditional HIF-1α depletion in myeloid cells abrogated 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced ear edema and leukocyte infiltration, exhibiting a similarly compromised immune response as in mice with myeloid HIF-2α deficiency (Cramer et al. 2003). Likewise, in several sepsis models, myeloid HIF-1α deficiency was demonstrated to reduce hypothermia and hypotension, and significantly extended animal survival (Peyssonnaux et al. 2007, Mahabeleshwar et al. 2012). Based on these data, I hypothesized that pan-HIF deficiency
in myeloid cells would also impair inflammatory responses and alleviate disease symptoms in these acute inflammation models.

In this chapter, I will summarize and discuss the observations made in one cancer and three acute inflammation models utilizing mice with individual HIF-α or pan-HIF loss in myeloid cells.

RESULTS

Loss of both HIF-α subunits worsens tumor burden and progression of murine colitis-associated colorectal cancer

To examine the individual and combined roles of myeloid HIF-1α and HIF-2α in inflammation-associated cancer, an azoxymethane (AOM)/DSS induced colitis-associated cancer (CAC) model was applied to 8-week old mice from the following four colonies: LysMCre, LysMCre;Hif1α<sup>fl/fl</sup>, LysMCre;Hif2α<sup>fl/fl</sup>, and LysMCre;Hif1α<sup>fl/fl</sup>;Hif2α<sup>fl/fl</sup>. As shown in Figure 20A, these animals were injected with 12.5 mg/kg AOM, a potent carcinogen, on Day 0, followed by three cycles of DSS treatment with two-week intervals of regular drinking water in between cycles. This experiment was designed to end on Day 98 (14 weeks) to assess tumor formation and colonic inflammation. All mice that survived until Day 98 developed polyps along the colon, with the majority located towards the distal end and rectum (Figure 20B). Compared with LysMCre controls, LysMCre;Hif1α<sup>fl/fl</sup> and LysMCre;Hif2α<sup>fl/fl</sup> mice exhibited slightly more polyps; however, LysMCre;Hif1α<sup>fl/fl</sup>;Hif2α<sup>fl/fl</sup> mice developed the most polyps per mouse (Figure 20C). Using the sum of polyp diameter as a measure of tumor burden, LysMCre;Hif1α<sup>fl/fl</sup>;Hif2α<sup>fl/fl</sup> mice also had a significantly greater tumor burden (Figure 20C). Of note, female animals generally suffered less tumor burden compared with male animals, especially within the LysMCre;Hif1α<sup>fl/fl</sup>;Hif2α<sup>fl/fl</sup> cohort (Figure 20D). On a per lesion basis, the average size of each polyp, indicated by its diameter, was again larger in LysMCre;Hif1α<sup>fl/fl</sup>;Hif2α<sup>fl/fl</sup> mice compared to LysMCre mice (Figure 21A). Unsurprisingly, LysMCre;Hif1α<sup>fl/fl</sup>;Hif2α<sup>fl/fl</sup> mice also had a higher percentage of larger polyps (Figure 21B). Histopathological evaluation of colonic sections from all four cohorts revealed more
Figure 20. Deficiency of both HiF-αs in myeloid cells worsens tumor burden of murine colitis-associated colorectal cancer. (A) Schematic of the experimental regimen in AOM/DSS induced CAC model. (B) Gross images of tumors in the colon and rectum. (C) Total polyp counts per mouse (right) and sum of polyp diameter per mouse (right) in mice survived until the end of the experiment. N=7 for LysMCre, n=11 for LysMCre;Hif1α<sup>fl/fl</sup>, n=2 for LysMCre;Hif2α<sup>fl/fl</sup>, and n=10 for LysMCre;Hif1α<sup>fl/fl</sup>;Hif2α<sup>fl/fl</sup>; mean ± s.e.m. (D) Sum of polyp diameter by gender and genotypes. Mice that developed tumors but did not survive until the end of the experiment were also included in this analysis. Two-way ANOVA, *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.
Figure 21. Deficiency of both HIF-αs in myeloid cells results in larger and more advanced tumors in murine CAC model. (A) Average polyp diameter per mouse of mice that survived until the end of the experiment. N=7 for LysMCre, n=11 for LysMCre;Hif1α<sup>+/−</sup>, n=2 for LysMCre;Hif2α<sup>+/−</sup>, and n=10 for LysMCre;Hif1α<sup>+/−</sup>;Hif2α<sup>+/−</sup>, mean ± s.e.m. (B) Total polyps per mouse based on polyp size in LysMCre and LysMCre;Hif1α<sup>+/−</sup>;Hif2α<sup>+/−</sup> colonies. (C) Percentage of each tumor stage in total lesions per mouse. Two-way ANOVA applied for (A and C); Student’s t-test applied for (B); *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.
Figure 22. Macrophage infiltration in murine CAC model is not altered upon loss of both HIF-αs in myeloid cells. (A) Representative immunohistochemistry images of F4/80 staining in tumor sections from LysMCre and LysMCre;Hif1α<sup>Δβ</sup>;Hif2α<sup>Δη</sup> colonies. (B) Quantification of F4/80<sup>+</sup> cells per field per lesion in stalk area and within tumor. N=3; mean ± s.e.m. Student’s t-test; *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.
advanced lesions in LysMCre;Hif1a\textsuperscript{fl/fl} and LysMCre;Hif1a\textsuperscript{fl/fl};Hif2a\textsuperscript{fl/fl} mice (Figure 21C), given that adenocarcinoma was only detected in these two cohorts. These results suggest that only the absence of both HIF-1α and HIF-2α promotes tumor burden and progression in murine CAC.

Given the important influence of TAMs on CAC, we further assessed macrophage infiltration in LysMCre and LysMCre;Hif1a\textsuperscript{fl/fl};Hif2a\textsuperscript{fl/fl} colonic sections by F4/80 immunohistochemistry. Accumulation of these cells was observed in both the stalk of each tumor and within tumors (Figure 22A). Stalk is the lamina propria area that is above the submucosa. Regardless of the large difference in tumor burden, no significant variance of macrophage infiltration was observed between LysMCre and LysMCre;Hif1a\textsuperscript{fl/fl};Hif2a\textsuperscript{fl/fl} cohorts (Figure 22B).

**Mice with myeloid HIF-2α deficiency exhibit more severe inflammation and a survival disadvantage in the murine CAC model**

One interesting observation made during this AOM/DSS induced CAC experiment was that LysMCre;Hif2a\textsuperscript{fl/fl} mice exhibited a survival disadvantage compared with other cohorts, with the majority (80%) of the mice dropping off the study during the three cycles of DSS stimulation (Figure 23A). Therefore, we suspected that mice with myeloid HIF-2α deficiency may be more susceptible to chronic intestinal inflammation. Indeed, the decrease in body weight upon each DSS treatment cycle (Figure 23B) and shrinkage of colon length (Figure 23C) at the end of the experiment were most drastic in the LysMCre;Hif2a\textsuperscript{fl/fl} cohort, indicating much worse inflammation throughout the experimental time course. Consistently, pathohistological grading of inflammation severity also suggested a higher percentage of LysMCre;Hif2a\textsuperscript{fl/fl} mice exhibited more severe inflammation (Figure 23D). These observations prompted us to examine whether loss of myeloid HIF-2α indeed renders mice more susceptible to chronic inflammatory stress.

**Myeloid HIF-2α is not required for DSS-induced chronic colitis pathology**

To determine whether deficiency of myeloid HIF-2α indeed worsens chronic inflammation in the gut, LysMCre and LysMCre;Hif2a\textsuperscript{fl/fl} mice were administered three cycles of 2% DSS for five days followed by regular drinking water for another five days after each DSS treatment.
Figure 23. Mice with myeloid HIF-2α deficiency are more susceptible to DSS-induced inflammation in AOM-DSS colitis-associated colorectal cancer model, displaying a survival disadvantage. (A) Kaplan-Meier survival curves during the 14-week long AOM/DSS colitis-associated colorectal cancer model. (B) Body weight change before and after three cycles of DSS treatment. (C) Colon length of mice that were sacrificed at end of the experiment. N=7 for LysMCre, n=11 for LysMCre;Hif1αα−/+, n=2 for LysMCre;Hif2αα−/+, and n=10 for LysMCre;Hif1αα−/+,Hif2αα−/+. mean ± s.e.m. Two-way ANOVA; *p<0.05. (D) Histological evaluation of inflammation severity. Y-axis represents percentage of mice from each cohort exhibiting three severity levels.
Similar to the DSS-induced acute colitis model, body weight change, Disease Activity Index (DAI), and colon length at the end of the experiment were used as parameters to determine disease severity. Neither body weight change nor DAI differed significantly between \textit{LysMCre} and \textit{LysMCre;Hif2a\textsuperscript{fl/fl}} mice over the experimental course (Figure 24A-B). It is worth noting that fluctuations in DAI correlated very well with treatment regimen, in the way that each DSS cycle increased DAI and was then reduced by regular drinking water (Figure 24B). Of note, during the first cycle, \textit{LysMCre;Hif2a\textsuperscript{fl/fl}} mice exhibited a higher DAI compared to \textit{LysMCre} mice when kept were on regular drinking water (Figure 24B). Even without statistical significance, this phenotype recapitulated the defective resolution in \textit{LysMCre;Hif2a\textsuperscript{fl/fl}} mice observed in the acute colitis model (Figure 8G). Even though the shorter colon lengths of \textit{LysMCre;Hif2a\textsuperscript{fl/fl}} mice would suggest more severe inflammation (Figure 24C), comparable gene expression of genes encoding pro-inflammatory cytokines in the gut (Figure 24D), body weight change, and DAI indicated a none-to-mild effect of myeloid HIF-2\(\alpha\) on the pathology of DSS-induced chronic colitis. This conclusion was further supported by flow cytometry analysis, which also did not detect any major differences in myeloid and lymphoid cell populations in the lamina propria between the two cohorts (Figure 25). Collectively, these data suggest myeloid HIF-2\(\alpha\) is not required for DSS-induced chronic colitis.

\textbf{Loss of myeloid ARNT mildly alleviates TPA-induced acute skin inflammation}

The phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) is a small molecule drug that activates the signal transduction enzyme protein kinase C (PKC) (Castagna et al. 1982, Blumberg 1988), and has been widely used as a carcinogen and inflammatory stimulus for mouse skin (Iversen 1985, Stanley et al. 1991). A previous study by our lab reported that myeloid HIF-2\(\alpha\) was required for TPA-induced skin inflammation (Imtiyaz et al. 2010). Therefore, we further examined the role of pan-HIF in this acute inflammation model using \textit{LysMCre} and \textit{LysMCre;Arnt\textsuperscript{fl/fl}} mice. Mice from these two cohorts were painted with 2.5\(\mu\)g of TPA on one ear and an equal-volume of acetone as vehicle control on the other. Tissue biopsy was taken 24 hours post-treatment for the assessment of tissue thickness, weight, histology, pro-inflammatory
Figure 24. Myeloid HIF-2α deficiency does not significantly affect disease onset and resolution in a DSS-induced chronic colitis model. (A) Graphical summary of body weight changes and (B) Disease Activity Index (DAI) of LysMCre (n=8, mean ± s.e.m.) and LysMCre;Hif2α<sup>−/−</sup> (n=7, mean ± s.e.m.) mice. Mice were given three cycles of 2% DSS for five days and regular water for another five days. (C) Colon length of LysMCre (n=8, mean ± s.e.m.) and LysMCre;Hif2α<sup>−/−</sup> (n=6, mean ± s.e.m.) mice sacrificed on Day 30. (D) RT-qPCR analysis of genes encoding proinflammatory cytokines from colon tissue of LysMCre (n=8, mean ± s.e.m.) and LysMCre;Hif2α<sup>−/−</sup> (n=6, mean ± s.e.m.) mice. Student’s t-test; *p<0.05.
Figure 25. Myeloid HIF-2α deficiency did not change the composition of lamina propria immune cell populations in a DSS-induced chronic colitis model. (A) Percentage of myeloid cells in live CD45+ cells (left) and cells counts normalized to colon tissue weight (right). (B) Percentage of lymphoid cells in live CD45+ cells (left) and cells counts normalized to colon tissue weight (right). N=8 for LysMCre and n=8 for LysMCre;Hif2a<sup>−/−</sup>; mean ± s.e.m.; Student’s t-test; *p<0.05.
cytokine gene expression, and leukocyte infiltration. Mice with myeloid ARNT deficiency exhibited a moderately lower fold increase in ear thickness and weight upon TPA treatment (Figure 26A-C), suggesting less edema at the inflamed site. TPA also induced the gene expression of several pro-inflammatory cytokines (IL-1β, IL-6, IL-12α and TNFα), which was suppressed for most when ARNT was depleted in myeloid cells (Figure 26D). However, similar to the mild alleviation of edema, suppression of pro-inflammatory cytokine gene expression was not remarkable, indicating HIFs may be minor contributors to the pathology of this skin inflammation model.

To examine whether myeloid cell infiltration was altered by the absence of ARNT, we applied flow cytometry analysis of immune cells isolated from inflamed ear tissue. Compared to acetone-treated ears, ears treated with TPA exhibited increased numbers of immune cells (CD45+) per mg of ear tissue (Figure 27A), and myeloid ARNT deficiency further promoted immune cell infiltration (Figure 27A). Among the TPA-induced leukocyte infiltration, neutrophils and monocytes showed significantly increased percentages in CD45+ cells (Figure 27B, 27D), while the percentage of dendritic cells remained comparable and the percentage of macrophages decreased significantly compared with acetone-treated controls (Figure 27C, 27E). This is consistent with acute inflammation where neutrophils and inflammatory monocytes are often the first line of cells recruited to the inflammation site. This trend was also observed when cells counts of each population were normalized to tissue weight (Figure 27B-E). Of note, LysMCre;Arntfl/fl mice had higher cell counts per mg of ear tissue of all four myeloid cell populations when compared to LysMCre mice upon TPA stimulation (Figure 27B-E), suggesting that loss of myeloid ARNT may promote myeloid cells infiltration in this model.

Interestingly, in this skin inflammation model, we noticed a considerable subpopulation of neutrophils were positive for CD11c, which can be further divided based on F4/80 expression (Figure 28A). Cells that were CD11c+ and F4/80+ exhibited decreased percentage upon TPA treatment (Figure 28A, 28B, and 28E). However, loss of myeloid ARNT not only prevented the percentage decline of these cells, but also elevated cell numbers per mg of ear tissue (Figure 28A, 28B, and 28E), bringing the ratio of CD11c+/F4/80+ vs. CD11c+/F4/80− populations similar to
Figure 26. Myeloid HIF deficiency mildly alleviates TPA-induced cutaneous inflammation. (A) Representative H&E images of mouse ear cross sections from LysMCre and LysMCre;Arnt<sup>fl/fl</sup> mice following acetone treatment on one ear and TPA treatment on the other ear for 24 hours. (B) Fold change in ear thickness normalized to acetone-treated ear 24 hours post-treatment. (C) Fold change in the weight of ear biopsy normalized to acetone-treated ear 24 hours post-treatment. (D) RT-qPCR analysis of pro-inflammatory cytokine gene expression in ear tissue from LysMCre (n=4, mean ± s.e.m.) and LysMCre;Arnt<sup>fl/fl</sup> (n=4, mean ± s.e.m.) mice. Student’s t-test applied for (C); Two-way ANOVA applied for (D); *p<0.05.
Figure 27. Myeloid HIF deficiency increases myeloid cell numbers at the site of TPA-induced cutaneous inflammation. (A) Counts of CD45+ immune cells normalized to weight of ear tissue. (B-E) Percentage and counts/mg of ear tissue of (B) neutrophils (CD45+, CD11b+, Ly6G+), (C) macrophages (CD45+, Ly6G+, CD11b+, F4/80+), (D) monocytes (CD45+, Ly6G+, CD11b+, Ly6C+, F4/80+), and (E) dendritic cells (CD45+, Ly6G+, CD11c+, F4/80+) from LysMCre (n=4, mean ± s.e.m.) and LysMCre;Arnt+/+ (n=4, mean ± s.e.m.) mice. Two-way ANOVA; *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.
Figure 28. Myeloid HIF deficiency significantly increases CD11c⁺, F4/80⁺ neutrophils during TPA-induced cutaneous inflammation. (A) Representative flow gating of neutrophil (CD45⁺, CD11b⁺, Ly6G⁺) subpopulations based on CD11c and F4/80 expression. (B) Breakdown of three neutrophil subpopulations as percentage out of total neutrophils. (C-D) Percentage in neutrophils (top) and cell counts normalized to weight of ear tissue (bottom) of the three subpopulations from LysMCre (n=5, mean ± s.e.m.) and LysMCre;Arntlox (n=5, mean ± s.e.m.) mice: (C) CD11c⁺, (D) CD11c⁺, F4/80⁺, and (E) CD11c⁺, F4/80⁻. Two-way ANOVA; **p<0.01, and ****p<0.0001.
Figure 29. Myeloid HIF deficiency does not affect recovery in the chronic cutaneous inflammation model induced by TPA. *LysMCre* (n=4, mean ± s.e.m.) and *LysMCre;Arnt<sup>−/−</sup>* (n=3, mean ± s.e.m.) mice were treated with 10 μg TPA on one ear for three times on Day 0, Day 4, and Day 8. (A) Fold change in ear thickness normalized to acetone-treated ear measured at five time points following the first TPA treatment. (B) Fold change in the weight of ear biopsy normalized to acetone-treated ear measured at five time points following the first TPA treatment.
that in acetone-treated mice (Figure 28B, 28D, and 28E). Whether this ratio is important for skin inflammation and the exact function(s) of these subpopulations remains unknown.

Given the mildly alleviated inflammation in the acute skin inflammation model, we next sought to examine the role of pan-HIF activity in chronic disease. LysMCre and LysMCre;Arnt^{f/f} mice were given 2.5µg TPA, with acetone as vehicle control, repeatedly for three times on Day 0, 4, and 8 before assessment of edema as well as inflammation on Day 12. In contrast to the acute model, myeloid ARNT deficiency did not provide any recovery benefit to these mice, manifested by comparable fold change in ear thickness and weight (Figure 29A-B). Collectively, these data suggest a minor role for myeloid HIF signaling solely in the acute phase of skin inflammation.

**Myeloid ARNT is dispensable for thioglycollate-induced peritonitis and LPS-induced endotoxemia**

Two additional acute inflammation models were tested to characterize the importance of pan-HIF activity in mediating inflammatory responses *in vivo*. We first induced peritoneal inflammation by injecting LysMCre and LysMCre;Arnt^{f/f} mice with thioglycollate (TG), and measured immune infiltrates in the peritoneal cavity four hours and four days post-injection. Consistent with other reports, neutrophils responded rapidly to the stimulus, shown by their high percentage of CD45+ cells four hours post-injection (Figure 30A), while macrophages peaked four days post-injection (Figure 30B). However, no significant difference was observed between LysMCre and LysMCre;Arnt^{f/f} mice. The lymphoid compartment also did not exhibit noticeable changes. Therefore, myeloid ARNT is not required for immune cell migration in this TG-induced peritonitis model.

The second model was an endotoxemia induced by lipopolysaccharide (LPS) injection, causing rapid systemic inflammation similar to sepsis. LysMCre;Arnt^{+/+} and LysMCre;Arnt^{f/f} mice were given 15 mg/kg of LPS by intraperitoneal injection and monitored closely. Complete loss of two Arnt alleles did not provide any survival advantages compared with mice with one Arnt allele
remaining (Figure 31), suggesting that myeloid ARNT may not be the determining factor for survival in this endotoxemia model.

DISCUSSION

In this chapter, we demonstrate that myeloid HIFs adopt different roles in various inflammatory diseases. Myeloid HIF deficiency exacerbates tumor burden and progression of murine colitis-associated cancer, and mildly suppresses TPA-induced skin inflammation. However, in other inflammatory models, myeloid HIFs are dispensable for leukocyte infiltration in TG-induced peritonitis and animal survival in LPS-induced endotoxemia. These data reinforce that function(s) of each HIF-α subunit in myeloid cells is disease-dependent and introduce many intriguing questions to be addressed further.

Based on the protective effects of myeloid HIF-2α loss and HIF inhibition by ACF treatment in the same AOM/DSS induced CAC model (Shay et al. 2014, Imtiyaz et al. 2010), we initially predicted that depletion of both myeloid HIF-αs would mitigate tumor growth and progression. Surprisingly, our study revealed greater tumor burden and more advanced tumor progression when both HIF-1α and HIF-2α were absent in myeloid cells. Since ACF treatment can inhibit HIF signaling in colonic cells other than myeloid cells, it is possible that myeloid HIF signaling acts to suppress tumor growth, but is not enough to override the pro-tumor effects of HIF signaling in other cells types, such as colonic epithelial cells (Ma et al. 2017, Xue and Shah 2013, Xue et al. 2012). Again, this highlights a similar theme as in the colitis model where the role of HIF signaling is highly cell type-specific. Additionally, myeloid HIF-2α deficiency did not limit tumor burden as previously described (Imtiyaz et al. 2010); instead it slightly increased tumor burden, although this was not statistically significant (Figure 20). The discrepancy may be attributed to the differences in genetic background. Rather than using mice with a mixed 129/Sv × C57BL/6 genetic background, we used mice with a pure C57BL/6 background, which are known to be more susceptible to DSS-induced colitis compared with 129/Sv mice (Knod et al. 2014).
Figure 30. Myeloid HIF deficiency does not affect immune cell infiltration in the thioglycollate-induced peritonitis model. (A) Percentages of lymphoids (left) and myeloids (right) in live CD45+ cells from LysMCre (n=5, mean ± s.e.m.) and LysMCre;ArntΔΔ (n=4, mean ± s.e.m.) mice 4 hours post-thioglycollate injection (30mg/mouse). (B) Percentages of lymphoids (left) and myeloids (right) in live CD45+ cells from LysMCre (n=5, mean ± s.e.m.) and LysMCre;ArntΔΔ (n=5, mean ± s.e.m.) mice 4 days post-thioglycollate injection (30mg/mouse). Student’s t-test; *p<0.05.
Figure 31. Survival in a LPS-induced endotoxemia model is not dependent on myeloid HIF signaling. Kaplan-Meier survival curve of LysMC;Armt<sup>flx<sup>+</sup></sup> (n=8) and LysMC;Armt<sup>l<sup>tr</sup></sup> (n=8) mice in LPS-induced endotoxemia model.
We found that the numbers of TAMs were comparable in control mice and mice with myeloid HIF deficiency, while ACF treatment decreased macrophage infiltration (Shay et al. 2014). This may indicate a cell-extrinsic control of TAM migration and invasion by HIF signaling in other colonic cells, and require further investigation. Additionally, the fact that significant differences in tumor burden and progression were accompanied by comparable TAM presence let us to wonder whether functional phenotypes of these cells were more important than numbers, similar to what was observed in the acute colitis model: numbers of macrophages were almost identical during colitis resolution in LysMCre and LysMCre;Arntfl/fl mice, while LysMCre;Arntfl/fl macrophages exhibited a less pro-resolving gene expression profile (Figure 13). Genetic profiling of these TAMs may help to confirm whether they are true contributors to CAC pathology and provide insights regarding how HIF signaling regulates their functions in this particular setting.

The specific survival disadvantage of LysMCre;Hif2αfl/fl mice in the AOM/DSS induced CAC model is an interesting observation. However, the result that myeloid HIF-2α was dispensable for DSS-induced chronic colitis suggests that myeloid HIF-2α deficiency did not contribute to the lower survival rate by exacerbating inflammation. Instead, loss of myeloid HIF-2α may render animals more susceptible to carcinogen, AOM, or the combination of AOM and DSS.

Consistent with previous studies about each HIF-α isoform (Imtiyaz et al. 2010, Cramer et al. 2003), we also found less severe skin inflammation when ARNT was depleted from myeloid cells, manifested by slightly reduced ear edema. However, in contrast to the decrease in leukocyte and neutrophil infiltration observed in these previous studies, mice with myeloid ARNT deficiency exhibited higher number of CD45+ cells and neutrophils normalized to the weight of ear tissue. Moreover, Ly6C+ monocytes, another pro-inflammatory myeloid population, also showed almost 2.5-fold increase in LysMCre;Arntfl/fl mice compared with control mice. Alleviated inflammation accompanied by a higher presence of pro-inflammatory myeloid cells is quite counterintuitive. Furthermore, a high number of neutrophils were CD11c+ in this skin inflammation model, which was not seen in the intestine or the spleen (data not shown). This CD11c+ subpopulation can be further segregated based on F4/80 expression, which have not been
reported in any literature so far. Interestingly, TPA treatment increased the proportion of CD11c⁺,F4/80⁻ cells and significantly decreased the proportion of CD11c⁺,F4/80⁺ cells out of total neutrophils, while their proportions in TPA-treated LysMCr;Arntfl/fl mice were comparable to acetone-treated mice. The ratio or balance of these two neutrophil subpopulations may be important for the in vivo inflammatory responses in this model and will require additional investigation.

Finally, we revealed that HIF-α/ARNT is dispensable for leukocyte infiltration in a peritonitis model and animal survival in an endotoxemia model. Myeloid HIF-2α deficiency has been shown to impair macrophage migration towards the site of peritonitis (Imtiyaz et al. 2010), which was not recapitulated in our study where myeloid pan-HIF activity was inhibited by ARNT depletion. HIF-1α in the myeloid compartment may oppose HIF-2α in these cells. However, the concomitant blockade of AhR activity by ARNT depletion is a more likely explanation for the discrepancy, given that AhR has been demonstrated to suppress Alum-induced peritonitis and the recruitment of neutrophils as well as Ly6C⁺ monocytes in this model (Huai et al. 2014). Similarly, the comparable survival of LysMCr;Arntfl/fl and LysMCr;Arntf⁰⁰ mice may be attributed to the partial loss of ARNT in heterozygous mice. However, the inhibition of AhR activity is more likely contributing to the phenotype. In the study by Imtiyaz and colleague, the control group was also heterozygous LysMCr;Hif2αfl/fl mice, but LysMCr;Hif2αfl/fl mice exhibited significantly better survival, with 40% of mice alive 5 days after LPS injection. Mice with myeloid HIF-1α deficiency showed even lower mortality rate (~25%) for almost 5 days (Peyssonnaux et al. 2007). Therefore, we believe the heterozygous control in our study was not sufficient to account for the lack of significant survival differences. As reduced AhR activation has been linked to higher mortality in the same endotoxemia model (Bessede et al. 2014), depletion of ARNT, which inhibits AhR signaling, may override the protective effects of impaired HIF activity, resulting in the 100% mortality we observed within 40 hours after LPS injection. Experiments utilizing LysMCr and LysMCr;Hif1αf⁰¹;Hif2αf⁰¹ mice will be able to address these hypotheses by teasing apart the contribution from HIF-αs vs. AhR.
In summary, this chapter further highlights the disease and tissue dependence of myeloid HIF-α functions. The opposing effects of AhR to HIFs in these models underscore the importance of developing more specific ways to target HIF pathways.
CHAPTER 4

Concluding Remarks

Myeloid cell hypoxic responses are a key component in the pathology of many inflammatory diseases, as inflammation is often accompanied by tissue hypoxia and dysregulated innate immunity (Eltzschig and Carmeliet 2011). Myeloid cells, essential contributors to innate immunity, preferentially accumulate within hypoxic regions and adapt to limited O₂ via activation of HIF signaling (Murdoch, Muthana, and Lewis 2005). However, these HIF-mediated responses in macrophages and neutrophils do not always promote or suppress inflammation; it depends on the disease type, cell type, and phases of inflammation, as revealed by this body of work. Moreover, common (Cramer et al. 2003, Imtiyaz et al. 2010, Walmsley et al. 2005, Thompson et al. 2014), non-redundant (Cramer et al. 2003, Imtiyaz et al. 2010), and opposing functions (Takeda et al. 2010, Keith, Johnson, and Simon 2011) of HIF-1α and HIF-2α in myeloid cells (Lin and Simon 2016) add to the complexity of predicting clinical outcomes of HIF inhibition or activation. In order to expand our understanding of myeloid HIF signaling in particular pathologic conditions, this thesis utilized genetically engineered mouse models driven by highly myeloid specific LysMCre as an approach to more precisely investigate the roles of myeloid HIF-αs in settings of several acute and chronic inflammatory diseases.

In Chapter 2, I demonstrated that myeloid HIF signaling promotes the resolution of acute intestinal inflammation. Depletion of ARNT from myeloid cells effectively repressed HIF-1α and HIF-2α mediated transcriptional activities in BMDMs. Disruption of myeloid HIF signaling had no effect on the onset of acute colitis, but resulted in significantly inefficient resolution of colonic inflammation in a DSS-induced acute colitis model. Importantly, this defect should be primarily attributed to HIF-1α and HIF-2α, given that the loss of either subunit in myeloid cells recapitulated the phenotype of myeloid ARNT depletion in this model. During the resolution phase, myeloid HIF deficiency led to increased infiltration of pro-inflammatory neutrophils and monocytes into diseased tissues. HIF-deficient macrophages also exhibited impaired functional conversion to a
protective, pro-resolving phenotype. Moreover, gut serum amyloid A levels were remarkably elevated upon loss of myeloid ARNT during the resolution phase of colitis. Collectively, these dysregulated processes may together contribute to defective resolution of colitis, providing additional insights into therapeutic approaches to treat IBD patients.

In Chapter 3, I extended the investigation of HIF inhibition to colitis-associated cancer (CAC) and other inflammatory diseases. In an AOM/DSS-induced CAC model, tumor burden and progression were exacerbated only when both HIF-1α and HIF-2α were depleted from myeloid cells. Additionally, pan-HIF inhibition mildly alleviated edema in a TPA-induced skin inflammation model. However, infiltration of immune cells in a TG-induced peritonitis model and animal survival in a LPS-induced endotoxemia model appeared to be completely independent of myeloid HIF signaling. Collectively, these data add to our current understanding of myeloid HIF in these pathological conditions, reinforce diverse roles of myeloid HIF-α’s in various inflammatory diseases, and highlight that their functions depend on disease type.

Here, I will summarize some key implications that arise from this body of work and propose future studies to further extend our understanding of the crosstalk between hypoxia, HIF, myeloid cells and inflammation.

Select the right HIF-α subunit

HIF-1α and HIF-2α are the two best characterized HIF-α subunits, and govern key biological processes in myeloid cells under hypoxic or inflammatory stresses (Lin and Simon 2016). Interestingly, these two subunits can share the same, but also sometimes elicit distinct effects in many pathological conditions (Keith, Johnson, and Simon 2012). For example, intestinal epithelial cell HIF-1α helps to ameliorate inflammation (Karhausen et al. 2004, Furuta et al. 2001, Hirota et al. 2010), while HIF-2α in the same cells worsens acute colitis (Xue et al. 2013). In a tumor setting, HIF-2α promotes neuroblastoma progression to an aggressive phenotype; however, high HIF-1α levels correlates with low tumor stage and a favorable patient prognosis (Holmquist-Mengelbier et al. 2006, Noguera et al. 2009). Consistently, HIF-1α and HIF-2α can also exhibit
antagonistic functions in macrophages, e.g. nitric oxide (NO) production (Takeda et al. 2010); or shared functions, e.g. cytokine production (Cramer et al. 2003, Imtiyaz et al. 2010). The notion that HIF-1α and HIF-2α can adopt distinctive roles is further demonstrated in vivo by this body of work. In Chapter 2, I showed that HIF-2α has a less dramatic induction under hypoxia in BMDMs compared to HIF-1α, which was further reflected in vivo as a less impressive contribution of HIF-2α, compared with HIF-1α, to colitis resolution. Regardless of HIF-1α being the dominant isoform, both subunits impact proper resolution of intestinal inflammation. TPA-induced skin inflammation is another inflammatory condition where inhibition of either HIF-1α or HIF-2α, or both, helped to mitigate edema of ear tissue. However, myeloid HIF-1α and HIF-2α displayed divergent roles in a CAC model, where myeloid HIF-1α loss correlated with more advanced disease progression, and HIF-2α deficiency exacerbated colonic inflammation. Future studies will be required to identify the disease type-specific factors dictating myeloid HIF-α functions and the underlying molecular programs through which the two subunits cast similar or distinct effects. Nevertheless, my in vivo data, together with previous studies, stress the importance of selecting the appropriate HIF-α subunit(s) to target in different types of disease for the optimal outcome.

**Intervene at the right time**

Inflammation is a highly dynamic and tightly regulated process, with onset of the reaction to clear pathogens and a resolution program to ensure restoration of homeostasis. Non-resolving acute inflammation could eventually develop into persistent chronic inflammation (Soehnlein and Lindbom 2010, Ortega-Gomez, Perretti, and Soehnlein 2013). Macrophages are particularly plastic and can adopt multiple functional phenotypes depending on their surrounding microenvironment (Sica and Mantovani 2012, Shapouri-Moghaddam et al. 2018, Galli, Borregaard, and Wynn 2011). Unsurprisingly, macrophages exhibit distinct phenotypes at different stages of inflammatory diseases (Soehnlein and Lindbom 2010). My work provides two great examples demonstrating how crucial it is to intervene with myeloid HIF signaling at the correct disease stage. In an acute colitis model, activation of myeloid HIF signaling is essentially dispensable for the onset of inflammation, but necessary for the proper resolution of colonic
inflammation. The requirement of HIF signaling for macrophages to convert to a pro-resolving phenotype in a timely manner is very likely underlying the temporal dependence of myeloid HIF in acute colitis. Another example is the TPA-induced skin inflammation, where myeloid HIF deficiency alleviates the acute disease but does not affect pathology of chronic skin inflammation. Future studies with careful temporal characterization of myeloid HIFs are needed to provide useful insights into the optimized timing of pharmacological intervention, and a more detailed understanding of how myeloid HIFs mediate molecular decisions of inflammation timing.

**Target the right cells**

An inflammatory site harbors myriad cells, all of which experience low O2 stress (Eltzschig and Carmeliet 2011). HIF-mediated hypoxic responses differ between cell types and do not necessarily dictate all to adopt a pathogenic or protective phenotype. For example, ACF, a potent inhibitor of HIF-α/ARNT dimerization, prevents tumor progression in a CAC model in vivo, and blocks HIF signaling in both macrophages and colorectal cancer cell lines in vitro (Shay et al. 2014). However, repression of HIF signaling in macrophages and cancer cells likely opposes each other in terms of disease outcome. Utilizing cell type-specific genetic mouse models, we and others have demonstrated that myeloid deficiency of HIF-1α and HIF-2α exacerbates CAC burden and progression, while loss of intestinal epithelial HIF-2α facilitates to suppress CAC growth (Ma et al. 2017, Xue and Shah 2013, Xue et al. 2012). In this particular situation, cancer cell specific-HIF inhibition may actually result in more effective tumor suppression than targeting all cells in the colon. Similarly in the setting of colitis, HIF-1α and HIF-2α antagonize each other in the intestinal epithelial cells (Karhausen et al. 2004, Furuta et al. 2001, Hirota et al. 2010, Xue et al. 2013); while pan-HIF activation in myeloid cells was shown in Chapter 2 to promote resolution of acute colitis. Therefore, a pan-HIF activating approach, i.e. administration of PHD inhibitors, specific to myeloid cells may possess greater potential to suppress inflammation. However, we should take into account the technical difficulty of cell type-specific drug delivery as a major hindrance to clinical practice.
Table 2. Significantly changed diseases and biological functions in ARNT-deficient lamina propria macrophages

<table>
<thead>
<tr>
<th>Diseases and biological functions</th>
<th>P value</th>
<th>Predicted activation state</th>
<th>Activation z-score</th>
</tr>
</thead>
<tbody>
<tr>
<td>transport of lipid</td>
<td>2.53E-03</td>
<td>Increased</td>
<td>2.26</td>
</tr>
<tr>
<td>fatty acid metabolism</td>
<td>4.06E-04</td>
<td>Increased</td>
<td>2.132</td>
</tr>
<tr>
<td>inflammation of body region</td>
<td>1.60E-08</td>
<td>Decreased</td>
<td>-2.02</td>
</tr>
<tr>
<td>differentiation of phagocytes</td>
<td>1.96E-03</td>
<td>Decreased</td>
<td>-2.132</td>
</tr>
</tbody>
</table>
AhR: confounding factor and alternative target

AhR is another ARNT binding partner that has been implicated in mediating inflammatory responses (Mulero-Navarro and Fernandez-Salguero 2016). It has the potential to contribute to the phenotypes observed in studies utilizing ARNT depletion in particular as an approach to study HIF-α functions. In the acute colitis model, we ruled out AhR as a major contributor to resolution of colonic inflammation, consistent with the finding from a previous study using mice with myeloid-specific depletion of AhR in the same model (Chinen et al. 2015). However, in the peritonitis and endotoxemia models, AhR may be the major confounding factor to the phenotypic discrepancy we observed between ARNT and individual HIF-α loss. AhR may represent as a more viable therapeutic target than HIF for these specific diseases, given its unique ligand binding ability towards a broad range of xenobiotics (Mulero-Navarro and Fernandez-Salguero 2016, Pandini et al. 2007). Future experiments using LysMCre;Hif1α\(^{fl/fl}\);Hif2α\(^{fl/fl}\) mice will help to address these hypotheses, and the comparison between ARNT- and pan-HIF depletion will prove useful in teasing apart the effects from HIF and AhR.

Lipid metabolism in colonic macrophages

Dysregulated lipid metabolism is another feature of some inflammatory scenarios. In particular, obesity and atherosclerosis are the two best studied diseases for crosstalk between lipid metabolism, macrophages and inflammation (Lin and Simon 2016). Lipid-laden macrophages, known as foam cells, facilitate initiation and progression of atherosclerosis (Moore, Sheedy, and Fisher 2013, Moore and Tabas 2011). Adipose tissue macrophages (ATMs) also adopt a pro-inflammatory phenotype in obese patients (Weisberg et al. 2003). Unsurprisingly, HIFs govern key molecular programs determining the phenotypes of these pathogenic macrophages in atherosclerosis and obesity (Lin and Simon 2016). In light of this evident link between inflammation and lipid metabolism, as well as the reported lipid abnormalities in IBD patients (Agouridis, Elisaf, and Milionis 2011, Ungaro et al. 2017), we are excited to discover “transport of lipid” and “fatty acid metabolism” as the two most significantly upregulated biological processes in LysMCre;Arnt\(^{fl/fl}\) lamina propria macrophages based on IPA of our microarray data.
Some of the upregulated genes under these two biological processes have been shown to facilitate inflammation. For example, *Fabp4* encodes a fatty acid binding protein, and deletion of this gene in macrophages is sufficient to protect mice from developing atherosclerosis and dyslipidemia (Makowski et al. 2001). We believe that disrupted lipid metabolism represents a highly promising mechanism through which myeloid HIF-α subunits contribute to the defective resolution of acute colitis.

Concerted efforts have enabled development of many HIF-α inhibitors (e.g. ACF) and stabilizers (e.g. PHD inhibitors). In recent years, more and more inhibitors that are highly specific to either HIF-1α or HIF-2α have made into preclinical or even clinical trials (Wigerup, Pahlman, and Bexell 2016). These achievements empower us with tools that can either suppress or activate HIF signaling in a broad range of diseases. I believe that disease-by-disease characterization of HIF functions in a temporally-regulated and cell type-specific approach would truly accelerate and expand the application of these elegantly designed drugs in the treatment of patients afflicted by chronic inflammation and cancer.
MATERIALS AND METHODS

Mice

The Arnt, Hif1α and Hif2α conditional alleles were crossed with the LysM-Cre allele (Clausen et al. 1999) to achieve myeloid-specific Arnt, Hif1α or Hif2α conditional knockout mice. Mice with Hif1α conditional allele on a C57BL/6 background were purchased from Jackson Laboratory (Bar Harbor, ME). LysMCre;Hif2α<sup>fl/fl</sup> mice were generated and described in a previous study (Imtiyaz et al. 2010). Ever since this study, we have backcrossed Hif2α<sup>fl/fl</sup> mice with C57BL/6 mice sufficiently to ensure a similar background to other strains. Arnt<sup>fl/fl</sup> mice with a mixed background of C57BL/6 and 129svJ were also backcrossed with C57BL/6 mice sufficiently before crossed with LysMCre mice. All animal procedures were performed in accordance with NIH guidelines and were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Induction of colitis and clinical scoring

For acute colitis model, dextran sulfate sodium (DSS) (MW 36-50 kDa, MP Biomedicals, Santa Ana, CA) was administered orally in drinking water at 3% (w/v) concentration for 5 days followed by normal drinking water for 3 days. For chronic colitis model, mice were given 3 cycles of 2% (w/v) DSS for 5 days followed by normal drinking water for another 5 days after each DSS treatment. Mice of both genotypes (LysMCre vs. LysMCre;Arnt<sup>fl/fl</sup> for acute colitis; and LysMCre vs. LysMCre;Hif2α<sup>fl/fl</sup> for chronic colitis) were housed in the same cages to minimize potential confounding influences from differing microbiomes. Body weight, stool consistency, and fecal blood were monitored and recorded daily for each mouse. Disease Activity Index (DAI) was calculated as the sum of scores for body weight loss, stool consistency, and fecal blood. These three parameters were scored as following (Melgar, Karlsson, and Michaelsson 2005, Qiu et al. 2011): 0, no weight loss or less than 1% weight loss, normal stool pellets, negative Hemoccult test (Beckman Coulter, Brea, CA); 1, 1%-5% weight loss, slightly loose feces; 2, 5%-10% weight
loss, lose feces, positive Hemoccult test; 3, 10%-20% weight loss, watery diarrhea; 4, more than 20% weight loss, positive Hemoccult test, and visible fecal and rectal blood.

**Histopathology assessment of DSS-induced acute colitis**

Colons ranging from cecum to rectum were cut longitudinally, fixed in 4% paraformaldehyde/PBS (4°C overnight), and embedded in paraffin for sectioning. Five-µm-thick sections were cut and stained with hematoxylin and eosin and scored in a double-blind manner. Tissue sections were scored for loss of mucosal architecture, cellular infiltration, crypt abscess formation, Goblet cell depletion, and tissue affected, yielding a total histopathology score. Loss of mucosal architecture was scored 0 to 3 for absent, mild, moderate, and severe with loss of entire crypts. Cellular infiltration was scored 0 to 3 for absent, mild, moderate, and extensive. Crypt abscess formation was scored 0 or 1 for absent or present. Goblet cell depletion was scored 0 or 1 for absent or present. Percentage of tissue affected was scored 0 to 3 for absent, >10%, 20-30%, and 40-50%. The sum of these values for each mouse gave a total histopathology score.

**Azoxymethane (AOM)/DSS induced colitis-associated colorectal cancer**

The AOM/DSS induced CAC model was performed as previously described (Hongxia). Eight- to nine-week-old male and female mice received a single intraperitoneal injection of 12.5 mg/kg AOM on Day 0 followed by 3 cycles of DSS in drinking water starting on Day 5 (cycle 1: 1.8% DSS for 5 days; cycle 2: 2.0% DSS for 7 days, cycle 3: 2.0% DSS for 7 days) with 2 weeks of regular drinking water in between each cycle and after the third cycle till the end of this experiment. The end point was set to 14 weeks, when mice were sacrificed. Some mice exhibiting severe symptoms were sacrificed along the course of experiment before 14 weeks. Colons were flushed with PBS and cut open longitudinally for counting and measurement of polyps. Polyps were counted across the whole colon from rectum to cecum. Their diameter was measured using a digital caliper (Thermo Fisher Scientific, Waltham, MA). Tumor grading was performed as previously described (Hongxia). Inflammation was graded as mild, moderate or
severe. Tumor-associated macrophages were counted as F4/80+ cells on a ×20 field for 3 fields using TMARKER (Nexus, Zürich, Switzerland).

**TPA-induced ear cutaneous inflammation**

TPA-induced ear cutaneous inflammation was conducted as described previously (Cramer et al. 2003). Male mice that were 9-week old were used in this experiment. Briefly, for each mouse, one ear was painted with 2.5 µg of TPA in acetone and the other ear was treated similarly with acetone as vehicle control. The mice were sacrificed 24 hours after the TPA application. Tissue biopsy with the same surface area was captured and used for assessment of edema (ear tissue thickness and weight), and then prepared for H&E, gene expression or leukocyte infiltration by flow cytometry.

**Thioglycollate-induced peritonitis**

Female mice that were 8-week old were injected intraperitoneally with 1 mL of 3% thioglycollate, and were sacrificed 4 hours and 4 days after the inject for peritoneal exudate cells harvest. These cells were immediately subject to flow cytometry analysis.

**LPS-induced endotoxemia**

LPS-induced endotoxemia was conducted as described previously (Peyssonnaux et al. 2007). Male mice that were 14-week old were injected intraperitoneally with 15mg/kg of LPS, and monitored for survival. Survival data were analyzed by the construction of Kaplan-Meier plots.

**Isolation of lamina propria cells**

Lamina propria cells were isolated using a modified version of previously described protocols (Weigmann et al. 2007, Zaph et al. 2007). Briefly, colons were cut open longitudinally and shaken in medium with 1 mM EDTA and 1 mM DTT twice for 20 minutes each at 37°C. The remaining tissue was further digested with 0.5mg/mL Collagenase/Dispase (Roche, Basel, Switzerland) and 0.05 mg/mL (92.15 Kunitz unit/mL) DNase I (Sigma-Aldrich, St. Louis, MO) for
40 minutes at 37°C with agitation. Cells were then harvested by passing the suspension through a 70-μm cell strainer (Corning, Corning, NY). Single cell suspensions were later analyzed ex vivo by flow cytometry.

**Flow cytometry**

Single cells suspensions were blocked with Mouse BD Fc Block™ (BD Biosciences, Franklin Lakes, NJ) for 10 minutes and then stained in FACS buffer (PBS with 4% FBS and 2 mM EDTA) with the following fluorochrome-conjugated antibodies: APC-conjugated anti-CD19 (1D3, #550992, 1:200), APC-Cy7-conjugated anti-CD4 (GK1.5, #552051, 1:200), PE-Cy7-conjugated anti-CD8a (53-6.7, #552877, 1:200), FITC-conjugated anti-CD45 (30-F11, #561088, 1:100), V450-conjugated anti-CD3e (500A2, 560801, 1:100), APC-Cy7-conjugated anti-Ly6C (AL-21, #560596, 1:100), PE-Cy7-conjugated anti-CD45 (30-F11, #552848, 1:100), V450-conjugated anti-CD11c (HL3, #560521, 1:100), PerCP-Cy5.5-conjugated anti-CD11b (M1/70, #561114, 1:100), AF700-conjugated anti-Ly6G (1A8, #561236, 1:100) (from BD Biosciences); PE-conjugated anti-F4/80 (BM8, #12-4801, 1:100) (from eBioscience, San Diego, CA). Viability was determined by staining cells with LIVE/DEAD™ Fixable Aqua Dead Cell stain, 1:300 (Thermo Fisher Scientific). Flow cytometry was performed on a LSR A flow cytometer (BD Biosciences), and data were analyzed using FlowJo software.

**Colonic explant supernatant collection and ELISA**

A 0.5 cm-long colon segment was collected about 1 cm from the rectum from each well-flushed mouse colon. These colon segments were cultured in 24-well plates containing 0.6 mL of complete tissue culture medium (DMEM with 25 mM HEPES, 0.05 mM 2-mercaptoethanol, 2 mM L-Glutamine, 100 U/mL Penicillin, 100 μg/mL Streptomycin, and 10% FBS) for 24 hours till cell-free culture supernatant was collected. The collected supernatants were then subject to quantification of cytokine levels using the following ELISA kits: Mouse IL-1 beta/IL-1F2 DuoSet ELISA kit (#DY401-05), Mouse IL-6 DuoSet ELISA (#DY406-05), Mouse CXCL1/KC DuoSet ELISA (#DY453-05), Mouse Serum Amyloid A DuoSet ELISA (#DY2948-05), Mouse Cytokine
Antibody Array, Panel A (#ARY006) (from R&D Systems, Minneapolis, MN), and Mouse SAA-3 ELISA (#EZMSAA3-12K) (from Millipore Sigma, Burlington, MA).

**Microarray analysis**

Total RNA was extracted from flow cytometry-sorted lamina propria macrophages from *LysMCre* and *LysMCre;Arnt<sup>fl/fl</sup>* mice using TRIzol™ LS Reagent (Thermo Fisher Scientific). Total RNA quality control tests were determined using BioAnalyzer 2100 (Agilent) and Nanodrop spectrophotometry (Thermo Fisher Scientific). The cDNA was prepared, labeled, and hybridized to Affymetrix GeneChip, mouse gene 2.0 using GeneChip WT PLUS Reagent Kit (Affymetrix, Santa Clara, CA). Hybridized chips were scanned with GeneChip™ Scanner 3000 7G (Affymetrix). Affymetrix Command Console and Expression Console (Thermo Fisher Scientific) were used to quantitate expression levels for targeted genes; default values provided by Affymetrix were applied to all analysis parameters. Transcriptomic analysis was carried out using Partek Genomic Suite 6.1 (Partek, Inc., St. Louis, MO). Robust MultiArray Average (RMA) (Irizarry et al. 2003) was used for normalization of the raw probe intensity data. Significance Analysis of Microarrays (SAM) (Tusher, Tibshirani, and Chu 2001) was applied to compare *LysMCre* and *LysMCre;Arnt<sup>fl/fl</sup>* samples. The magnitude of d score, the *T*-statistic value used in SAM, scales with statistical significance. A false discovery rate (q-value) was estimated by SAM based on a null distribution for the d score by permuting the samples with respect to *LysMCre* and *LysMCre;Arnt<sup>fl/fl</sup>* classes. Differentially expressed genes were defined as those having fold change above or below 1.5 and q value < 0.05.

**Generation and culture of bone marrow-derived macrophages (BMDMs) and neutrophils (BMDNs)**

Bone marrow cells were isolated from femurs and tibias of *LysMCre* and *LysMCre;Arnt<sup>fl/fl</sup>* mice. After a quick incubation in ammonium-chloride-potassium (ACK) lysing buffer (Lonza, Basel, Switzerland) to remove red blood cells, the remaining bone marrow cells were cultured in BMDM medium (DMEM with 20% heat-inactivated HyClone FBS, 30% L929 conditioned medium, 1X
Antibiotic-Antimycotic, 2 mM L-Glutamine, and 0.055 mM 2-mercaptoethanol) for 5 day on non-treated tissue culture plates before passaging. To obtain BMDNs, EasySep™ Mouse Neutrophil Enrichment Kit (STEMCELL Technologies, Vancouver, Canada) was used for negative selection of neutrophils from bone marrow cells after lysis by ACK lysing buffer. BMDNs were analyzed immediately or cultured in neutrophil culture medium (RPMI 1640 with 10% FBS, 100 U/mL Penicillin and 100 μg/mL Streptomycin) for up to 24 hours before analysis.

**Neutrophil viability/apoptosis assessments**

Right after isolation of neutrophils from bone marrow cells using EasySep™ Mouse Neutrophil Enrichment Kit (STEMCELL Technologies), total number of viable cells was determined by cell counting with Trypan Blue. Same number of viable neutrophils was then cultured in neutrophil culture medium (RPMI 1640 with 10% FBS, 100 U/mL Penicillin and 100 μg/mL Streptomycin) for 24 hours under normoxia (21% O₂) or hypoxia (0.5% O₂) before another viability analysis. Percentage viability was taken as percentage of viable neutrophils after 24-hour culture out of viable neutrophils seeded. Caspase-Glo® 3/7 assay (Promega, Madison, WI) was used with these cells at these two time points to assess apoptosis.

**Immunohistochemistry**

Immunohistochemistry was performed on sections from paraffin-embedded samples using VECTASTAIN Elite ABC HRP Kit (#PK-6100, Vector Laboratories, Burlingame, CA). Rat anti–mouse F4/80 primary antibody (#ab6640, Abcam Inc., Cambridge, MA) was used at a 1:100 dilution (10 µg/mL). Vector Laboratories biotinylated anti-rat IgG was used as the secondary antibody at a 1:200 dilution. Sections were incubated with DAB substrate (Vector Laboratories) and counterstained with hematoxylin for nuclei.

**Quantitative RT-PCR**

Total RNA was isolated from colon tissues, macrophages derived from colon, BMDMs, and BMDNs using the RNeasy Mini Kit (Qiagen, Hilden, Germany). For colon tissues, BMDMs,
and BMDNs, cDNA was synthesized using a High-Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Foster City, CA). PCR reactions were performed using Taqman Universal PCR reagents mixed with indicated cDNAs and Taqman primers in a ViiA7 Real-Time PCR system (Applied Biosystems). Expression levels were normalized to \textit{Hprt} (Mm01318743\_m1). The following Taqman primers were used in this study: \textit{Arnt} (Mm00507836\_m1), \textit{Adm} (Mm00437438\_g1), \textit{Vegfa} (Mm01281449\_m1), \textit{Ldha} (Mm01612132\_g1), \textit{Pgk1} (Mm00435617\_m1), \textit{Arg1} (Mm00475988\_m1), \textit{Serpine1} (MM00435860\_M1), \textit{Il1b} (Mm00434228\_m1), \textit{Il6} (Mm00446190\_m1), \textit{Il12a} (Mm00434169\_m1), \textit{Tnf} (Mm00443258\_m1), \textit{Cxcl10} (Mm00445235\_m1), \textit{Cxcl12} (Mm00445553\_m1), \textit{Cxcl13} (Mm04214185\_s1), \textit{Ccl4} (Mm00443111\_m1), \textit{Ccl5} (Mm01302427\_m1), \textit{Cyp1a1} (MM00487218\_M1), \textit{Ugt1a1} (Mm02603337\_m1), \textit{Cxc2r} (MM99999117\_S1), \textit{Il23a} (Mm01160011\_g1), \textit{Chi3l3} (Mm00657889\_mH), \textit{Mrc1} (Mm00485148\_m1), and \textit{Retnla} (Mm00445109\_m1). For macrophages isolated from colon, due to limited amount of mRNA, anti-sense RNA (cRNA) generated in preparation for microarray analysis using GeneChip WT PLUS Reagent Kit (Affymetrix) was used to generate cDNA. PCR reactions were performed in a ViiA7 Real-Time PCR system using Sybr Green PCR Master Mix (Invitrogen, Carlsbad, CA) with following primers: mouse \textit{Saa1} (Forward 5' to 3': ACACCAGGATGAAGCTACTCACCA; Reverse 5' to 3': CCCTTGGAAAGCTCTCGTAACCA), mouse \textit{Saa2} (Forward 5' to 3': TGGCTGGAAAGATGGAGACAA; Reverse 5' to 3': AAAGCTCTCTTTGATCATCTG), mouse \textit{Saa3} (Forward 5' to 3': TGCCATCATCTTTGCATCTTG; Reverse 5' to 3': CCGTGAACCTGCTGCAACG), mouse \textit{Ptges1} (Forward 5' to 3': GGATGCGCTGATGACGCA; Reverse 5' to 3': CAGGAATGAGTACGCAGG), mouse \textit{Ptges2} (Forward 5' to 3': AAGGCCATGAATGACGAGG; Reverse 5' to 3': TTCTGGGTACAGCTTGGAG), and mouse \textit{Ptgs2} (Forward 5' to 3': TTCAACACACTCTATCCTGGC; Reverse 5' to 3': AGAAGCGCTTTCGTTACTCAT).

\textit{Western blot analysis}
BMDMs were lysed with RIPA buffer containing protease inhibitor (Thermo Fisher Scientific). Cells lysates were boiled in SDS sample buffer for 10 minutes, separated by SDS-PAGE, transferred to nitrocellulose membranes, probed with primary antibodies overnight at 4°C, and then detected with horseradish peroxidase-conjugated secondary antibodies (Vector Laboratories) followed by exposure to ECL (PerkinElmer, Waltham, MA). The following antibodies were used at indicated concentration: rabbit anti-ARNT (#5537, 1:1000, Cell Signaling Technology, Danvers, MA), rabbit anti-HIF-1α (#10006421, 1:1000, Cayman Chemical, Ann Arbor, MI), rabbit anti-HIF-2α (#PA1-16510, 1:1000, Thermo Fisher Scientific), and mouse anti-β-actin (#SC-47778, 1:4000, Santa Cruz Biotechnology, Dallas, TX).

Eicosanoids analyses by liquid chromatography–mass spectrometry

The analysis of eicosanoid metabolites in colonic explant supernatants and BMDMs/BMDNs culture media was performed as described previously for human plasma (Mazaleuskaya et al. 2016) with a few modifications. Culture medium samples (450 μl) were spiked with stable isotope-labeled internal standards ([d4]-PGE2 [5 ng]; [d4]-PGF2α [2.5 ng]; [d4]-TxB2 [10 ng]; [d4]-LTB4 [1 ng]; [d5]-LTE4 [2.5 ng]; [d8]-5-HETE [2.5 ng]; [d8]-12-HETE [25 ng]; [d8]-15-HETE [1 ng]; [d8]-AA [2,500 ng]) (Cayman Chemical) in 1350 μl of acetonitrile. The samples were cleaned up by using Phree cartridges for phospholipid and protein removal (#8B-S133-TAK from Phenomenex, Torrance, CA). Samples were then dried under a gentle stream of nitrogen at the ambient temperature and reconstituted with 30 μl of methanol. Before injection, 30 μl of water was added to each sample and an aliquot of 20 μl was injected into a C18 UPLC column (ACQUITY UPLC BEH 2.1 × 150 mm × 1.7 μm) (Waters Corporation, Milford, MA) and eluted at a flow rate of 350 μl/min, with a linear gradient from 20% solvent B to 90% in 20 minutes. Mobile phase A consisted of water/mobile phase B, 95:5 (v/v), with 0.5% formic acid; mobile phase B consisted of acetonitrile/methanol, 95:5 (v/v), with 0.5% formic acid. Mass spectrometer parameters were optimized to obtain maximum sensitivity for respective product ions as described previously (Mazaleuskaya et al. 2016). The analysis was performed on a Waters ACQUITY Ultra Performance Liquid Chromatography system in-line with a Waters Xevo TQ-S
Triple Quadrupole Mass Spectrometer (Waters Corporation). The UPLC system directly interfaced with the negative-mode electrospray ionization (Lin et al.) source of the mass spectrometer using multiple reaction monitoring (MRM). Quantitation was done by peak area ratio and results were normalized to the sample volume.

**Statistical analysis**

Data were analyzed with GraphPad Prism 7. Unpaired, two tailed t test was used for all single comparisons, and two-way ANOVA followed by Bonferroni’s correction was used for multiple comparisons. Data are presented as mean ± standard error of the mean (S.E.M.); values of p < 0.05 were considered statistically significant.


Hutter, R., W. S. Speidl, C. Valdiviezo, B. Sauter, R. Corti, V. Fuster, and J. J. Badimon. 2013. "Macrophages transmit potent proangiogenic effects of oxLDL in vitro and in vivo


Prolyl 4-Hydroxylase-2 Inhibition Protects Against Development of Atherosclerosis.


