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Hypoxia Inducible Factors in Cancer and Inflammation

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Abstract

Hypoxia-inducible factors (HIFs) mediate adaptation to low O2, or hypoxia, are important at every stage of tumor initiation, and impact the progression of a variety of diseases, including colorectal cancer. This body of work investigates the role of hypoxia and HIF-mediated signaling in both tumor cells and macrophages across the natural history of inflammation-induced cancers. First, the effect of HIF inhibition in tumor parenchyma and stroma in extant colitis-associated colon carcinomas (CAC) is investigated using acriflavine (ACF), a naturally occurring compound known to repress HIF transcriptional activity. Pharmacologic HIF inhibition represents a novel therapeutic strategy for cancer treatment and data indicates ACF treatment halts the progression of an autochthonous model of established CAC in immunocompetent mice and does so largely through HIF-dependent means. These results suggest pharmacologic HIF inhibition in multiple cell types, including epithelial and innate immune cells, significantly limits tumor growth and progression.

Second, myeloid specific deletion (LysMCre) of the HIF constitutive binding partner ARNT is studied in the setting of acute and chronic inflammatory responses that eventually result in inflammation-associated cancer development. Data indicates loss of ARNT results in severe macrophage defects including decreased edema and inflammatory infiltrate in an acute model of skin inflammation and lower stage disease and decreased tumor inflammation in a model of CAC. Collectively, these data suggest the hypoxic response is necessary for sustained inflammation and tumor progression and may provide a link between chronic inflammatory conditions and cancer development.

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ABSTRACT

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Hypoxia-inducible factors (HIFs) mediate adaptation to low O_2, or hypoxia, are important at every stage of tumor initiation, and impact the progression of a variety of diseases, including colorectal cancer. This body of work investigates the role of hypoxia and HIF-mediated signaling in both tumor cells and macrophages across the natural history of inflammation-induced cancers. First, the effect of HIF inhibition in tumor parenchyma and stroma in extant colitis-associated colon carcinomas (CAC) is investigated using acriflavine (ACF), a naturally occurring compound known to repress HIF transcriptional activity. Pharmacologic HIF inhibition represents a novel therapeutic strategy for cancer treatment and data indicates ACF treatment halts the progression of an autochthonous model of established CAC in immunocompetent mice and does so largely through HIF-dependent means. These results suggest pharmacologic HIF inhibition in multiple cell types, including epithelial and innate immune cells, significantly limits tumor growth and progression.

Second, myeloid specific deletion (LysMCre) of the HIF constitutive binding partner ARNT is studied in the setting of acute and chronic inflammatory responses that eventually result in inflammation-associated cancer development. Data indicates loss of ARNT results in severe macrophage defects including decreased edema and inflammatory infiltrate in an acute model of skin inflammation and lower stage disease and decreased tumor inflammation in a model of CAC. Collectively, these data suggest the hypoxic response is necessary for sustained inflammation and tumor progression and may provide a link between chronic inflammatory conditions and cancer development.

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Chapter One

Hypoxia-inducible factors and the myeloid response to oxygen deprivation in settings of inflammation

SUMMARY

Cellular access to oxygen is a critical component of many physiologic and pathologic processes. The hypoxia-inducible factors (HIFs) are oxygen-sensitive transcription factors that are responsible, in large part, for the transcriptional regulation of cells experiencing hypoxia. Although the HIF response continues to be characterized within the tumor parenchyma, it is clear that HIF-1α and HIF-2α exhibit both overlapping and non-redundant roles and contribute to distinct aspects of cancer stem cell behavior, cell survival and proliferation, tumor metabolism, angiogenesis, invasion and metastasis.

Similarly, although the tumor parenchyma is of obvious importance in tumor progression, the influence and importance of recruited and resident stromal cells is being elucidated. As the complexity of the tumor microenvironment continues to be appreciated, hypoxic and HIF-mediated responses in the tumor stroma are proving to be important. In large part, HIFs mediate the response to hypoxia in inflammatory conditions, the innate immune system at large, and recruited macrophages within the tumor microenvironment as well as physiologic roles of HIF activity in inflammation and wound healing.

INTRODUCTION

All multicellular, eukaryotic organisms require oxygen (O_2) (Semenza 2007). Decreased O_2 availability (hypoxia) necessitates cellular and organismal adaptation to maintain energetic and biosynthetic homeostasis. Cells adapt to hypoxia in many different ways including the secretion of pro-angiogenic factors, decreasing bioenergetic requirements, and increasing anaerobic metabolic flux. These responses occur as a result of many different cellular pathways. The hypoxia-inducible factors (HIFs) are oxygen-sensitive transcription factors that are responsible, in large part, for the transcriptional regulation of cells experiencing hypoxia (Semenza et al. 1992; Semenza 2012). HIFs are important regulators of the physiologic and pathologic consequences of hypoxia. In this section, I shall discuss the role of HIFs in cancer, inflammation, the innate immune system, and crosstalk between these components.

HYPOXIA INDUCIBLE FACTORS

HIFs are responsible for metabolic reprogramming and changes in gene expression that are necessary for adaptation to decreased O_2 availability (Ema et al. 1997; Tian et al. 1997). These transcription factors are widely appreciated as key regulators of cellular adaptation to hypoxic stress (Majmundar et al. 2010). HIFs are heterodimeric proteins within the basic helix-loop-helix/PER-ARNT-SIM (bHLH/PAS) family of transcription factors and are primarily regulated through post-translational modification and stabilization (Figure 1). They are comprised of an O_2-labile α subunit and constitutively expressed β subunit (Qing et al. 2009). There are currently three identified α subunits: HIF-1α, HIF-2α, and HIF-3α. As very little is known about the role of HIF-3α, the following will focus exclusively on the overlapping and differential roles of HIF-1α and HIF-2α. Hydroxylation of two conserved proline residues (P402/P405 and
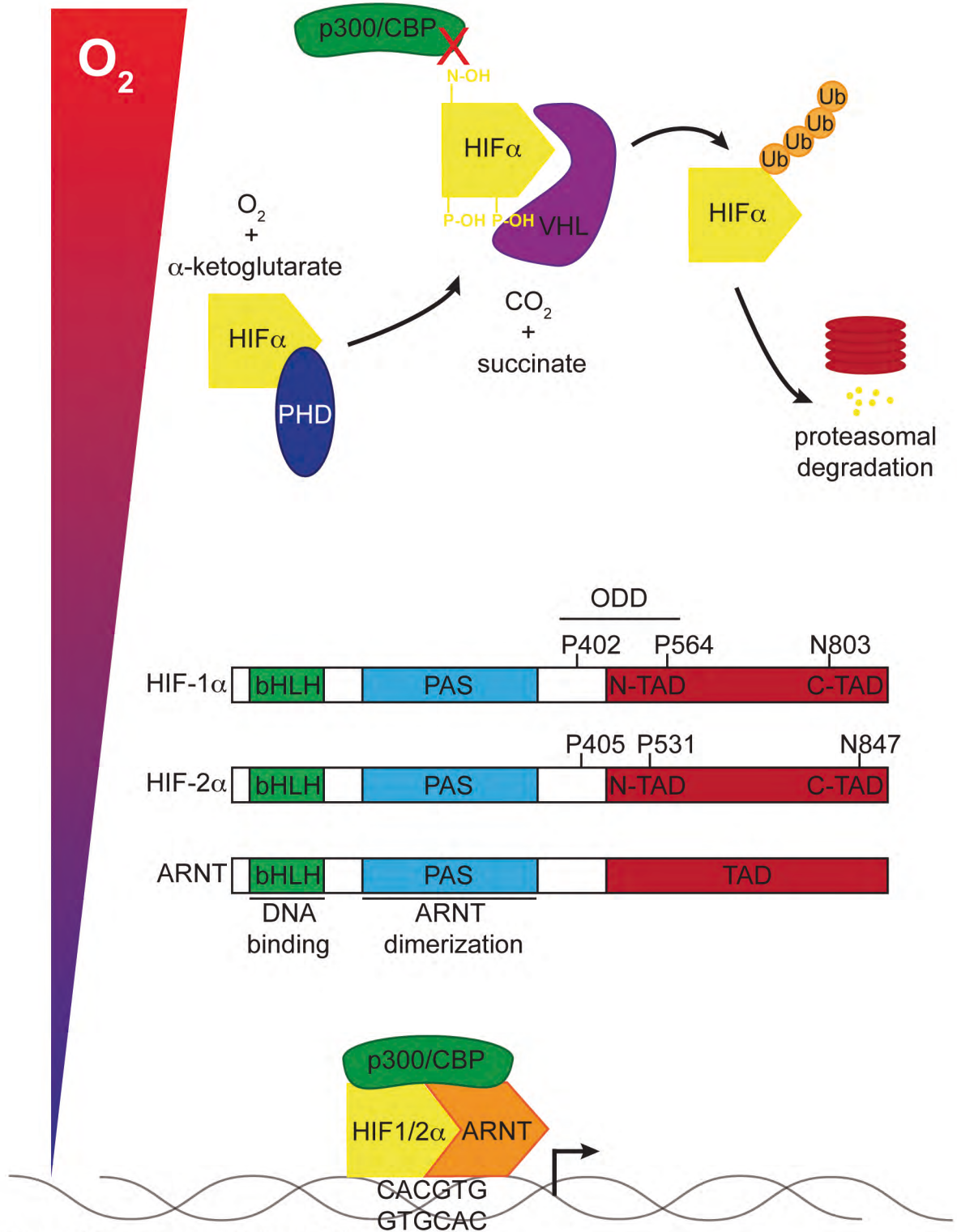
Figure 1. Oxygen regulation of HIF stabilization.

P564/P531 for HIF-1α/HIF-2α respectively) within the O_2-dependent degradation domain (ODD) of the α subunit occurs by prolyl hydroxylase domain proteins (PHDs) under normal O_2 tensions (Figure 1). Following hydroxylation, polyubiquitination by the von Hippel-Lindau (VHL) tumor suppressor E3 ubiquitin ligase complex occurs and is eventually followed by degradation via the 26S proteasome (Maxwell et al. 1999; Cockman et al. 2000; Jaakkola et al. 2001).

Regulation by O_2 availability. PHDs are members of the Fe(II) 2-oxoglutarate-dioxygenase family and require oxygen as a substrate for activity (Kaelin et al. 2008). One atom of O_2 is used as a substrate for the decarboxylation of 2-oxo-glutarate to succinate and CO_2 while the other atom is directly incorporated into the oxidized residue (in this case a prolyl residue) of the HIFα subunit (Kaelin 2005). As such, under hypoxia, PHDs cannot hydroxylate either of the proline residues on the HIFα subunit, as access to the substrate (O_2) is limited. When stabilized under low O_2, HIFs are no longer modified by PHDs and targeted for proteasomal degradation, but instead translocate to the nucleus, dimerize with their obligate partner ARNT/HIF-1β through interaction of bHLH and PAS domains, and recruit coactivators such as CBP and p300 (Bertout et al. 2008; Patel et al. 2008; Majmundar et al. 2010; Keith et al. 2011). HIF heterodimers drive gene transcription involved in adaptation to hypoxic stress through binding and recognition of hypoxia-response elements (HREs), with the consensus sequence G/ACGTG, within the promoter regions of target genes (Talks et al. 2000; White et al. 2004; Semenza 2007; Imtiyaz et al. 2010).

More recently, other HIF-regulating oxygen-dependent enzymes have been identified. Factor-inhibiting HIF (FIH1) is an Fe(II) 2-oxoglutarate-dioxygenase that can hydroxylate an asparaginyl residue in the c-terminal transactivation (CTAD) domain of the HIFα subunit (N803/N847 for HIF-1α/HIF-2α respectively) in a mechanism very

similar to PHDs (Lando et al. 2002; Kaelin et al. 2008). Unlike prolyl hydroxylation, FIH1 activity does not directly lead to VHL recruitment, but instead prevents recruitment and interaction of coactivators CBP and p300 when asparaginyl hydroxylation is present (Lando et al. 2002; Kaelin et al. 2008). Interestingly, recent work has suggested HIF-1α is more sensitive to FIH-1 activity than is HIF-2α (Kaelin et al. 2008).

HIF regulation apart from O$_2$ levels. HIF hydroxylases can be inhibited by TCA intermediates such as citrate, isocitrate, succinate, fumarate, and pyruvate (Kaelin et al. 2008). Whereas PHDs are more sensitive than fumarate and succinate levels, FIH1 activity can be inhibited by citrate and oxaloacetate. Cells that lack intermediate enzymes may experience a buildup of succinate or fumarate leading to PHD inhibition and HIF stabilization. Indeed heterozygous mutants are predisposed to tumors following loss of heterozygosity (LOH) of succinate dehydrogenase or fumarate hydratase (Kaelin et al. 2008). Similarly, iron chelators and ascorbate levels can also influence rates of HIF hydroxylation. Lastly, accumulation of nitric oxide (NO) can also lead to HIF accumulation, even under normoxic conditions (Kaelin 2005).

Regulation of HIF accumulation and hydroxylation is an ongoing area of study as O$_2$ levels and metabolic intermediates become increasingly important. Importantly, intracellular and extracellular changes that characterize the tumor microenvironment are responsible for increasing HIF activity. The role of HIF and cancer development and progression will be discussed further in the following section.

HIF AND CANCER

Rapid proliferation of tumor cells can outpace existing or new vascular networks and thus results in decreased oxygen supply. Regions within the tumor microenvironment may be characterized by hypoxia, secondary to necrosis or aberrant neovascularization. Cancer cells may also proliferate faster than their blood supply and

thus result in regions that lack perfusion (Majmundar et al. 2010). Similarly, recruited or resident stromal cells may also impact regional O_2 availability. HIF-mediated transcriptional and metabolic changes accompany many distinct parts of tumor initiation and progression (Keith et al. 2007; Bertout et al. 2008; Keith et al. 2011).

HIFs and cancer stem cells. Recent studies have demonstrated the importance of Oct4 and c-Myc in allowing differentiated fibroblasts to regain stem cell like behavior. Both Oct4 and c-Myc have been identified as HIF-2α targets (Keith et al. 2007) and have been independently implicated in multiple cancer types. Similarly, HIF activity may regulate the Notch pathway with specific roles in tumor initiating cells. Human telomerase has also been demonstrated as hypoxia inducible in a HIF-dependent manner (reviewed in (Keith et al. 2007). Although the cancer stem cell hypothesis remains somewhat controversial, it is clear there are key genetic alterations that occur very early in tumorigenesis. Hypoxia and HIF mediated changes may influence the self-renewal and 'stem cell-like' nature of cells undergoing the earliest genetic modifications that accompany tumor initiation. Together these findings provide evidence of HIF-mediated transcriptional changes that may control the earliest stages of tumorigenesis.

Metabolic reprogramming. As previously referenced, tricarboxylic acid cycle (TCA) intermediates such as fumarate, succinate, citrate, and oxaloacetate can inhibit PHDs and FIH1, leading to HIFα stabilization (Kaelin et al. 2008). In the absence of O_2, aerobic glycolysis is severely limited. Likewise, highly proliferative cells, including tumor cells, exhibit large increases in anaerobic glycolysis (Vander Heiden et al. 2009). The Warburg effect, so named for Otto Warburg, the scientist who first described the paradoxical increase in anaerobic glycolysis observed in tumor cells even under settings of adequate oxygen access, can result in stabilization of HIFα, in part through accumulation of lactate and pyruvate (Hirschhaeuser et al. 2011).

Recently, TCA enzymes have been directly implicated in tumorigenesis and hypoxia-driven tumor cell metabolism. Mutations in isocitrate dehydrogenase 1/2 (IDH1/2) can indirectly upregulate HIF-1α by decreasing α-ketoglutarate availability, a known PHD substrate (Zhao et al. 2009). IDH mutations have been identified in many different tumors including gliomas, leukemias, and most recently, sarcomas (Zhao et al. 2009; Figueroa et al. 2010; Ward et al. 2010; Lu et al. 2013; Ward et al. 2013). Interestingly, IDH1/2 mutants can function in reductive carboxylation whereby α-ketoglutarate is converted to 2-hydroxyglutarate (2HG), a metabolite associated with glioblastomas but otherwise uncharacterized (Dang et al. 2010) until quite recently. Lu et al. identified the byproduct of mutant IDH1/2, 2HG, as functionally able to impair histone demethylation and thereby acts to block cell differentiation (Lu et al. 2012). Similarly, normal IDH1 can function in reverse however 2HG is not produced as a byproduct. Rather, reductive carboxylation of α-ketoglutarate to citrate can occur under hypoxia when IDH1 functions in the reverse. Under hypoxia, when decreased forward TCA flux occurs, reductive glutamine metabolism allows for lipogenesis and is at least partly dependent on HIF activity (Metallo et al. 2011; Wise et al. 2011).

Differential interactions of HIF-1α and HIF-2 with oncogenes and tumor supressors. While the HIF-1α subunit is expressed ubiquitously, HIF-2α is selectively expressed in a much more tissue-restricted manner but can be found at high levels in vascular endothelial cells and myeloid-derived cells (Patel et al. 2008). Both α subunits however, can exhibit potent effects on various oncogenes and tumor suppressors implicated in tumor progression. The different effects of the HIFα subunits have been most closely studied in the setting of VHL-deficient clear cell renal cell carcinomas (ccRCC) (Gordan et al. 2007; Gordan et al. 2008). Patient samples clustered into two main groups, those that express both HIF-1α and HIF-2α, and those that express HIF-

2α alone. Previous work has demonstrated the difference may reside in the differential interactions with MYC. Myc is a proto-oncogene that can direct metabolic and biosynthetic changes that enhance cell proliferation (Gordan et al. 2007). Furthermore, HIF-1α may be deactivated by the stress-sensor Sirtuin 1 (Sirt1) in tumor cells whereas HIF-2α is activated by Sirt1 and appears to play a larger role in c-Myc driven tumor cell proliferation. Whereas HIF-2α appears to promote MYC activity, HIF-1α inhibits MYC function (Gordan et al. 2007; Gordan et al. 2008). The evidence of opposing roles for the HIFα subunits is further supported by the fact that tumor samples expressing both α subunits proliferate slower than those expressing HIF-2α alone.

Likewise, HIF-1α and HIF-2α exhibit opposing roles in interactions with p53 (Keith et al. 2011). Trp53 (p53) is a tumor suppressor that is often lost or mutated in human cancers and is responsible for coordinating cellular responses to DNA damage and repair, cell cycle arrest and more. The tumor suppressor p53 can be stabilized through interaction with HIF-1α, which may or may not be through a direct interaction with MDM2. Similarly, ionizing radiation can increase HIF-1α accumulation and leads to increased phosphorylation and activity of p53 (Bertout et al. 2009; Keith et al. 2011). Unlike HIF-1α, HIF-2α does not interact with MDM2 and has been implicated in inhibiting p53 activity, perhaps by inhibiting reactive oxygen species (ROS) accumulation. Loss of HIF-2α results in increased p53 activity suggesting HIF-1α and HIF-2α exhibit opposing roles in p53 regulation (Bertout et al. 2009). These findings have far reaching consequences, as MYC and p53 are some of the most important oncogenes and tumor suppressors in human cancers.

Angiogenic activity secondary to HIF signaling. There is a finite distance across which O_2 can diffuse to metabolically active cells. To compensate, regions of hypoxia may stimulate the development of new blood vessels, also known as

angiogenesis (Pugh et al. 2003). HIFs function as key transcriptional regulators of developmental and tumor angiogenesis (Pugh et al. 2003). Multiple pro-angiogenic factors including vascular endothelial growth factor (VEGF), nitric oxide synthases (iNOS, eNOS), platelet-derived growth factor (PDGF), and various angiopoietins, have been previously demonstrated as direct or indirect HIF targets (reviewed in Pugh et al. 2003). Pro-angiogenic signaling can come from many sources, including tumor cells, endothelial cells, infiltrating leukocytes, and more. Importantly, HIFs have been implicated in stimulating angiogenesis or secreting angiogenic factors in all of these cell types.

In particular, HIF-1α and HIF-2α are co-expressed in endothelial cells (ECs) with largely non-overlapping roles (Skuli et al. 2009; Skuli et al. 2012). Interestingly, in models of tumor angiogenesis, endothelial expression of both HIF-1α and HIF-2α correlate with vessel density. However, whereas HIF-1α has been implicated in the proliferation, survival and metabolism of hypoxic ECs, HIF-2α expression has been associated with EC migration and vessel integrity (Skuli et al. 2009). Once again, HIF-1α and HIF-2α exhibit overlapping but not entirely redundant roles in tumor progression – in particular in the setting of tumor angiogenesis through intrinsic effects on ECs.

Direct regulation of tumor cell metastasis and extracellular remodeling by HIF activity. Invasion of surrounding tissue and colonization/seeding of distant sites characterize metastatic disease. In head and neck cancers, HIF-1α was shown to directly regulate TWIST expression by binding to an HRE site in the proximal promoter. Upregulation of TWIST secondary to HIF-1α stabilization resulted in increased epithelial-mesenchymal transition (EMT), an early feature of invasive and metastatic cells, and metastasis (Yang et al. 2008). HIF activity within the hypoxic tumor microenvironment can therefore drive cell intrinsic changes that result in EMT and metastatic disease.

Recently, HIFs have been implicated in direct modulation of the extracellular matrix to allow for invasion and metastatic dispersal. In particular, enzymes responsible for collagen modification and remodeling the extracellular matrix, procollagen-lysine 2-oxoglutarate 5-dioxygenase 2 (PLOD2) and lysyl oxidase (LOX), have come to light as direct HIF targets as well as major players in metastatic disease in both sarcoma and breast carcinoma (Wong et al. 2011; Eisinger-Mathason et al. 2013; Gilkes et al. 2013; Gilkes et al. 2013; Gilkes et al. 2013). In a particularly elegant model, Eisinger-Mathason et al. demonstrated PLOD2 activity is hypoxia and HIF-dependent and is responsible for modifying the collagen network that is surrounds and encompasses tumor cells in an autochthonous model of sarcoma. A direct consequence of these collagen modifications is maturation of collagen and changes in tissue/tumor stiffness. These changes ultimately support or inhibit (based on the specific modifications) cell invasion and metastasis. In particular, it appears these collagen modifications may allow vascular intravasation. Similarly, Wong et al. have demonstrated LOX as a HIF-1α target that can also remodel the extracellular matrix and support breast cancer metastasis.

Cellular access to oxygen is a critical component of many physiologic and pathologic processes. HIF stabilization may occur secondary to hypoxia in development or within the tumor microenvironment. Although the HIF response continues to be characterized within the tumor parenchyma, it is clear that HIF-1α and HIF-2α exhibit both overlapping and non-redundant roles and contribute to distinct aspects of cancer stem cell behavior, cell survival and proliferation, tumor metabolism, angiogenesis, invasion and metastasis. The hypoxic tumor microenvironment is an exciting area of new development as HIF-mediated effects on cell extrinsic features come to light. In particular, modification of the extracellular matrix secondary to hypoxia and HIF-stabilization, is now accepted as a driving force in metastasis and is uncovering new

therapeutic targets. HIF activity within ECs and other recruited cell types are also proving to impact tumor progression.

Although the tumor parenchyma is of obvious importance in tumor progression, the influence and importance of recruited and resident stromal cells is being elucidated. As the complexity of the tumor microenvironment continues to be appreciated, hypoxic and HIF-mediated responses in the tumor stroma are proving to be important. In the following section I will discuss in further detail the role of hypoxia and HIFs in inflammatory conditions, the innate immune system at large, and recruited macrophages within the tumor microenvironment as well as physiologic roles of HIF activity in inflammation and wound healing. Hypoxia is a key feature of development and tumor biology however, low O_2 tensions are also often observed in regions of intense inflammation such as sites of infection, within arthritic joints, and atherosclerotic plaques (Murdoch et al. 2005). Similarly, the hypoxic response is a physiologic adaptation of macrophages that may ultimately be co-opted by tumor-infiltrating cells to drive tumor progression.

INFLAMMATION

Inflammation is a biologic response to harmful stimuli, either infection or tissue injury, in an effort to restore tissue homeostasis (Barton 2008; Medzhitov 2008). In the case of infection, the innate immune system mediates the initial immune response by stimulating the release of chemokines, cytokines, and multiple vasoactive compounds in addition to a number of other secreted factors (Medzhitov 2008). This signaling cascade quickly results in an inflammatory exudate. Extravascular tissue at the site of injury or infection becomes edematous as a result of selective extravasation of leukocytes occuring across activated endothelium along with plasma proteins (Kumar et al. 2005).

Once the infection or tissue damage has been successfully contained, recruited and resident macrophages mediate resolution, wound repair, and return to homeostasis.

Inflammation is often marked by hypoxia (Eltzschig et al. 2011). Hypoxia may result from decreased O_2 availability secondary to decreased perfusion as a result of thrombosis, trauma, and edema. Similarly, increased metabolic activity of both foreign pathogens and recruited leukocytes can also contribute to hypoxic conditions observed within the inflammatory microenvironment (Karhausen et al. 2005; Nizet et al. 2009). As such, the hypoxic response under inflammatory conditions is a necessary step to restore tissue homeostasis. HIF activity regulates the inflammatory response in multiple cell types in the setting of infection or tissue damage. The following section will focus primarily on the myeloid and macrophage components of the innate immune system, their role in inflammation and, eventually, their tumor-promoting properties.

MYELOID ROLES IN INFLAMMATION

The innate immune system is the first line of defense against infection and tissue damage and can be found in all multicellular eukaryotes (Barton 2008). Identification of pathogens occurs via invariant pattern recognition receptors on the cell surface of the innate immune system. Following activation of innate immunity an inflammatory cascade is initialized that can help resolve the infection and/or any associated tissue damage. Furthermore, the innate immune system also functions to prime and activate the adaptive immune system, however the hallmarks of inflammation are almost invariably initiated by the innate immune system.

Components of the innate immune system. As described in *Robbins Pathologic Basis of Disease*, the innate immune system is comprised largely of phagocytic cells (including neutrophils and macrophages), dendritic cells, and natural killer cells (Kumar et al. 2005). Although neutrophil recruitment occurs quite rapidly after

the onset of edema and inflammation, it is tissue-resident macrophages that mediate initial recognition of infection or tissue damage (Medzhitov 2008). Monocyte-derived macrophages also play a large role in containing infection and can quickly comprise the majority of infiltrating leukocytes (Davies et al. 2013). Macrophages may phagocytose pathogens and then present pathogen-specific antigens on the cell surface to activate adaptive immunity. Macrophages thus comprise initial host defense and act as antigen-presenting cells (APCs).

Differentiation and maturation. Macrophages are derived from a common myeloid progenitor (CMP). CMPs give rise to all myeloid lineages including either the megakaryocyte/erythrocyte branch or granulocyte/macrophage progenitors (Akashi et al. 2000). Tissue-resident macrophages are highly diverse cell types depending on the specific microenvironment: skin (Langerhans cells), liver (Kuppfer cells), and brain (microglia) all contain exceptionally specialized macrophages (Davies et al. 2013). Interestingly, resident macrophages exhibit self-renewal capacity to maintain populations within the microenvironment. Alternatively, other resident macrophages, such as those within the gastrointestinal tract, are continually repopulated by peripheral circulating monocytes.

Macrophage polarization. Environmental cues influence macrophage polarization (Sica et al. 2012). In response to classical pro-inflammatory signals, such as LPS and IFNγ, macrophages undergo M1 activation akin to Th1 skewing of T cells (Figure 2). Other signals, such as IL-4 and IL-13, result in alternative or M2 macrophage polarization, similar to Th2 T cells. M1 polarized macrophages are responsible for initiating an inflammatory response: they secrete high levels of proinflammatory cytokines, reactive oxygen species (ROS), and nitric oxide (NO) and stimulate a Th1 response from the adaptive immune system. In contrast, M2 polarized macrophages influence tissue remodeling and wound repair, are immunosuppressive, and can

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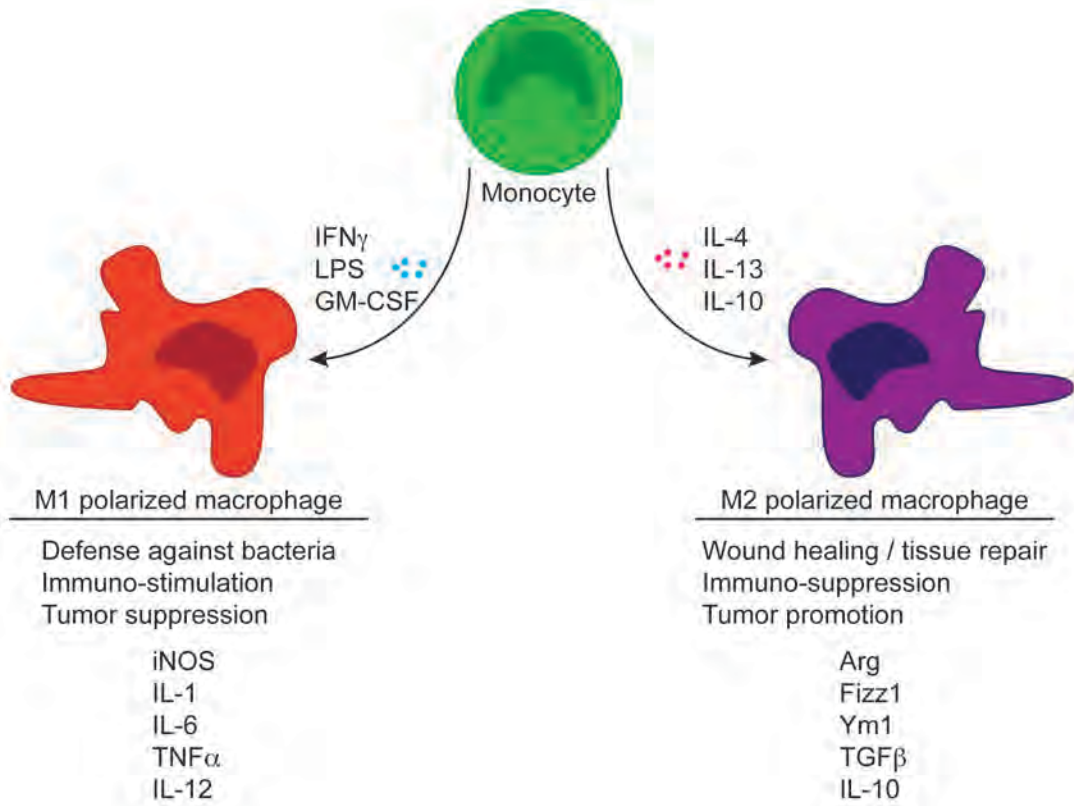
Figure 2. Macrophage polarization.

promote tumor progression. Interestingly, over the course of infection or tissue damage, macrophages may switch from an M1 phenotype to an M2 phenotype as tissue requirements change from controlling infection to tissue repair (Kumar et al. 2005; Sica et al. 2012). Because of the complexity of signaling cues within the microenvironment, it is likely macrophages exhibit intermediate phenotypes that contain attributes of M1 and M2 polarization.

Role in chronic inflammation. Macrophages play a large role in chronic inflammation. When inflammation is not contained, macrophages persist – either through local proliferation or continual recruitment from the circulation (Barton 2008). Tissue injury characterizes chronic inflammation and is due, in part, to continuous macrophage activation. Activated macrophages control and contain infection by releasing ROS, NO, stimulating fibroblast proliferation and angiogenesis. However, these responses can also damage surrounding, healthy tissue. In addition, release of certain cytokines will recruit other classes of leukocytes for a continued inflammatory response (Kumar et al. 2005). Continued, inappropriate macrophage activation and tissue destruction are hallmarks of chronic inflammatory conditions and the resulting regeneration can, over time, result in increased risk of cancer development (Vakkila et al. 2004). Similarly, infiltrating macrophages are a key feature of most solid tumors (Balkwill et al. 2001; Pollard 2009). Inflammation-associated cancers are likely a consequence of chronic inflammation and will be examined more closely in the following section.

INFLAMMATION ASSOCIATED CANCER

In 1863 Rudolf Virchow first described the presence of infiltrating leukocytes within a solid tumor. He hypothesized that the presence of immune cells amongst cancer cells, as well as the fact that tumors often arise at sites of infection or tissue damage, indicated that cancer itself was a form of chronic inflammation (Virchow 1863). Ever

Table 1 | Chronic inflammatory condition and corresponding cancer

Inflammation	Associated cancer	Stimulus
Asbestosis	Mesothelioma	Asbestos
Bronchitis	Lung cancer	Tobacco smoke
Gastritis	Gastric cancer	*H. pylori* infection
Barrett's metaplasia	Esophageal cancer	Gastroesophageal reflux disorder
Hepatitis	Hepatocellular carcinoma	Hepatitis B virus, Hepatitis C virus
Cervicitis	Cervical cancer	Human papillomavirus
Pancreatitis	Pancreatic cancer	Alcohol, Tobacco
Inflammatory bowel disease	Colorectal cancer	Commensal bacteria, permeability
Cholecystitis	Gall bladder cancer	Bacterial infection, cholelithiasis
Cystitis	Bladder cancer	Bacterial infection
Mononucleosis	Lymphoma	Epstein-Barr virus
Sunburn	Melanoma, BCC, SCC	UV light exposure

since this initial observation and hypothesis, researchers have attempted to explain the relationship between chronic inflammation and tumorigenesis (Virchow 1863; Balkwill et al. 2001; Vakkila et al. 2004). A complex association between chronic inflammatory states and cancer clearly exists. Although chronic inflammation may be due to a number of inciting agents (infectious, chemical, etc.), all appear to play a role in tumorigenesis. Today there is an obvious relationship between chronic inflammatory states and cancer (Table 1). Chronic exposure to asbestos fibers results in an inflammatory condition known as asbestosis and can lead to mesothelioma. Similarly, infectious agents such as Human Papillomavirus, Hepatitis B and C, and *Helicobacter pylori* have been linked to cervical cancer, hepatocellular carcinoma, and gastric cancer respectively (Balkwill et al. 2001; Vakkila et al. 2004).

Inflammatory bowel disease. In particular, inflammatory bowel disease (IBD) has been linked to an increased risk of colorectal cancer (CRC). Ulcerative Colitis (UC) and Crohn's Disease (CD) are states of chronic colitis and are subsets of IBD; while lesions associated with CD can be found throughout the gastrointestinal tract, UC starts in the rectum and progresses in a proximal fashion (Rhodes et al. 2002). Similarly, whereas histopathologic analysis of UC demonstrates large numbers of infiltrating neutrophils within the lamina propria and crypts, CD lesions exhibit macrophage aggregates in the form of non-caseating granulomas (Xavier et al. 2007).

According to the Center for Disease Control (CDC), peak age of IBD onset and diagnosis is between 18 and 30 years of age. Whereas UC exhibits a slight male predominance, CD is somewhat more frequent in females. It is currently thought that 1.4 million people in the United States and 2.2 million people in Europe currently suffer from IBD. Although smoking and other lifestyle factors are known risk factors, genetic factors are most important as history of first-degree relatives with IBD remains the largest single risk factor (Loftus 2004; Hanauer 2006).

The innate immune system is thought to play a role in IBD pathogenesis (Figure 3). Recently, the importance of commensal gut bacteria in IBD has come to light. As the microbiome continues to be investigated, further insights into the relationship between commensal bacteria and physiologic and pathologic interactions with host immune defenses will be discovered (Xavier et al. 2007). Mutations that result in dysfunctional macrophage pathogen recognition have recently been revealed as a genetic inducer of CD (Karin et al. 2006). As a result of this defect, intestinal resident macrophages secrete elevated levels of the pro-inflammatory cytokine IL-1β leading to increased local and systemic inflammation. Interestingly, IL-1 secretion is also elevated at tumor sites and can promote tumor growth and invasion (Lin et al. 2007). Similarly, defects in epithelial barrier function can result in overactive macrophage responses in inflamed colonic tissue (Mahida 2000). Inflammatory cytokine release by myeloid-derived cells mediates wound-healing responses in intestinal epithelial cells (Elinav et al. 2013). Importantly, patients with Crohn's or UC have an elevated incidence of developing CRC when compared to the general population (Danese et al. 2010). In particular, after 30 years of UC, a patient's cumulative probability of developing CRC is 18% (Eaden et al. 2001). Current understanding suggests the microbiome, host defects in innate immune recognition of commensal and pathogenic gut bacteria, and flaws in epithelial barrier function together result in chronic inflammation that leads to tissue damage and continued repair and regeneration that can, over time, ultimately lead to acquired cellular mutations and tumorigenesis.

Sustained immune activation in the setting of chronic inflammatory states can promote malignancy through angiogenic signaling, inhibition of apoptosis, proliferative cues, and dysfunctional immune surveillance (O'Byrne et al. 2001). Similar to Virchow's initial findings 150 years ago, colitis-associated cancer (CAC) is correlated with the infiltration of macrophages and other leukocytes into the tumor stroma. Because of their

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NORMAL   INFLAMMATION   CANCER

Increasing Oxygen Tension

Epithelial cell
Goblet cell
T cell
Macrophage
Dendritic cell
Bacteria
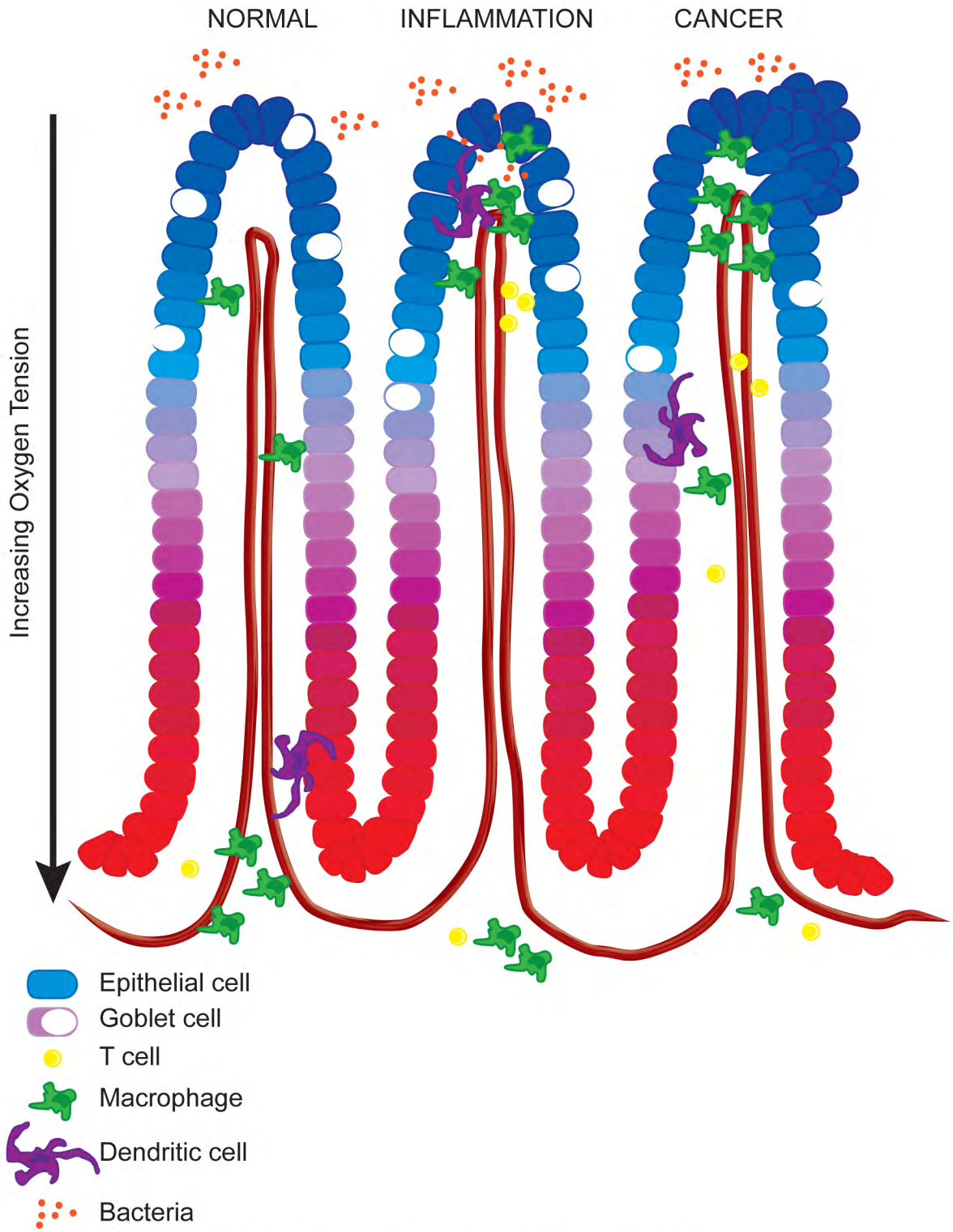
Figure 3. Colon physiology, inflammation, and cancer development.

highly plastic and heterogeneous nature, macrophages provide an intriguing link between chronic inflammation and cancer (Tanner et al. 1984). The following section will focus primarily on the multifaceted roles of infiltrating macrophages, both in the setting of inflammation-associated cancers and cancer-associated inflammation.

MYELOID ROLES IN CANCER

As previously mentioned, tumor-associated macrophages (TAMs) have been implicated in connecting the innate immune system, chronic inflammation and tumorigenesis (Solinas et al. 2009; O'Connor et al. 2010; Saleh et al. 2011), as the appearance of macrophages in tumors is positively correlated with poor patient prognosis, increased lymph node involvement, and distant metastases (Leek et al. 1996; Bingle et al. 2002; Fang et al. 2009; Kang et al. 2010). TAMs are a heterogeneous myeloid population that infiltrates predominantly hypoxic regions within solid tumors, where they secrete growth factors and cytokines that stimulate angiogenesis and facilitate invasion and/or metastasis (Leek et al. 2002; Pollard 2004; Condeelis et al. 2006; Jedinak et al. 2010; Qian et al. 2010).

Prognostic implications. Clinical studies show a strong association between TAM number in the primary tumor, lymph node involvement, and metastasis (Fang et al. 2009; Kang et al. 2010). The presence of TAMs in the tumor microenvironment correlates with poor prognosis (Bingle et al. 2002). Tumor cells and stromal cells within the tumor microenvironment are thought to secrete inflammatory cytokines that act to recruit circulating monocytes and polarize TAMs. High expression of specific tumor-secreted cytokines is also associated with poor prognosis in colon cancer (Qian et al. 2010). Interestingly, culturing human colon cancer cells in activated macrophage-conditioned media (AMCM), which contains increased levels of IL-6 and other cytokines, results in increased proliferation and migration of tumor cells (Jedinak et al. 2010). This

finding is clinically relevant as increased mucosal and serum IL-6 levels are seen in UC and colon cancer patients, and correspond with more advanced disease (Knupfer et al. 2010; O'Connor et al. 2010). Similarly, IL-1β has been implicated in promoting tumor cell migration (Naldini et al. 2010).

In particular, CSF-1, considered the main macrophage growth factor, promotes recruitment, survival and proliferation of macrophages. Inhibition of CSF-1 in a mouse model of breast cancer resulted in decreased macrophage infiltration into the tumor microenvironment as well as decreased tumor burden and slowed progression and invasion. Conversely, increased expression of CSF-1 in tumors correlates with augmented macrophage infiltration and accelerated tumor progression (Lin et al. 2001). Importantly, CSF-1 knockout in a murine colon cancer model prevented macrophage recruitment and significantly inhibited tumor formation (Oguma et al. 2008). Additionally, immunocompromised mice with xenografted human colon cancer treated with an antisense oligonucleotide against *Csf-1* exhibited decreased macrophage recruitment and 50% decrease in tumor growth (Pollard 2004).

Macrophage depletion in mice shows a similar correlation (Lin et al. 2001; Pollard 2004; Oguma et al. 2008). Use of clodronate liposomes to deplete mice of circulating monocytes resulted in decreased TAM infiltration in murine models of teratocarcinoma and rhabdomyosarcoma and resulted in significantly decreased tumor growth and angiogenesis (Zeisberger et al. 2006). In short, the presence of TAMs within the tumor microenvironment is strongly associated with tumor growth in human cancer samples as well as multiple murine cancer models. TAMs are thus an intriguing area of exploration as a possible link between chronic inflammation and cancer induction. Ongoing research suggests TAMs may assist tumor progression by promoting angiogenesis and invasion and metastasis.

Effects on tumor angiogenesis. TAMs are thought to promote tumor progression, in part, through their positive effects on angiogenesis. In particular, TAMs are recruited to and accumulate in avascular hypoxic regions within tumors. Hypoxia in turn triggers increased expression of genes that promote angiogenesis. *In vitro* analysis of macrophages under hypoxia has shown that pro-angiogenic factors such as VEGF, bFGF, and COX2 are upregulated (Murdoch et al. 2008). Similarly, TAMs are known to release pro-angiogenic factors and cytokines such as VEGF, bFGF, TNF-α, thymidine phosphorylase, and insulin-like growth factor I (Leek et al. 1996; Lin et al. 2006; Coffelt et al. 2009). TAM secretion of VEGF is likely secondary to hypoxic adaptation, may trigger the angiogenic switch, and is likely involved in the transition to malignancy (Lin et al. 2006). Other factors such as IL-1β, IL-8, and MMP-9 have also been implicated in TAM-supported angiogenesis (Dirkx et al. 2006). These pro-angiogenic macrophages may be the reason current anti-angiogenic therapy fails as investigators have noted a correlation between specific myeloid cells and tumor refractoriness to anti-VEGF treatment (Shojaei et al. 2007; Coffelt et al. 2010).

Recent evidence indicates the presence of a subset of TAMs that express Tie2, an angiopoietin 2 (Ang2) receptor initially thought to only be expressed on the surface of endothelial cells (ECs) and hematopoietic stem cells (HSCs) (De Palma et al. 2003). These Tie2-expressing monocytes (TEMs) are recruited to tumor sites and have been shown to be necessary for angiogenesis as TEM-knockout prevented neovascularization in a murine glioma model (De Palma et al. 2005). Furthermore, gene expression comparisons indicates TEMs are highly related to TAMs but retain a specific gene signature of enhanced pro-angiogenic activity (Pucci et al. 2009). Upregulation of Tie2 increases TEMs response to Ang2, promoting angiogenesis. Indeed, Ang2 blockade limits angiogenesis and prevents progression and metastasis in multiple murine cancer models (Mazzieri et al. 2011).

Interestingly, Tie2 expression is upregulated in TEMs under hypoxic conditions such as highly angiogenic regions of the tumor microenvironment (Lewis et al. 2007). Thymidine phosphorylase, a pro-angiogenic factor known to be upregulated in TAMs in hypoxic regions of tumor samples, is also upregulated by Ang2 in TEMs (Leek et al. 2002; Coffelt et al. 2010). This further suggests a possible role for Tie2/Ang2 expression in pro-angiogenic tumor-associated macrophages under hypoxic conditions. In summary, TAMs express and secrete a variety of pro-angiogenic factors that are in part hypoxia-induced and have been shown to be important in tumor angiogenesis and progression. Furthermore, TAMs may be a promising target for future anti-angiogenic therapies as their presence strongly associates with failure of anti-VEGF treatment.

Effects on invasion and metastasis. TAMs are also thought to promote tumor progression, in part, through their positive effects on invasion and metastasis. Studies have shown a strong association between TAM number in the primary tumor and lymph node and distant metastases (Coffelt et al. 2009; Kang et al. 2010). Furthermore, overexpression of CSF-1 in tumors resulted in increased macrophage infiltration, accelerated tumor progression and increased metastasis while inhibition of CSF-1 resulted in the exact opposite (Lin et al. 2001; Pollard 2004; Oguma et al. 2008).

Local growth, invasion, and metastasis require proteolytic degradation of the extracellular matrix (ECM). TAMs secrete pro-metastatic factors such as TNF-α, MMP-2, MMP-9, IL-1β, and IL-6 amongst others. These molecules are thought to increase proliferation and aid in the disintegration of surrounding tumor stroma and connective tissue (Allavena et al. 2008). In fact, TAMs can be found at the invasive leading edge of advanced tumors and amongst regions of basement membrane breakdown in early stages of malignancy (Condeelis et al. 2006). Furthermore, TAMs are thought to promote tumor cell intravasation as part of the progression from primary tumor to metastatic spread (Wyckoff et al. 2004; Condeelis et al. 2006). Additionally, culturing

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human colon cancer cells in macrophage-conditioned media results in increased proliferation and migration of tumor cells (Jedinak et al. 2010). TAMs may also promote dispersal and seeding by conditioning the pre-metastatic niche (Mantovani et al. 2010). These results support the idea that TAMs may play a direct role in promoting tumor proliferation and metastasis.

  Hypoxic recruitment. TAMs are recruited to regions of solid tumors through various chemokines and other secreted factors. Sphingosine-1-phosphate and transforming growth factor-β (TGF-β) are derived from apoptotic cells and recruit macrophages. This process also upregulates HIF-1α, indicating a mechanism through which TAMs may infiltrate the tumor microenvironment in a HIF and hypoxia-dependent manner (Herr et al. 2009). VEGF has been shown to increase TAM recruitment and is a well-documented HIF-dependent hypoxia-induced target. Hypoxia appears to both regulate the expression of and play a role in modifying chemoattractant receptors on the surface of tumor-associated macrophages (Murdoch et al. 2004). For example, CXCL12 expression in tumor-associated fibroblasts appears to be induced under hypoxia and act to recruit tumor-associated macrophages. It is therefore possible that various elements within the tumor microenvironment may act to recruit macrophages in a hypoxia-dependent manner. Additionally, hypoxic down-regulation of a number of receptors and chemokine production in TAMs and tumor cells respectively, may, in fact, act to entrap infiltrating macrophages at sites of hypoxia and necrosis (Murdoch et al. 2005). These findings support the notion that release of hypoxia-promoted inflammatory signals within the tumor microenvironment recruit TAMs. TAMs are recruited by hypoxia-dependent factors and once localized in hypoxic domains, undergo adaptive gene expression changes that in turn promote tumorigenesis and tumor progression.

The association of TAMs with hypoxic tumor domains suggests that hypoxic responses in these cells are critical to their function. Several studies have implicated HIFs in controlling TAM gene transcription in hypoxic tumors (Wiesener et al. 1998; Talks et al. 2000; White et al. 2004; Imtiyaz et al. 2010), with different adaptive functions regulated by the closely related subunits HIF-1α and HIF-2α (Majmundar et al. 2010). Multiple strategies for macrophage recruitment to and retention in hypoxic regions of the tumor microenvironment have been proposed and previously described (Murdoch et al. 2004). Importantly, macrophages accumulate in hypoxic, avascular regions within tumors and upregulate both HIF-1α and HIF-2α transcription factors.

HIF and NF-κB interaction. Although various proteins within the nuclear factor κB (NF-κB) pathway (a key transcriptional regulator of inflammatory responses) are phosphorylated under hypoxia, the NF-κB pathway on its own is not implicated in the transcriptional regulation of macrophages experiencing/adapting to hypoxia (Fang et al. 2009). However, there appears to be a complex feedback loop between HIF-1α stabilization and expression, and the NF-κB pathway (Figure 4). Importantly, NF-κB is closely intertwined with hypoxic and HIF-mediated responses (Eltzschig et al. 2011). Under hypoxia, the NF-κB activator IKK-β is stimulated and the NF-κB component RelA translocates to the nucleus. HIF-1α is able to activate NF-κB and, conversely, NF-κB can influence HIF-1α transcription. In fact, basal NF-κB activity in macrophages is suggested to be required for HIF-1α stabilization and protein accumulation under low O_2 conditions such as that seen in settings of inflammation (Rius et al. 2008).

Inflammatory hypoxic crosstalk through HIFs. Just as redox balance affects HIF activation, so too do certain inflammatory signals. HIF-1α and HIF-2α are both upregulated in macrophages cultured under hypoxia. As previously described, the different HIFα subunits can exhibit overlapping and distinct roles that are important in
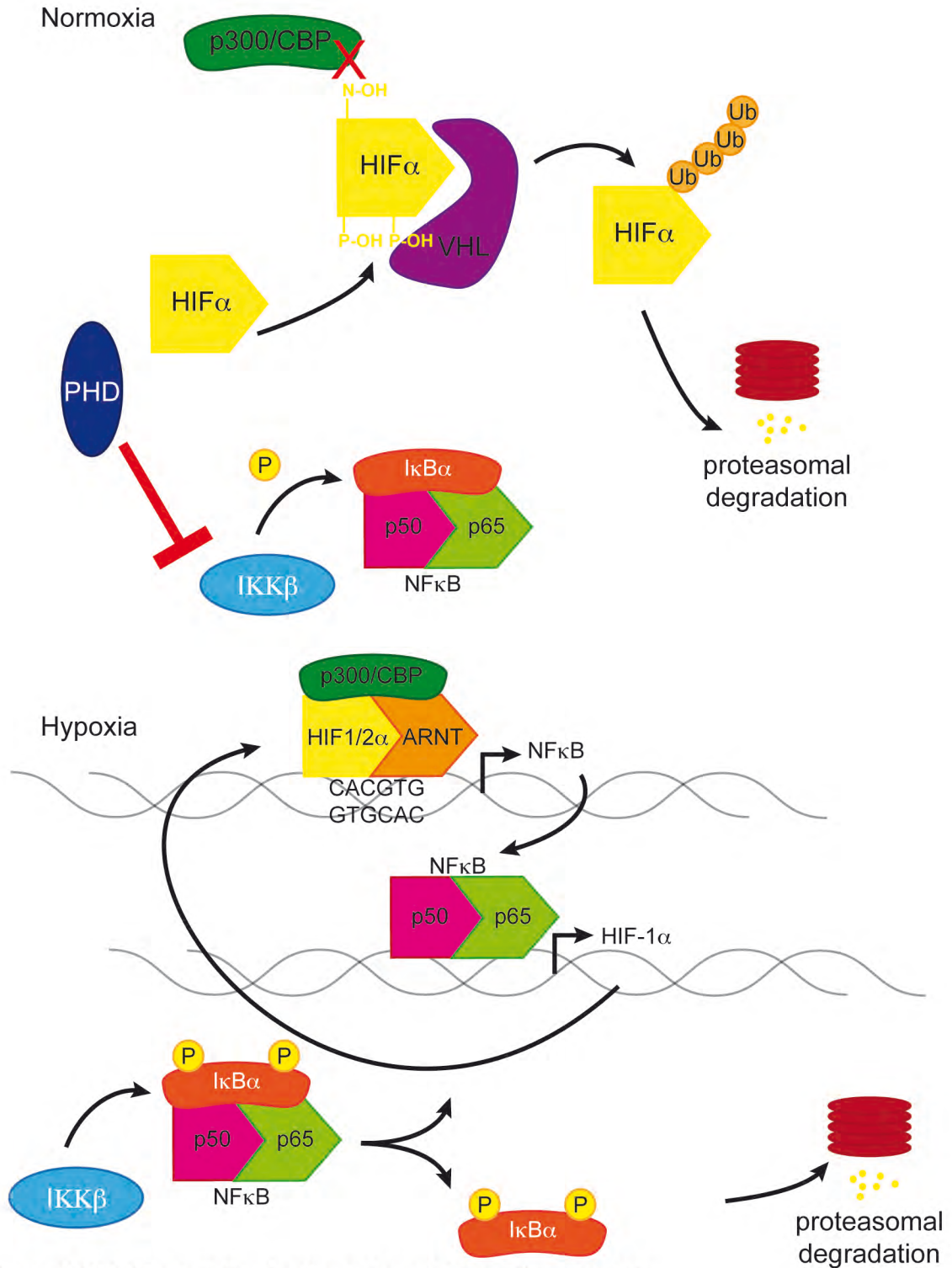
Figure 4. NF-κB regulation by hypoxia and interaction with HIF.

tumor cells and recruited stromal cells. The same is true of macrophages. In the following section, the distinct and occasionally redundant roles of the HIFα subunits will be discussed in greater detail (Figure 5).

HIF-1α. Perhaps more important than the recruitment of TAMs to low O_2 environments however, is the fact that macrophages undergo specific adaptive changes in gene expression as a response to hypoxia. Under prolonged hypoxic conditions (0.2% O_2), macrophages exhibit HIF-1α RNA upregulation and an extreme increase in *Vegf* expression (Staples et al. 2011). Hypoxic adaptation requires changes in metabolism and results in a very rapid switch from aerobic to anaerobic glycolysis in macrophages (Kawaguchi et al. 2001). This is partly due to HIF-1α dependent expression of glucose transporter 1 (GLUT-1) and phosphoglycerate kinase (PGK) as well as a switch to the more active isoenzyme of phosphofructokinase that results in an increase in fructose-2,6-bisphosphate concentration and overall glycolytic flux under hypoxic conditions (Rodriguez-Prados et al. 2010). Indeed, macrophages that lack HIF-1α expression have decreased cellular ATP. Loss of HIF-1α changes the metabolic status of myeloid-derived cells and results in decreased recruitment, as well as decreased migration and invasion (Cramer et al. 2003).

Recently the importance of HIF-1α signaling in controlling metabolic flux of macrophages has come to light. Lipopolysaccharide (LPS), a characteristic feature of gram-negative bacteria, strongly polarizes macrophages towards an M1 phenotype. A consequence of this reaction is an increase in glycolysis, an accumulation of the TCA intermediate succinate, and stabilization of HIF-1α leading to increased secretion of IL-1β (Tannahill et al. 2013). HIF-1α expression is therefore an important mediator of the metabolic changes the innate immune system undergoes in response to infection.

Inflammation-associated hypoxia:

Infiltrating macrophage

HIF-1α	HIF-2α
ATP generation	Cytokine production
Cytokine production	Acute inflammation
Acute inflammation	Tumor inflammation
Migration/invasion	Migration/invasion
Bactericidal activity	
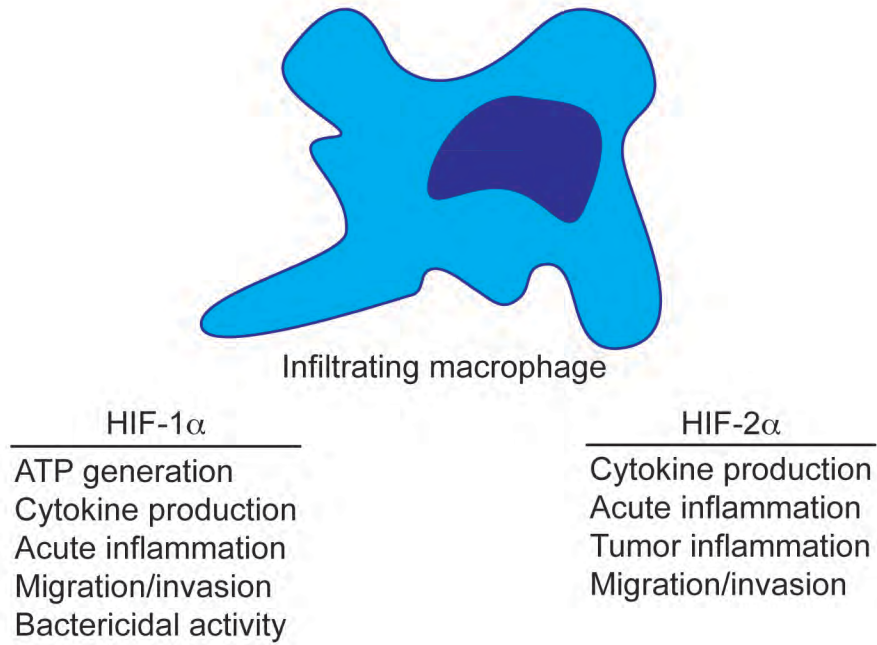
Figure 5. Role of HIF-1α and HIF-2α in macrophages.

HIF-1α in particular has been previously implicated in instigating release of known inflammatory cytokines in stimulated macrophages (Barnes et al. 1997; Peyssonnaux et al. 2007). Interestingly, HIF-1α expression influences macrophage toll-like receptor (TLR) expression. In particular, TLR4 is upregulated in ischemic inflamed tissues and is expressed in macrophages under hypoxic stress secondary to HIF stabilization (Kim et al. 2010). Furthermore, macrophage HIF-1α expression has been implicated in suppressing T cell activation in the tumor microenvironment in part through inducible nitric oxide synthase (iNOS) expression (Doedens et al. 2010).

NO metabolism. Nitric oxide (NO) metabolism and homeostasis is of great functional importance for macrophages experiencing hypoxia (Takeda et al. 2010). Similar to previous findings, HIF-1α and HIF-2α have distinct and somewhat opposing roles in NO regulation. Nitric oxide production is controlled differently depending on macrophage polarization. While HIF-1α is upregulated in classically activated, pro-inflammatory macrophages, HIF-2α activation corresponds with an alternatively activated macrophage phenotype (Takeda et al. 2010). HIF-1α promotes *iNOS* expression while HIF-2α promotes Arginase 1 (*Arg1*) expression. Thus each HIF subunit is an antagonist for the other and may play a role in NO homeostasis. When HIF-2α is present, *Arg1* is expressed and suppresses NO production. This contrasts with HIF-1α which, when present, promotes *iNOS* expression and increases NO production. The HIF isoforms are primarily expressed in differently activated macrophages and have opposing roles in NO production. Thus NO production and HIF isoform expression is closely aligned with macrophage polarization (Takeda et al. 2010).

HIF-2α. Whereas HIF-1α expression appears to be upregulated in macrophages stimulated by classic inflammatory signals, HIF-2α may play a larger role in the immunosuppressive effects of TAMs (Takeda et al. 2010). Although both HIF-1α and

HIF-2α are expressed in macrophages, elevated expression of HIF-2α in TAMs corresponds directly with clinical severity of many different human cancers (Talks et al. 2000; Hu et al. 2003; Hu et al. 2007; Qing et al. 2009). TAM presence within the microenvironment is strongly associated with tumor growth in human cancer samples as well as multiple murine cancer models (Lin et al. 2001; Pollard 2004; Zeisberger et al. 2006; Oguma et al. 2008). Similarly, overexpression of HIF-2α in normoxic macrophages results in upregulation of a variety of pro-angiogenic factors (White et al. 2004).

In many instances, HIF-2α expression has been noted to be upregulated in tumor-infiltrating stromal cells as opposed to tumor cells themselves. In particular, early studies demonstrated stromal cells with appreciably higher levels of HIF-2α expression co-stained for macrophage specific markers. These tumor-associated macrophages are therefore specific regions within the tumor microenvironment with increased levels of HIF-2α expression. Interestingly, HIF-2α was not detected in normal human macrophages (Talks et al. 2000). Although both HIF-α proteins are expressed in macrophages, elevated expression of HIF-2α in TAMs corresponds with poor prognosis and high-grade tumors in a variety of human cancers (Talks et al. 2000). HIF-2α expression could therefore be specific to TAMs and may play a role in tumor progression. Hypoxia upregulates HIF-2α expression in macrophages and in turn promotes the release of *Vegf* (Pollard 2004). Importantly, HIF-2α expression is prolonged when compared to HIF-1α and can persist under re-oxygenation (Elbarghati et al. 2008).

Previous work from the Simon lab has demonstrated the importance of HIF-2α expression in macrophages in multiple murine models of systemic inflammation, hepatocellular carcinoma, and colitis-associated cancer (Imtiyaz et al. 2010). Deletion of HIF-2α in macrophages results in decreased IL-6, IL-1β, VEGF, and adrenomedullin

production, indicating the importance of HIFs in regulating macrophage adaptation to hypoxia (Imtiyaz et al. 2010). Importantly, *Pgk1* levels and ATP production were unchanged in the loss of HIF-2α. These results support the idea that TAM-specific HIF expression may play a direct role in promoting tumor proliferation and metastasis. Surprisingly, studies have indicated stabilization of HIF-2α in TAMs treated with GM-CSF can result in increased secretion of the soluble form of the VEGF receptor (sVEGFR-1) resulting in decreased angiogenesis and tumor growth in a murine melanoma model (Roda et al. 2012). The outcome of HIF signaling is thus dependent on the environmental cues influencing and polarizing tumor-associated and inflammatory macrophages.

Effect of hypoxia and HIFs on chronic inflammation. HIFs have recently come to light as important mediators in myeloid-driven inflammation and tumor progression (Cramer et al. 2003; Imtiyaz et al. 2010). Like most solid tumors, colorectal tumors exhibit regions of hypoxia. HIF-2α is present at increased levels in subsets of patients with colorectal cancer as measured by immunohistochemistry (IHC). These patients have significantly decreased survival compared with patients whose tumor samples have undetectable levels of HIF-2α (Jubb et al. 2009). Interestingly, HIF-2α expression appears to be upregulated in the surrounding stroma as opposed to the tumor cells in some studies (Talks et al. 2000). Macrophages within the tumor stroma exhibited appreciably higher levels of HIF-2α expression than the surrounding tissue. In particular, tissue samples from patients with UC exhibited macrophage infiltrates that co-stained positively for HIF-2α, thymidine phosphorylase (TP), and VEGF albeit at low levels. Interestingly, the intestinal tissue from affected regions did not stain for HIF-2α. Additionally, normal tissue samples were unreactive for any of the previously mentioned markers (Giatromanolaki et al. 2003). Surprisingly, HIF-2α expression was undetectable

in normal human macrophages, indicating that the presence of HIF-2α in tumor infiltrating macrophages may be a response to tumor signals (Talks et al. 2000).

Macrophages are recruited to sites of infection and tissue damage, which are often marked by regional hypoxia. HIF-1α and HIF-2α are both upregulated in macrophages in response to hypoxia and mediate a number of different functions. Similar to what was observed in ccRCC and in interactions with c-Myc, p53, and Sirt1, HIF-1α and HIF-2α exhibit surprisingly distinct roles in macrophage adaptation to hypoxia. What is clear based on genetic studies however, is that each HIFα subunit is important for cellular responses to hypoxia and interactions with surrounding environment. What remains to be studied however, is the complete hypoxic response of macrophages in the setting of acute, chronic, and tumor-associated inflammation. Understanding the result of complete loss of HIF activity will be vital to studying HIF pharmacologic inhibition.

PHARMACOLOGIC HIF MANIPULATION

Because HIF activity influences physiologic functions of various tissues and impacts multiple facets of tumor initiation and progression their manipulation is of continued therapeutic interest (Semenza 2003; Semenza 2007). Inhibitors may affect HIF expression, synthesis, stability, ability to dimerize with ARNT, or DNA binding (Figure 6) (Semenza 2012). Alternatively, inhibiting PHDs will result in stabilized HIF activity and increases in angiogenesis, erythropoiesis, hypoxic metabolic adaptation, and more. In particular, PHD inhibition has proven effective at minimizing tissue ischemia through HIF effects on angiogenesis and erythropoiesis production (Nangaku et al. 2007; Adamcio et al. 2010).
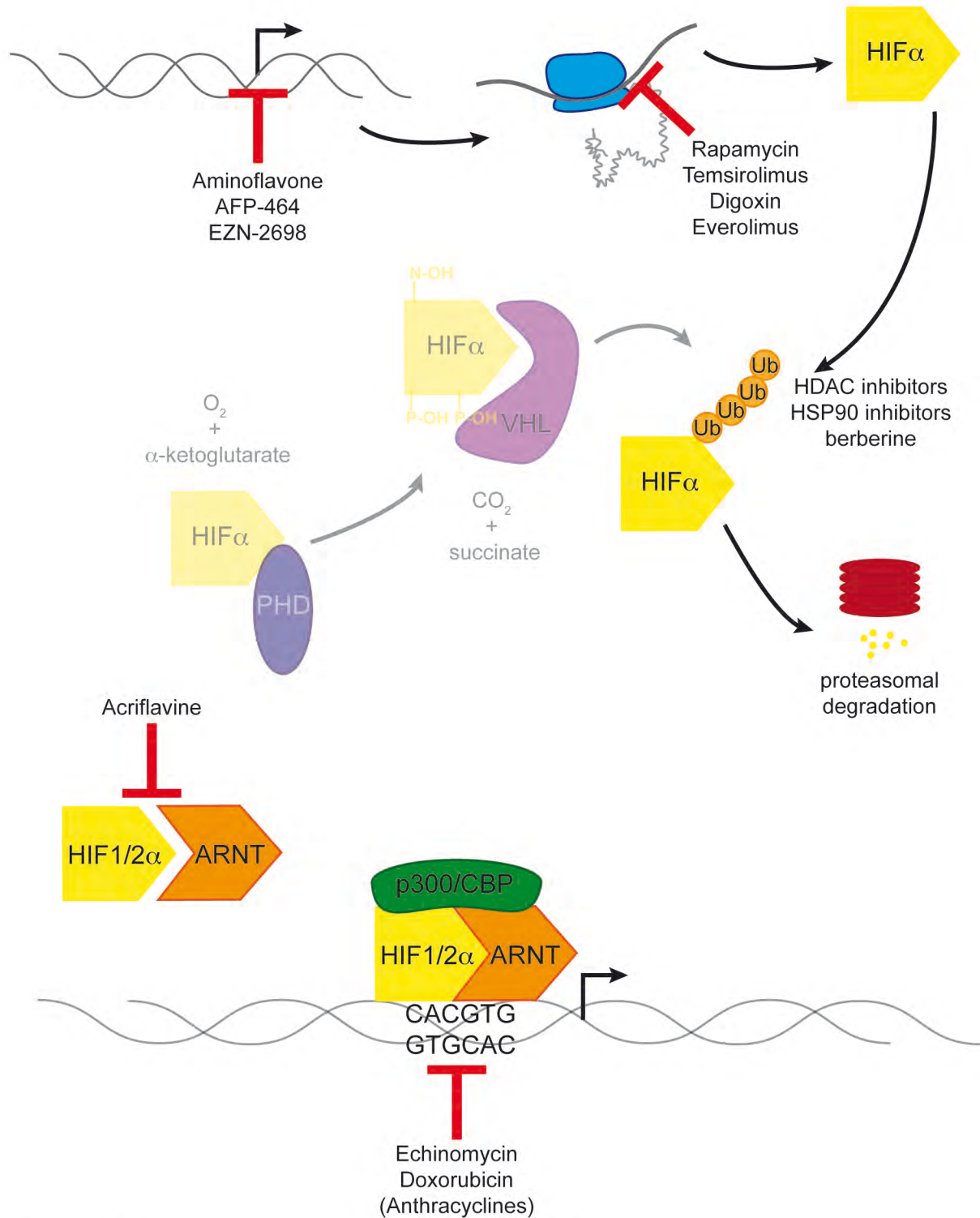
Figure 6. Mechanisms of pharmacologic inhibition of HIF activity.

To date, no specific HIF-inhibitors have been identified and the lack of specificity has made validating potential inhibitors difficult (Onnis et al. 2009). HIF inhibitors, although non-specific, generally result in decreased tumor growth and angiogenesis in multiple xenograft models. Aminoflavone, a constituent of AFP-464, partially inhibits HIF-1α transcription and completely inhibits translation (Semenza 2012). Similarly, the antisense oligonucleotide EZN-2698 inhibits HIF-1α transcription (Onnis et al. 2009). Multiple drugs, including rapamycin, temsirolimus, digoxin, and everolimus, inhibit HIF-1α translation (Onnis et al. 2009). The HIFα subunit can be targeted for degradation through multiple pathways such as HSP90 inhibitors, histone deacetylase inhibitors which stimulate ubiquitination of HIF-1α, and natural compounds such as berberine (Semenza 2012). Similarly, the HIF-α/β (HIF/ARNT) heterodimer can be destabilized through molecules that bind the PAS domain of the HIFα subunit. Acriflavine is a naturally occurring compound that inhibits HIF dimerization and can minimize tumor growth and angiogenesis in multiple tumor models (Lee et al. 2009; Semenza 2012; Wong et al. 2012). Lastly, anthracycline compounds like doxorubicin can bind to DNA and inhibit HIFα binding and transcriptional effects in cultured cells (Lee et al. 2009).

HIF-1α and HIF-2α exhibit overlapping and distinct roles. Additionally, depending on tissue type and the influencing tumor microenvironment, HIF activity may be beneficial for the host or detrimental. Targeting HIF activity for therapeutic treatment of cancers requires careful understanding of possible outcomes. Depending on individual host factors or specificities of cancer type, HIF inhibition may negatively impact disease progression. Use of HIF inhibitors, while encouraging, must proceed with caution. The area of pharmacologic HIF inhibition also brings to light the importance of continued investigation into the multifaceted roles of HIF activity.

CONCLUSION

The physiologic and pathologic adaptation to hypoxia can be appreciated in settings of acute, chronic, and tumor-associated inflammation. HIFs are O_2 sensitive transcription factors that allow transcriptional adaptation to hypoxic environments. It is becoming increasingly apparent however, that HIFs are regulated (both at the level of transcription and post-translationally) by other stress-sensors. HIF regulation incorporates oxygen availability, redox status, nutrient availability, and certain inflammatory signals. Recent work indicates differential effects of HIF-α subunit expression in endothelial cell nitrogen metabolism and resulting metastatic success. Similarly, HIF activity is being identified as driving factors in modifying the ECM that also influence resulting tumor cell invasion, intravasation and distant seeding. As previously described, inflammation often coincides with tissue hypoxia and the innate immune system has evolved to function in a highly specialized manner under low O_2 conditions (Nizet et al. 2009). Hypoxic responses of the innate immune system are a critical element in inflammation. The relationship and crosstalk between HIFs, hypoxia, the innate immune system, and cancer is epitomized in the setting of inflammation-associated cancers.

TAMs are recruited to hypoxic regions within the tumor microenvironment where they play a critical role in driving tumorigenesis. Within TAMs, HIF-1α again plays a large role in glycolytic changes that occur under hypoxia as well as promoting NO production. Interestingly, while HIF-1α may be induced by Th1 cytokines within the inflammatory component, HIF-2α is stimulated by Th2 cytokine-signaling and inhibits NO production – highlighting the occasionally opposing roles of each HIF subunit. Additionally, TAM HIF-2α expression has a minimal metabolic effect but an impressive effect on TAM infiltration within the tumor microenvironment. Prior work from this lab has demonstrated the

importance of TAM-specific HIF-2α expression in primary tumor burden. These findings indicate a broader role for HIF-regulated hypoxic metabolic adaptation. Lastly, pharmacologic inhibition of HIF activity is an ongoing area of research with many profound consequences as it is becoming increasingly apparent that HIFs are one common link between hypoxia, chronic inflammation, and tumor progression through roles in reprogramming tumor cells, macrophages and other cells within the microenvironment during cancer development.

Chapter Two

Inhibition of Hypoxia-Inducible Factors Limits Tumor Progression in a Mouse Model of Colorectal Cancer

SUMMARY

Hypoxia-inducible factors (HIFs) accumulate in both neoplastic and inflammatory cells within the tumor microenvironment, and impact the progression of a variety of diseases, including colorectal cancer. Pharmacological HIF inhibition represents a novel therapeutic strategy for cancer treatment. We show here that acriflavine (ACF), a naturally occurring compound known to repress HIF transcriptional activity, halts the progression of an autochthonous model of established colitis-associated colon cancer (CAC) in immunocompetent mice. ACF treatment resulted in decreased tumor number, size, and advancement (based on histopathological scoring) of CAC. Moreover, ACF treatment corresponded with decreased macrophage infiltration and vascularity in colorectal tumors. Importantly, ACF treatment inhibited the hypoxic induction of M-CSFR, as well as the expression of the angiogenic factor VEGF, a canonical HIF target, with little to no impact on the NF-κB pathway in bone marrow-derived macrophages (BMDMs). These effects likely explain the observed *in vivo* phenotypes. Finally, an allograft tumor model further confirmed that ACF treatment inhibits tumor growth through HIF-dependent mechanisms. These results suggest pharmacological HIF inhibition in multiple cell types, including epithelial and innate immune cells, significantly limits tumor growth and progression.

INTRODUCTION

Chronic inflammation increases an individual's risk of cancer, as exemplified by the well-established relationship between ulcerative colitis and the development of colorectal cancer (Virchow 1863; Balkwill et al. 2001; Eaden et al. 2001; Rhodes et al. 2002; Vakkila et al. 2004; Danese et al. 2010; O'Connor et al. 2010). Inflammatory lesions and solid tumors are similar in that both contain regions of varying oxygen (O_2) levels and are comprised of complex, highly heterogeneous cell populations (Bertout et al. 2008; Ruan et al. 2009). Hypoxic domains within tumors are characterized by the infiltration of certain bone marrow-derived cells that act to promote disease progression (Murdoch et al. 2004). In particular, tumor-associated macrophages (TAMs) have been implicated in promoting tumorigenesis, often as a result of chronic inflammation (Solinas et al. 2009; O'Connor et al. 2010; Saleh et al. 2011). Hypoxia-driven inflammatory intracellular and cytokine signaling and macrophage infiltration clearly enhance tumor progression (Tanner et al. 1984; O'Connor et al. 2010). Because both tumor cells and infiltrating TAMs must adapt to the unique stress of survival and proliferation under low O_2 concentrations, hypoxic responses in these cell types directly impact tumor growth, local invasion, and metastasis (Crowther et al. 2001; Bertout et al. 2008; Fang et al. 2009). As such, targeting the hypoxic response in either or both population(s) could have a beneficial effect on cancer therapy (Bingle et al. 2002; Waldner et al. 2010).

The transcriptional response to O_2 deprivation is mediated, in large part, by hypoxia-inducible factors (HIFs) (Semenza 2007; Majmundar et al. 2010). HIFs are composed of an O_2-sensitive α subunit, and a constitutively expressed β (HIF-1β/ARNT) subunit (Majmundar et al. 2010). The α subunit is regulated by the von Hippel-Lindau (VHL) E3 ligase complex and degraded by the 26S proteasome under elevated O_2 tensions (Cockman et al. 2000). Low O_2 levels stabilize HIF-α subunits by inhibiting

prolyl hydroxylases (PHDs) that modify HIF-α proteins and promote their degradation (Jaakkola et al. 2001). Once stabilized, HIF-α subunits translocate to the nucleus, form heterodimers with ARNT, and bind hypoxia-response elements (HREs) to promote gene expression devoted to adaptation to hypoxic stress (Talks et al. 2000; White et al. 2004; Semenza 2007; Semenza 2007; Imtiyaz et al. 2010). Three α subunits (HIF-1α, HIF-2α, and HIF-3α) have been identified; however, HIF-1α and HIF-2α appear to account for the majority of HIF-mediated transcriptional responses (Keith et al. 2007; Keith et al. 2011). Whereas the HIF-1α subunit is expressed in virtually all cells, HIF-2α has a more restricted expression profile, including components of the liver, kidney, lung, intestine, and brain (Wiesener et al. 2003). HIF-1α and HIF-2α possess distinct and occasionally overlapping roles; however, both have been suggested to actively promote the progression of a variety of cancers, including clear cell renal carcinoma, neuroblastoma, hepatocellular carcinoma, and colorectal cancer (Keith et al. 2011; Mucaj et al. 2012). HIFs play an important role in neoplastic and inflammatory cells within the tumor microenvironment, and crosstalk between these populations has clear effects on tumor growth (Lewis et al. 1999; Burke et al. 2002; Burke et al. 2003; Murdoch et al. 2004; Murdoch et al. 2005; Imtiyaz et al. 2010). Both HIF-α isoforms are expressed in TAMs, but have different downstream functions. For example, in the setting of nitric oxide (NO) metabolism, HIF-1α and HIF-2α elicit differential effects on arginase and inducible nitric oxide synthase (iNOS) activity respectively (Takeda et al. 2010; Keith et al. 2011). Both isoforms have been implicated in bone marrow-derived macrophages (BMDMs), mature macrophages, and the pro-tumorigenic and pro-angiogenic signaling observed in TAMs (Cramer et al. 2003; Fang et al. 2009). Importantly, whereas HIF-1α expression in macrophages has been implicated in modulating the switch from aerobic to anaerobic metabolism, as well as classical activation via Th1 cytokines, HIF-2α expression in

TAMs has been associated with an alternative activation via Th2 cytokines (Fang et al. 2009; Imtiyaz et al. 2010; Takeda et al. 2010; Shay et al. 2012). It is becoming increasingly apparent that HIFs are a common link between hypoxia, chronic inflammation, and tumorigenesis through their activity in macrophages during cancer development.

Pharmacological HIF inhibition as a novel therapeutic strategy is an active area of ongoing research (Semenza 2006; Semenza 2012; Semenza 2012). In particular, targeting HIF is well suited to colorectal cancer, as the HIF pathway has been repeatedly implicated in colorectal cancer pathogenesis (Waldner et al. 2010). Acriflavine (ACF), a mixture of trypaflavin and proflavine, inhibits HIF-α:ARNT dimerization, has shown promise in xenograft models of human cancers, and may be a viable source for future therapeutic interventions aimed at targeting HIF-1α and HIF-2α (Lee et al. 2009). Recently, ACF has also been shown to inhibit the recruitment of CD11b+ bone marrow-derived cells to the tumor microenvironment in an orthotopic model of breast cancer (Wong et al. 2012). Importantly, ACF does not appear to elicit any adverse side effects when administered to patients for extended periods of time (Wainwright 2001). Whereas ACF has proven effective in subcutaneous and orthotopic models, it has yet to be evaluated in an autochthonous tumor model in immunocompetent mice, which more accurately mimics the cellular complexity observed in clinical disease. Here we demonstrate that ACF limits tumor progression in murine models of colitis-associated colon cancer (CAC), and use *in vitro* cellular assays to assess underlying mechanisms in both macrophages and malignant colonic epithelial cells.

MATERIALS AND METHODS

Autochthonous and subcutaneous colorectal cancer models

8-week-old female Balb/C mice were purchased from Jackson Laboratory. Briefly, mice received a single intraperitoneal (i.p.) injection of 12.5mg/kg azoxymethane (AOM) at 8 weeks of age followed by 4 cycles of 2% dextran sulfate sodium (DSS) in their drinking water (cycle 1: 5 days, cycle 2: 4 days, cycle 3: 4 days, cycle 4: 4 days) with two weeks of regular water between each cycle for autochthonous induction of colitis-associated colon cancer. For subcutaneous experiments, 1×10^6 CT26 cells containing either shSCR or shARNT were injected subcutaneously into the left or right flank of 8-week-old female Balb/C mice respectively. For all *in vivo* experiments mice received acriflavine (Sigma M.W. 259.7) via daily i.p. injections at 2 mg/kg dissolved in PBS or an equivalent volume of PBS alone for the control cohort. The laboratory animal program is accredited by the American Association for Accreditation of Laboratory Animal Care. Animal health, well-being, and comfort were monitored constantly by certified veterinary staff. Every effort to minimize discomfort, stress, pain, and injury to the mice and the mice was maintained in accordance with the Animal Welfare Act and the DHHS Guide for the care and use of laboratory animals. These procedures were performed according to the protocols reviewed by the Institutional Animal Care and Use Committee (IACUC).

Cell lines and cell culture

CMT93 (ATCC CCL-223) and CT26 (ATCC CRL-2638) cell lines were purchased from ATCC and cultured according to instructions. Cells were cultured under normoxia (21% O_2) or hypoxia (0.5% O_2) using a Ruskinn InvivO$_2$ 400 workstation. Acriflavine (Sigma M.W. 259.7) was administered at 5μM in DMSO.

Bone marrow-derived macrophages

Generation of *VavCre* and *Arnt^{fl/fl}* mice has been previously discussed (Tomita et al. 2000; Stadtfeld et al. 2005). VavCre*;Arnt* mice were created by crossing VavCre mice (obtained as a gift from Speck lab) to *Arnt^{fl/fl}* mice on a mostly C57BL/6 background. Macrophages were isolated from C57BL/6, *VavCre;Arnt^{fl/+}* or *VavCre;Arnt^{fl/fl}* (littermates) mice by removing the long bones and flushing the marrow followed by red blood cell lysis. BMDMs were cultured in DMEM containing 20% Hyclone serum, 30% LCM, 1% L-glutamine, 1% Anti-Anti, and 0.1% beta-mercaptoethanol and stimulated with 5 ng/ml LPS (Sigma L3024) and 20 ng/ml IFN-γ(R&D 485-MI). For hypoxia induction, BMDMs were cultured under normoxia (21% O_2) or hypoxia (0.5% O_2 or 3% O_2). Acriflavine (Sigma M.W. 259.7) was administered at 1μM in DMSO.

Luciferase assay

CMT93 and CT26 cells were transfected according to Fugene protocols (Roche) with PGL3 plasmids containing firefly luciferase under control of either a wild type HRE promoter from the human PGK gene or a mutant HRE promoter along with renilla control, or an pGL4.32[*luc2p*/NF-κB-RE/Hygro] vector (Promega E849A) also with renilla control. 24 hours after transfection, media was changed to either DMSO or ACF and then cells were placed under normoxia or hypoxia. Luciferase activity was read on a luminometer 16 hours after addition of DMSO or ACF (Promega E1960). Firefly activity from the wildtype HRE plasmid was normalized to renilla and mutant HRE activity.

RT-qPCR

RNA was isolated from tumor tissue or cells using the RNAeasy minikit (Qiagen #74106). RNA concentration was quantified using the Nanodrop with equal amounts of mRNA used for reverse transcription to cDNA using the High-Capacity RNA-to-cDNA kit

(ABI #4387406). Expression was determined by quantitative PCR of synthesized cDNA using the Applied Biosystems 7900HT system and $\Delta\Delta$CT program settings. Target cDNA amplification was measured using the following TaqMan primers: VEGF (Mm00437304_m1), IL-1β (Mm00434228_m1), IL-6 (Mm00446190_m1), CXCR4 (Mm01292123_m1), COX-2 (Mm00478377_g1), SDF-1 (Mm0044552_m1), ARNT (Mm00507836_m1), PGK (Mm00435617_m1), HPRT (Mm01318743_m1), HIF-1α (Mm01283758_g1), HIF-2α (EPAS Mm00438717_m1), iNOS (Mm00440502_m1), ANG4 (Mm03647554_g1), RETNLB (Mm00445845_m1). Results were analyzed with HPRT as an endogenous control.

Production of shRNA containing lentiviruses and transduction

HEK-293T cells were used for lentiviral production using the following constructs: pLKO.1 scrambled shRNA (Addgene 1864), pLKO.1 *ARNT* shRNA (ThermoScientific TRCN0000079931), pLKO.1 *HIF1α* shRNA (ThermoScientific TRCN0000054448), and pLKO.1 *HIF-2α* shRNA (ThermoScientific TRCN0000082307), G protein of the vesicular stomatitis virus (VSV-G), pMDLG, and pRSV-rev. 293T cells were transfected according to the Fugene (Roche) protocol. 24 and 48 hours after transfection, supernatant was collected and concentrated using Amicon centrifugal filter units (Millipore). As the pLKO.1 shRNA constructs contain a puromycin resistance gene, transduction was followed by puromycin selection. CT26 cells were transduced with lentiviral particles containing copGFP in the form of the pCDH-CMV-EF1-copGFP vector (System Biosciences).

Immunostaining and Imaging

Immunohistochemistry was performed using enzymatic Avidin-Biotin Complex (ABC)-diaminobenzidine (DAB) staining (Vector Labs) with hematoxylin used for counterstaining of nuclei. Stained sections were visualized using an Olympus IX81 microscope. CD68 1:100 (Abcam ab955) used according to instructions (Vector PK-2200), CD31 1:50 (Abcam ab28364), Ki67 (Novocastra NCL-Ki67-MM1) used according to instructions (Vector PK-2200), TUNEL staining done according to instructions (Millipore ApopTag S7111), and copGFP staining performed using anti-TurboGFP antibody (Evrogen AB514). Staining was quantified using ImageJ software.

Immunoblot assays

Whole cell extracts were isolated in SDS/Tris pH 7.6 lysis buffer. Subcellular fractionation was performed as previously described (Pan et al. 2004). Protein was quantified using BCA and equal protein amounts were run on an 8% or 10% SDS-PAGE gel, transferred to nitrocellulose, and probed with the following antibodies: HIF-1α 1:1000 (Cayman 10006421), ARNT 1:1000 (Cell Signaling #5537), GAPDH 1:1000 (Cell Signaling #2118), NF-κB (Cell Signaling #3034), p-NF-κB (Cell Signaling #3039), IκBα (Cell Signaling #4814) p-IκBα (Cell Signaling #2859), M-CSFR (Cell Signaling #3152), DNMT1 (Cell Signaling #5032), AKT (Cell Signaling #9272), HDAC1 (Cell Signaling #5356). Representative western blots from multiple independent experiments are presented.

Flow Cytometry and Sorting

Subcutaneous tumors were grossly dissected, minced, collagenase-treated, and run through a 70μm cell-strainer to generate a single cell suspension. Live cells were run on a FACSVantage SE and sorted based on GFP staining. GFP negative parent cells

were run to set up GFP+ and GFP- gates. Acquired data was analyzed using FlowJo software.

Statistical Analysis

Unless otherwise indicated, data is shown as mean ± SEM. GraphPad Prism software was used to conduct statistical analyses and graph data. Unless otherwise indicated, unpaired 2-tailed Student's *t* test was performed to evaluate statistical differences between control and experimental groups. In situations where more than two groups were compared, a one-way Anova was used followed by post-test Tukey analysis. Significance is demonstrated by "#" indicating $p > 0.05$, "*" representing $p < 0.05$, "**" representing $0.001 < p < 0.01$, and "***" representing $p < 0.001$.

RESULTS

Acriflavine limits tumor burden in an autochthonous murine model of colitis-associated colon cancer. To analyze the effect of ACF treatment in the setting of colitis-associated cancer (CAC), eight week-old female Balb/C mice were subjected to a single intraperitoneal (i.p.) injection of the pro-carcinogen azoxymethane (AOM), followed by repeated treatments of 2% dextran sulfate sodium (DSS) to induce autochthonous CAC (Okayasu et al. 1990; Okayasu et al. 1996; Okayasu et al. 2002) (Figure 7A). Prior to initiating ACF treatment, we confirmed that mice exhibited gross intestinal polyp formation, with hyperplastic lesions making up approximately 60% of tumor burden and adenomas making up the remaining 40%, based on histologic examination of mice sacrificed at baseline (Figure 9A-C). The AOM/DSS treated mice were then separated into two cohorts: an experimental group that received daily i.p. injection of 2 mg/kg ACF for four weeks, and a control group that received daily

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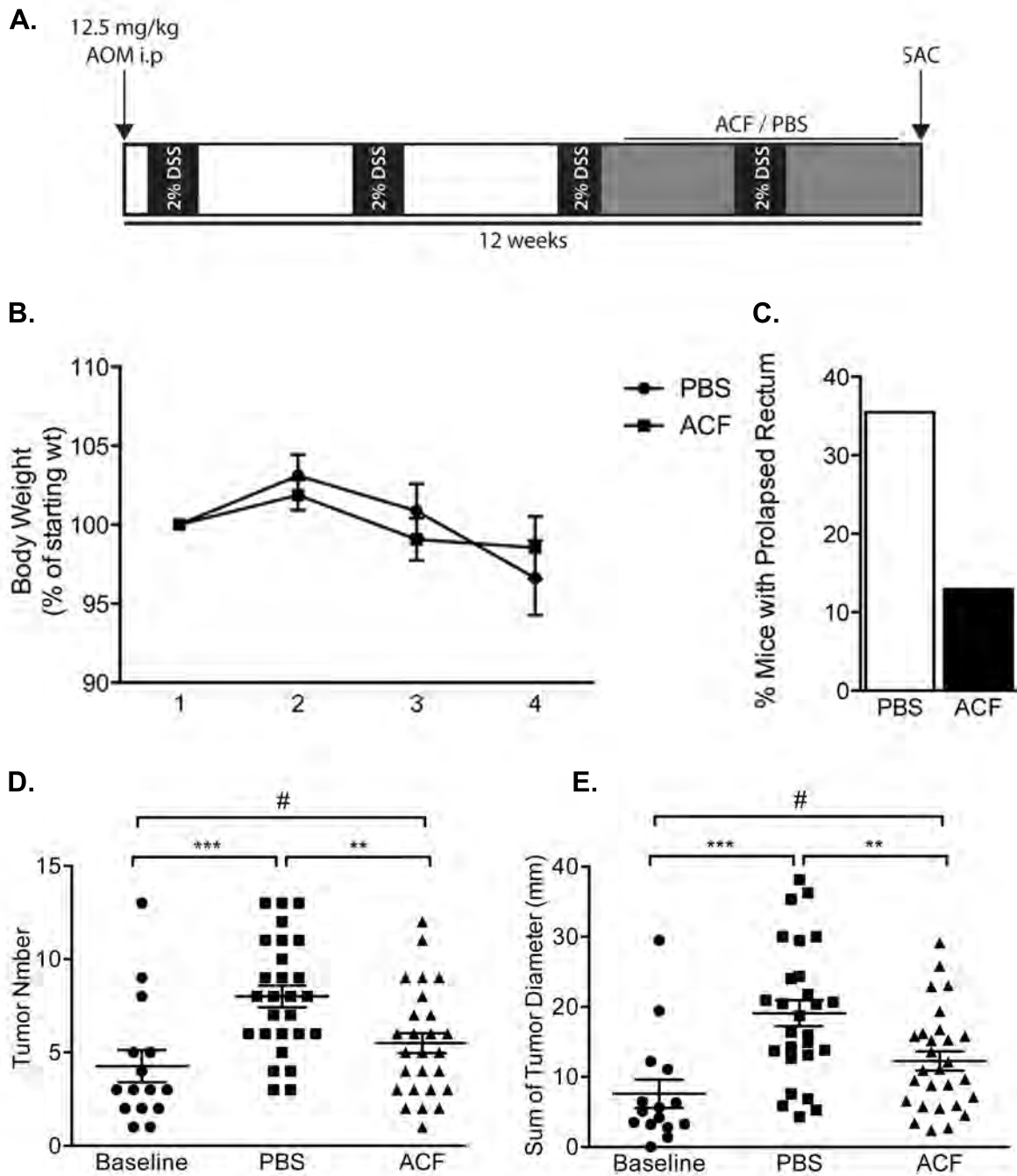
Figure 7. Gross analysis of autochthonous tumor development. (A) Schematic model of AOM/DSS treatment followed by 28 days of i.p. ACF or PBS administration. (B) Weight change in mice over course of treatment shown as percent change from weight at the beginning of daily injections - measured weekly. (C) Percentage of each cohort that exhibited prolapsed rectum based on visual observation. (D-E) Tumor number and tumor size upon gross examination either at the end of the third DSS administration (baseline), or at the end of either PBS or ACF treatment. (mean ± SEM, PBS: $n = 27$, ACF: $n = 28$, ** $p < 0.01$, *** $p < 0.001$)

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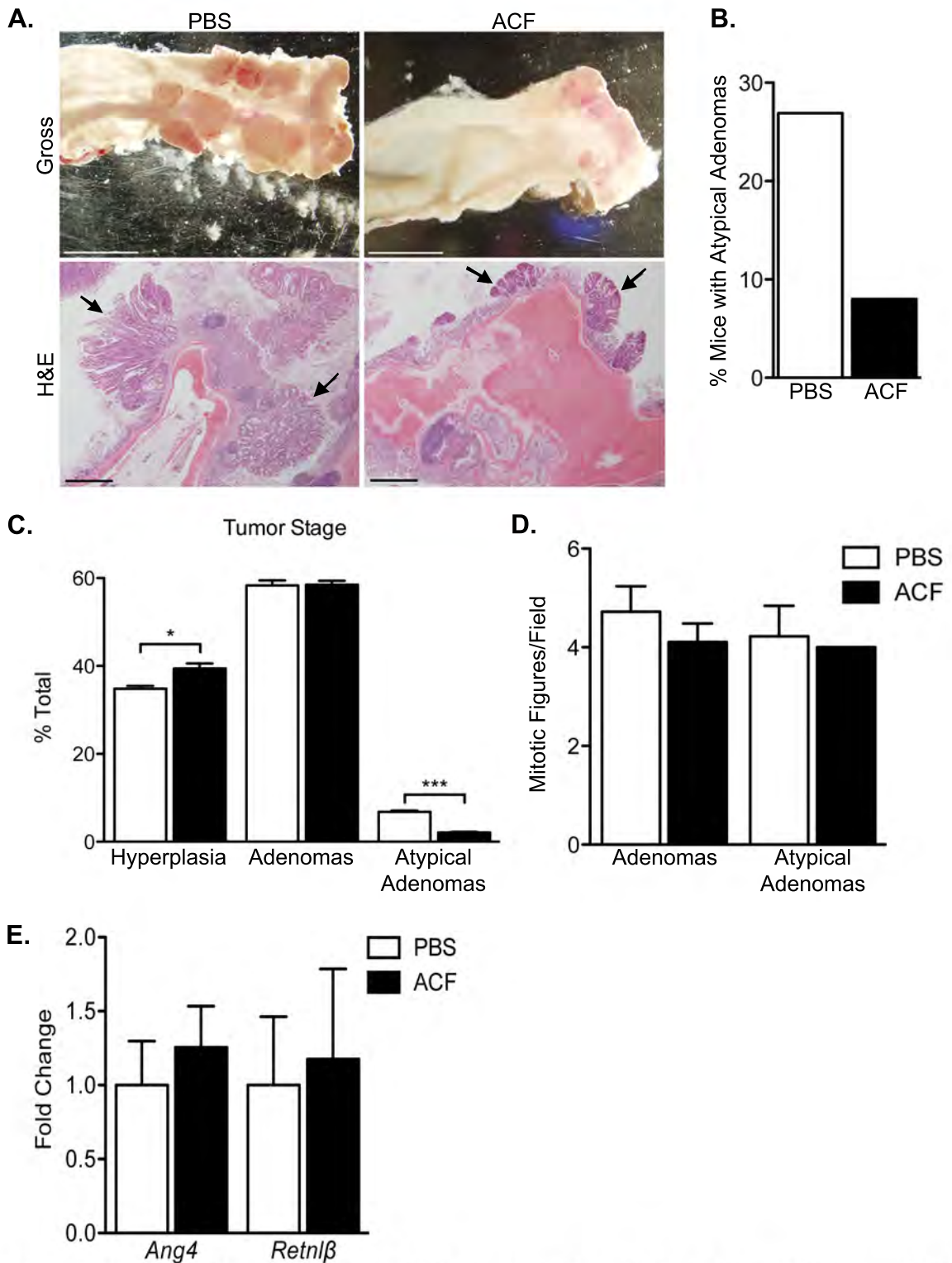
Figure 8. Decreased tumor staging in ACF mice based on H&E analysis. (A) Gross and H&E pictures of representative samples from PBS and ACF treatment cohorts - images acquired on dissecting microscope. (B) Tumor staging based on H&E slides from each mouse for either PBS- or ACF-treated cohorts. (C) Percent of each treatment cohort with at least 1 atypical adenoma, based on tumor staging. (D) Number of mitotic figures between either cohort. (E) RNA analysis of PBS- and ACF- treated tumors evaluating the expression of a variety of inflammatory regulated genes. (mean ± SEM, PBS: $n = 27$, ACF: $n = 28$, ** $p < 0.01$, *** $p < 0.001$)

injections of PBS. Over the course of treatment, no significant difference in mouse weight was observed between experimental and control groups (Figure 7B); however, the control cohort exhibited increased incidence of prolapsed rectum, indicative of underlying pathology (Figure 7C).

After one month, mice were euthanized, colons dissected, and tumor burden analyzed. Whereas the control cohort exhibited significant disease progression compared to the baseline group, the ACF-treated cohort did not (Figure 7D-E). Mice in the control group, on average, developed increased numbers of colorectal tumors (Figure 7D). Similarly, there was an overall greater tumor burden, with a greater proportion of large tumors, in the control cohort when compared to ACF treated or baseline groups (Figure 8A-D). The control cohort also displayed a larger fraction of high-grade lesions, based on nuclear pleiomorphism and atypia, than the corresponding ACF-treated group (Figure 8C). In particular, the most highly pleiomorphic lesions in this model (atypical adenomas), were observed at nearly three-fold higher frequency in the control group than in the ACF-treated cohort (Figure 8B). Although increased nuclear atypia were detected, no significant decreases in number of mitotic figures were noted in the control group compared to the ACF-treated group (Figure 8D). Lastly, we observed a trend towards decreased expression of multiple HIF-associated inflammatory molecules (Figure 9D) in RNA isolated from unstaged and unmatched individual polyps from ACF and PBS treated mice respectively. *Resistin like beta* (*Retnlβ*) and *Angiogenin 4* (*Ang4*) expression corresponds with colonic inflammation and bacterial influx (Hooper et al. 2003; Hogan et al. 2006). Importantly expression of both *Ang4* and *Retnlβ* was unchanged, suggesting ACF does not alter bacterial influx into the colonic epithelial cells (Figure 8E). As such, any observations in ACF-treated mice are unlikely to be due to the antimicrobial effects of acriflavine. Although previous studies have shown that intestinal microflora may contribute to the pathogenesis of colitis, and antibiotics are effective at

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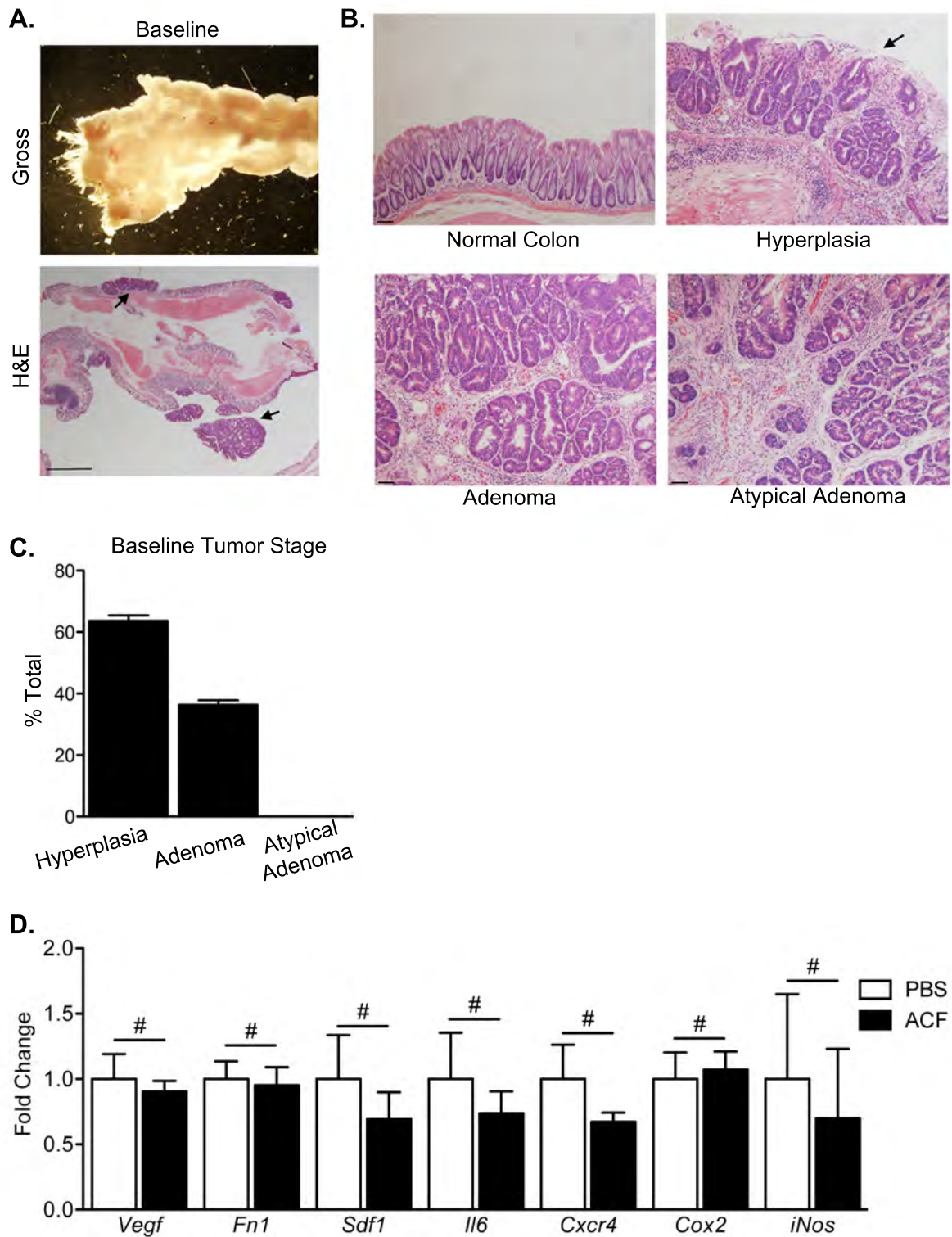
Figure 9. Baseline tumor analysis. (A) Representative gross and H&E images of tumor burden in baseline cohort. (B) Tumor staging of baseline cohort. (C) H&E images of normal colon, hyperplasia, adenoma, and atypical adenoma. (D) RNA analysis of PBS- and ACF- treated tumors evaluating the expression of a variety of inflammatory and HIF-regulated genes. (mean ± SEM, PBS: n = 27, ACF: n = 28, ** $p < 0.01$, *** $p < 0.001$)

A.

Bone Marrow

Spleen

B.

Bone Marrow

Spleen
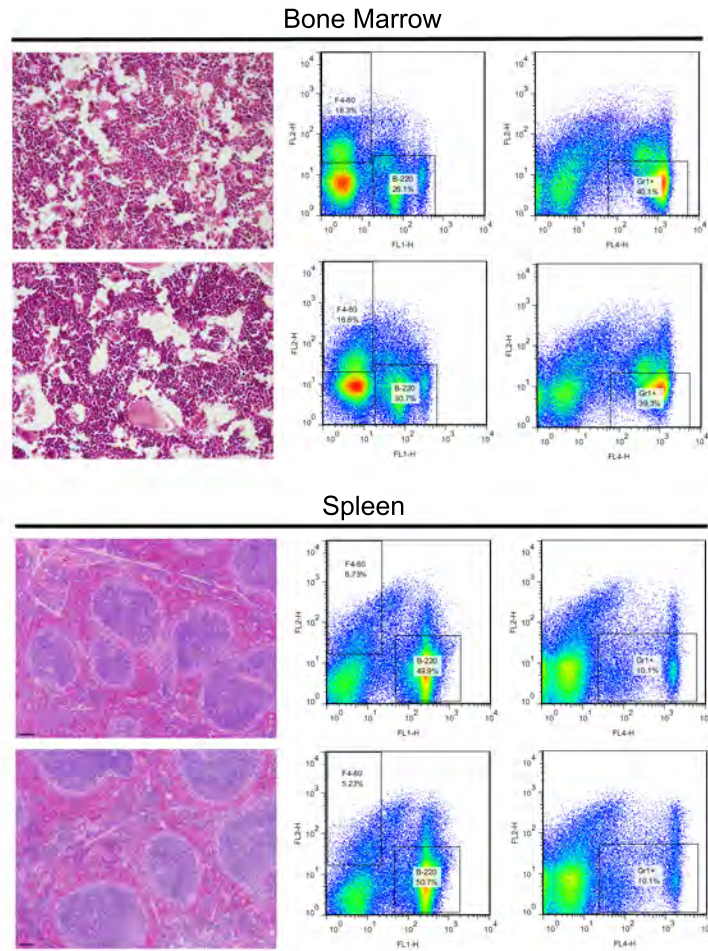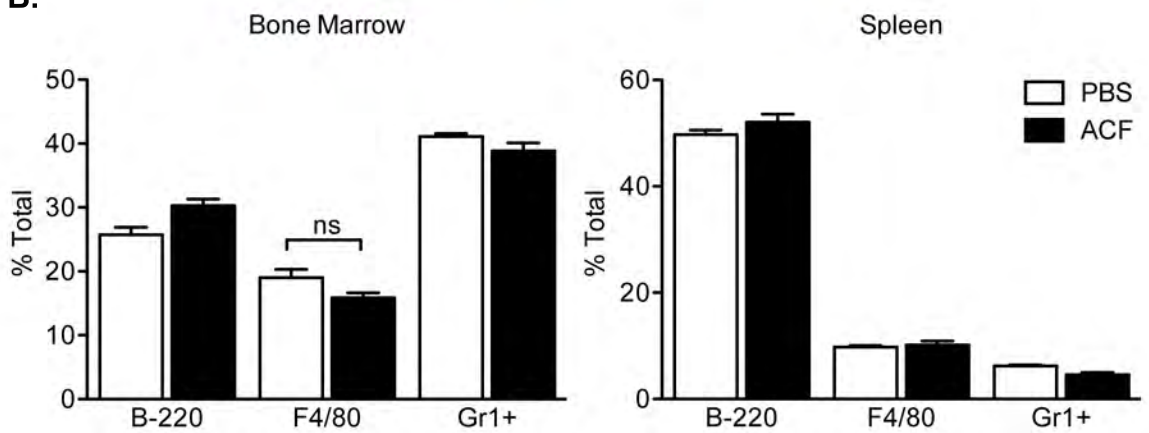
Figure 10. ACF has no effect on hematopoietic compartment of healthy mice. (A) Representative H&E and flow cytometry graphs of bone marrow and splenic compartments of control and experimental groups respectively with quantification (B) of B-cell (B220+), macrophage (F4/80+), or granulocyte (Gr1+) populations in the bone marrow or spelnic compartments of PBS- or ACF-treated mice.

minimizing disease in acute models of DSS-induced colitis, antibiotic treatment appears to be ineffective in models of chronic DSS-induced colitis (Hans et al. 2000; Hooper et al. 2003; Hogan et al. 2006). We concluded that ACF limits tumor progression in an autochthonous model of CAC, and may be doing so through HIF-dependent mechanisms.

**Effects on tumor vascularity and proliferation corresponding to acriflavine treatment.** Previous work has demonstrated an effect on tumor growth along with decreased vascularity and infiltrating CD11b+ cells in mice treated with ACF (Lee et al. 2009; Wong et al. 2012). Similarly, while a trend towards decreased mitotic figures was detected upon H&E analysis, further investigation revealed that tumors from ACF-treated mice exhibited approximately 30% fewer proliferating (Ki67-positive) cells in stage-matched sections (Figure 11A-B). In contrast, no difference in apoptotic cell numbers was noted between the two cohorts, based on TUNEL staining (Figure 11A-B).

Moreover, ACF-treated tumors exhibited significantly reduced vessel density and size, with vessel area nearly three times greater in PBS-treated mice, as assessed by CD31 staining (Figure 12A-B). In the setting of an inflammation-driven cancer, such as the AOM/DSS model of colitis-induced colorectal cancer, ACF appears to limit tumor burden through effects on tumor growth and progression, correlated with lower rates of tumor cell proliferation and decreased angiogenesis. ACF may also have an effect on the inflammatory component of this model.

Acriflavine-treated tumors exhibit decreased macrophage infiltration. We hypothesized that the effects of ACF on the growth and progression of AOM/DSS-induced colorectal tumors were caused by altered HIF activity in TAMs, transformed colonic epithelial cells, or both. To investigate ACF-mediated effects on macrophage

A.
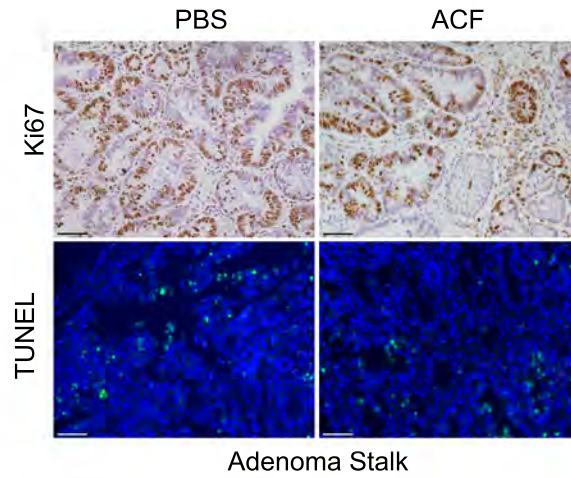
B.
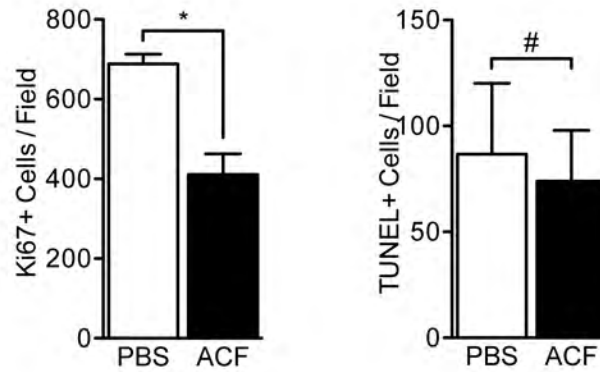
Figure 11. Decreased tumor proliferation corresponding to acriflavine treatment. (A) Representative examples of Ki67 and TUNEL staining. (B) Ki67+ and TUNEL+ cells quantified from stage-matched samples. Images taken at 200x magnification. (mean ± SEM, PBS: $n = 6$, ACF: $n = 6$)

A.
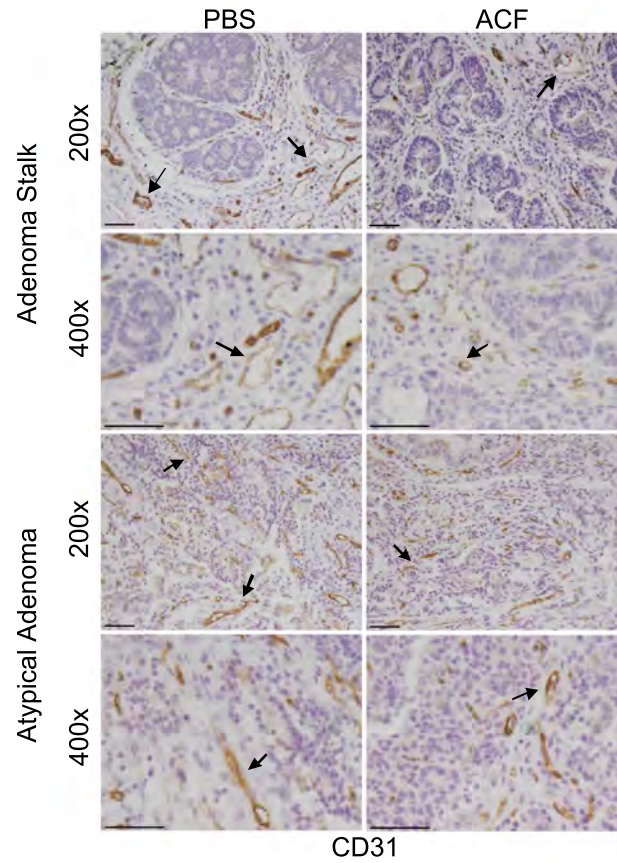
B.
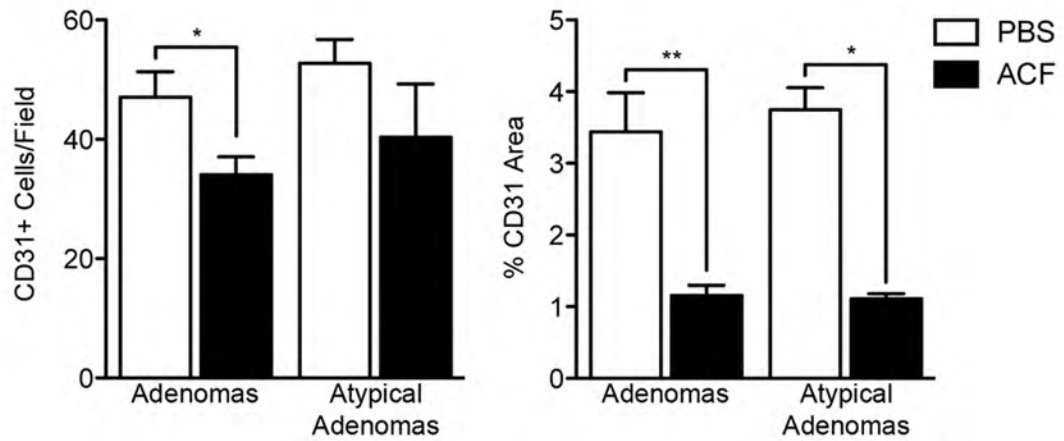
Figure 12. Decreased tumor vascularity in ACF treated samples. (A) Representative images and (B) quantification of CD31+ cells in both adenomas and atypical adenomas for stage-matched samples - images taken at 400x and 200x magnification. (mean ± SEM, PBS: n = 6, ACF: n = 6 for adenomas and PBS: n = 4, ACF: n = 2 for atypical adenomas, * p < 0.05, ** p < 0.01)

recruitment, CD68+ cells were counted in stage-matched tumor sections from PBS- and ACF-treated mice, respectively. Adenomas and atypical adenomas in control animals exhibited significantly greater numbers of infiltrating macrophages than corresponding tumors from the ACF-treated cohort (Figure 13A-B, 17A). These results confirm previous work in different non-inflammation-driven tumor models, and are unlikely to be a consequence of general myelosuppressive effects of ACF treatment, as experimental animals displayed no discernible changes in the number of B220+, F4/80+, and Gr1+ cells in bone marrow or spleen, following one month of treatment (Figure 10 A-B) (Wong et al. 2012).

Because regulatory T cells possess anti-inflammatory functions and have been implicated in CAC, we stained for CD3 and Foxp3 in sections from ACF-treated and control mice (Ullman et al. 2011). However, no differences were observed in Foxp3+ or CD3+ T lymphocyte numbers among control and ACF-treated cohorts, indicating that ACF treatment primarily affects innate immune cells associating with colonic tumors in this setting (Figure 14A-B). Collectively, these findings, along with previously published data, underscore the importance of infiltrating macrophages in inflammation-driven cancers, and provide a possible mechanism to explain the less aggressive CAC observed in ACF-treated mice.

Acriflavine inhibits HIF signaling in macrophages. To evaluate the general impact of ACF treatment on macrophages, we investigated HIF-dependent responses in bone marrow-derived macrophages (BMDMs) obtained from wild type C57BL/6, *VavCre;Arnt$^{fl/+}$*, or *VavCre;Arnt$^{fl/fl}$* mice (also on a C57BL/6 background). To confirm efficacy of *Arnt* deletion, BMDMs were purified and whole cell lysates analyzed for ARNT protein levels. As shown in Figure 15B, no detectable ARNT protein was observed in macrophages isolated from *VavCre;Arnt$^{fl/fl}$* mice (Figure 15B) (Krock et al. 2013). *Arnt$^{\Delta/\Delta}$*

A.
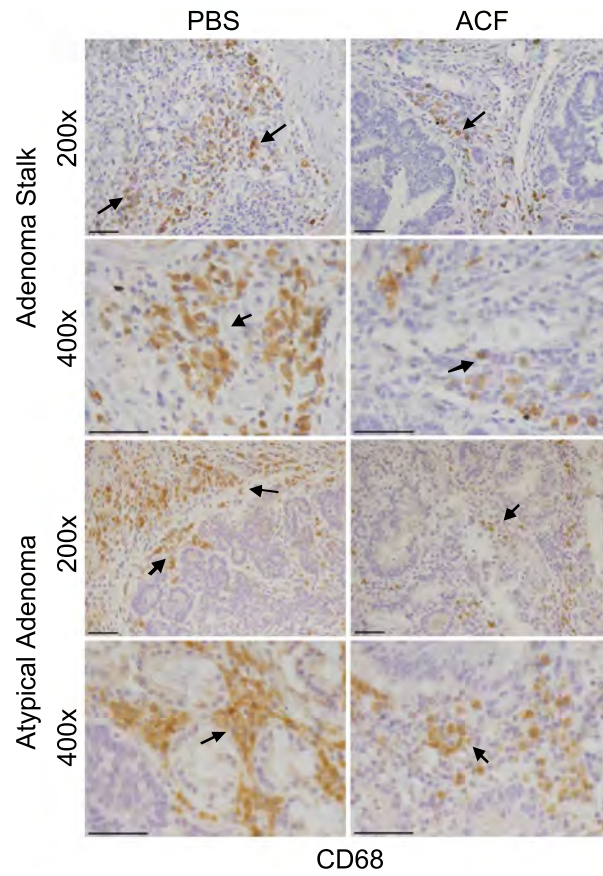
CD68

B.
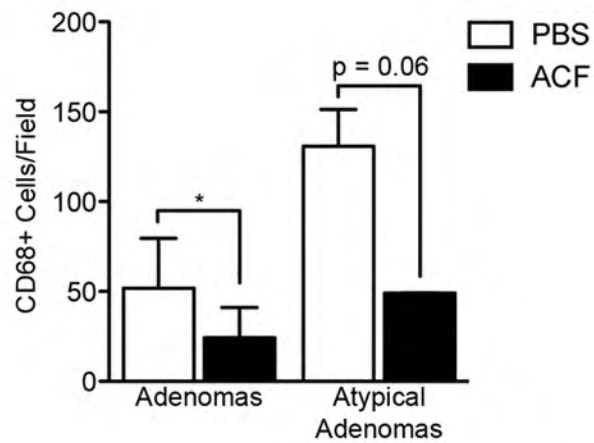
Figure 13. Tumors treated with acriflavine exhibit decreased macrophage infiltration. (A) CD68 staining in PBS- and ACF-treated tumors, with representative images and quantification of both adenomas and atypical adenomas respectively - images taken at 400x and 200x magnification. (mean ± SEM, PBS: n = 6, ACF: n = 6 for adenomas and PBS: n = 4, ACF: n = 2 for atypical adenomas, * p < 0.05)

A.
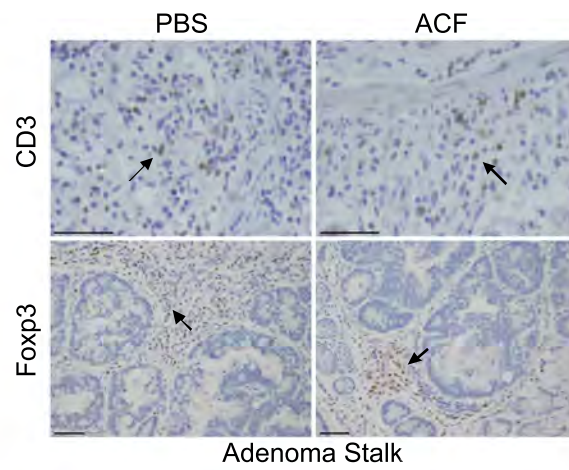
B.
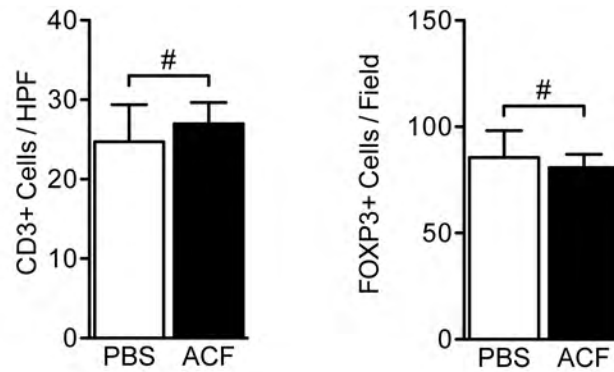
Figure 14. No difference observed in infiltrating T cells. (A) CD3 and Foxp3 staining with (B) respective quantifications of PBS- and ACF-treated tumors from stage-matched tumors - images taken at 400x and 200x magnification. (mean ± SEM, PBS: *n* = 6, ACF: *n* = 6)

macrophages are therefore deficient in the obligate HIF-α dimerization partner, and consequently fail to engage either HIF-1α and HIF-2α responses. We propose that this genetic model is similar to pharmacological HIF inhibition by ACF. To mimic the hypoxic microenvironment, macrophages were cultured in complete media at 3% O_2 and growth compared to that at 21% O_2. Macrophage proliferation was unaffected by ACF treatment under normoxia or hypoxia in complete media (21% or 3% $O_2,$ respectively, Figure 17C), suggesting that the observed decrease in CAC infiltrating macrophages is secondary to decreased recruitment rather than an effect on resident macrophage numbers.

In contrast, ACF treatment significantly inhibited the expression of genes encoding interleukin 1β (IL-1β) and VEGF, both HIF targets, in $Arnt^{\Delta/+}$ macrophages (Figure 15C). Interestingly, $Il1\beta$ and $Vegf$ transcript levels in ACF-treated $Arnt^{\Delta/+}$ macrophages are similar to those observed in HIF-deficient $Arnt^{\Delta/\Delta}$ macrophages. Furthermore, ACF treatment failed to substantially further reduce the expression of either gene in $Arnt^{\Delta/\Delta}$ macrophages, confirming that ACF is acting via a predominantly HIF-dependent pathway. We also observed that hypoxic stimulation of M-CSFR, a principal receptor for the macrophage growth factor and chemoattractant M-CSF, was ablated upon ACF treatment (Figure 16A). This is consistent with our previous observation that macrophage M-CSFR expression is regulated by HIF-2α, and suggests a mechanism by which ACF treatment limits macrophage recruitment to, and infiltration of, inflammation-associated tumors (Imtiyaz et al. 2010). Interestingly, when macrophages are cultured without M-CSF under 21%, 1.5%, or 0.5% O_2 and treated with DMSO or ACF, there appeared to be a specific proliferative effect on macrophages treated with ACF under hypoxic conditions. This may be secondary to decreased M-CSFR expression, as the proliferative defect is recapitulated with macrophages cultured in the absence of M-CSF (Figure 16B). We concluded that ACF treatment limits hypoxic

A.

MΦ

B.

C.
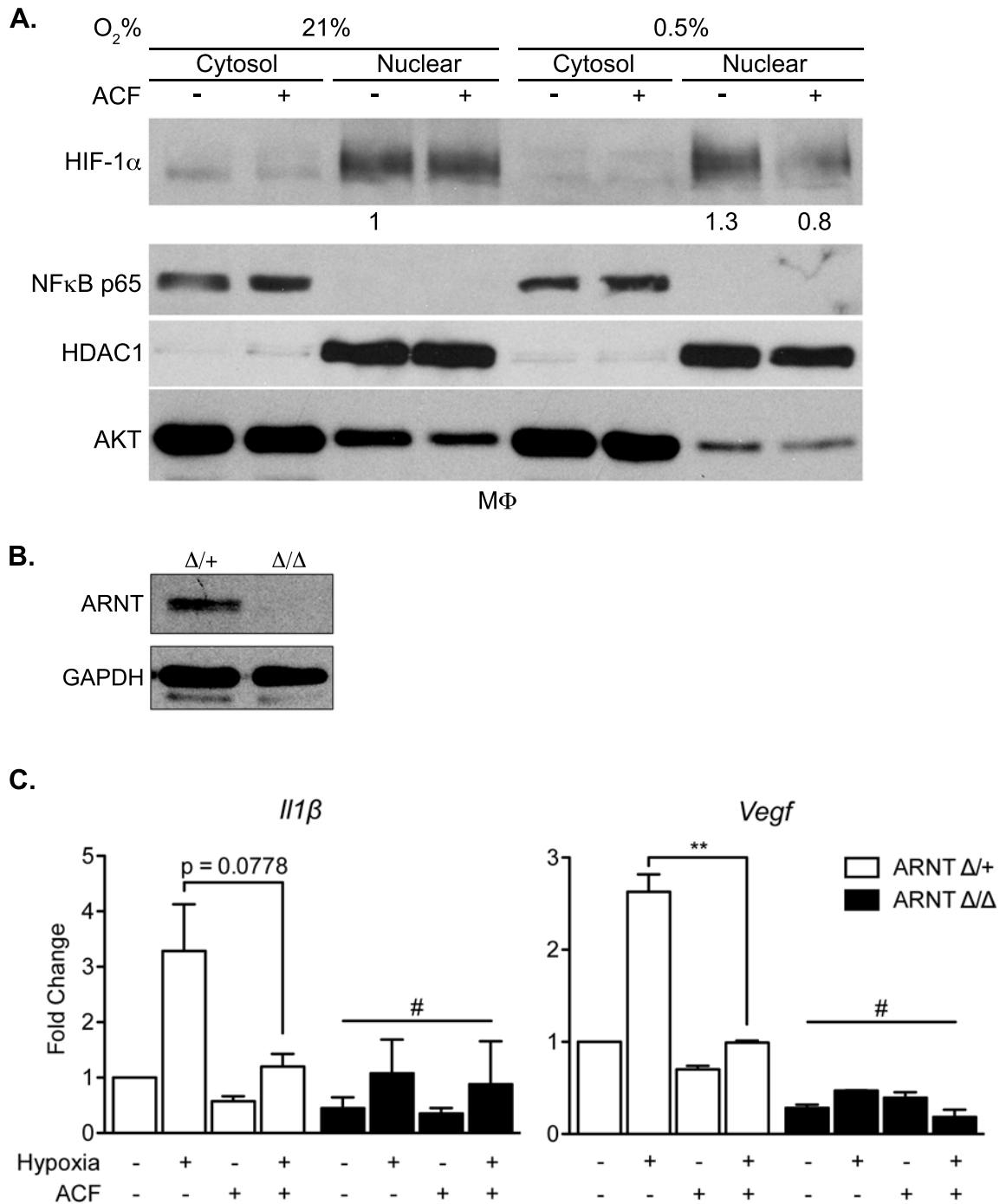
Figure 15. Effects of acriflavine on macrophage transcription are largely HIF-dependent. (A) Cytoplasmic/nuclear subcellular fractionation of macrophage lysates under normoxia (21% O_2) and hypoxia (0.5% O_2) with or without ACF treatment and immunoblotting for HIF-1α, NFκB p65, HDAC1, and AKT. (B) Western blot validation of ARNT ablation in macrophages purified from *VavCre;Arntfl/fl* mice. (C) Expression of pro-inflammatory (*Il1β)* or pro-angiogenic (*Vegf*) genes upon ACF treatment or *Arnt* deletion. (mean ± SEM, PBS: *n* = 3, ACF: *n* = 3 # p > 0.05, * p < 0.05**, p < 0.01, *** p < 0.001)

induction of M-CSFR expression, minimizing macrophage stimulation by M-CSF under low O_2. This effect was also detected during macrophage motility, as demonstrated by M-CSF-mediated migration of seeded macrophages in a modified Boyden chamber migration assay (Figure 16C). Although no difference between either WT, $Arnt^{\Delta/+}$, or $Arnt^{\Delta/\Delta}$ cells was noted under normoxia, migration under 0.5% O_2 was limited in ACF-treated WT and $Arnt^{\Delta/+}$ cells and in both DMSO-treated and ACF-treated $Arnt^{\Delta/\Delta}$ macrophages. These results indicated ACF treatment acts upon macrophages in a hypoxia and HIF-dependent manner, in large part through the expression of M-CSFR.

NF-κB (Nuclear factor kappa-light-chain-enhancer of activated B cells) is a central regulator of the inflammatory response and has an established role in inflammation-associated cancers (Barnes et al. 1997). Moreover, ACF has been suggested to have possible effects on this pathway (Barnes et al. 1997; Lee et al. 2009). NF-κB transcription complexes are generally maintained in an inactive state in the cytoplasm and only translocate to the nucleus when dissociated from inhibitors such as IκBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha) (Pahl 1999). As such, NF-κB nuclear localization is a strong indicator of transcriptional activity. We investigated whether ACF treatment alters NF-κB nuclear transit in BMDMs cultured under normoxia or hypoxia. Of note, ACF treatment had no appreciable effect on NF-κB subcellular localization or IκBα phosphorylation (Figure 15A), a necessary step to release NF-κB and allow nuclear entry. However, the same lysates clearly demonstrate decreased hypoxia-induced nuclear localization of HIF-1α (Figure 15A). AKT and HDAC-1 immunoblotting indicate the purity of cytosolic and nuclear fractions, respectively. Similarly, ACF treatment had no detectable effect on the expression of transcripts encoding COX-2, an inflammatory protein regulated by multiple stimuli, including NF-κB (Figure 17B). Taken together, these findings indicate ACF inhibits
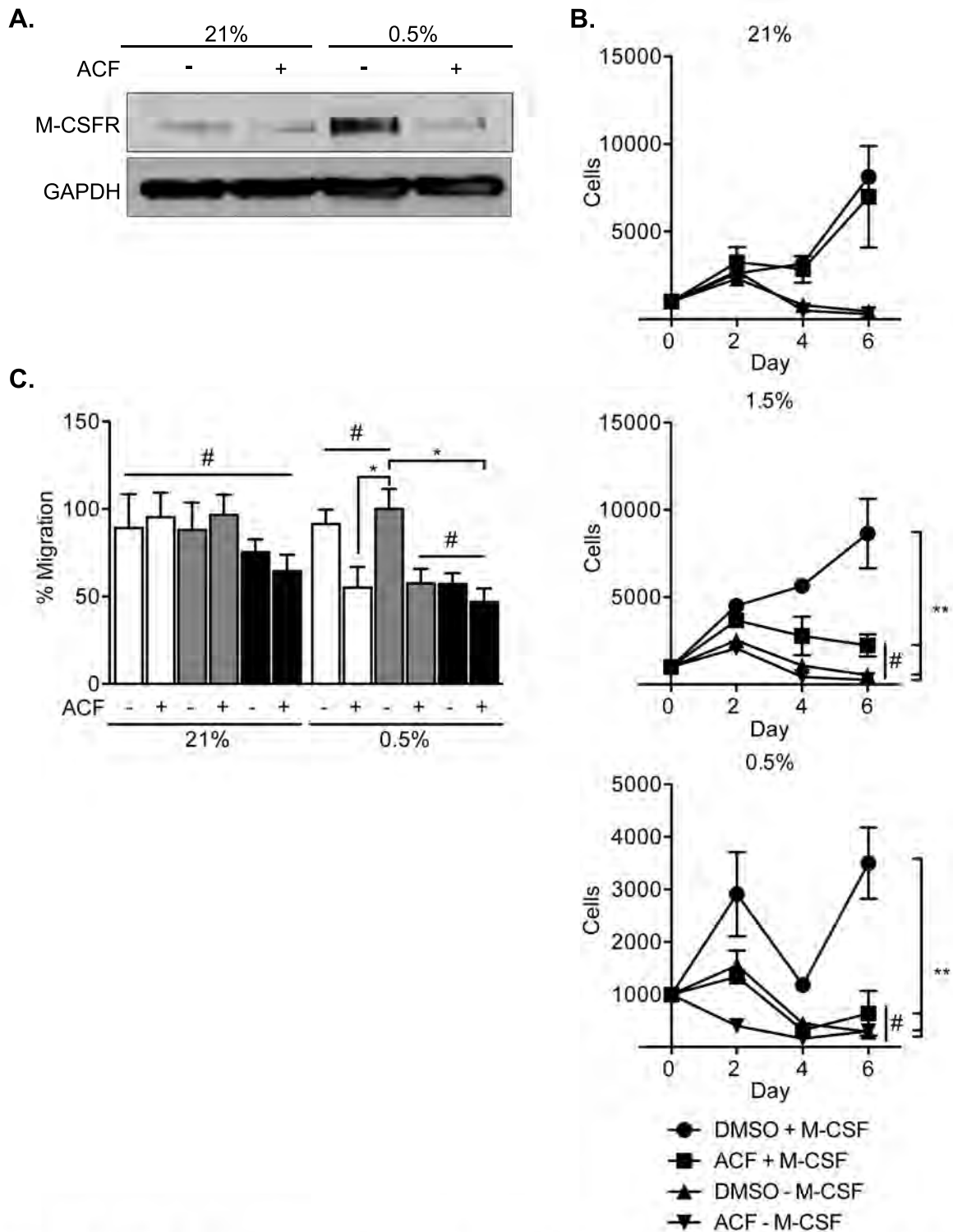
Figure 16. Effects of acriflavine on macrophage M-CSFR expression under hypoxia. (A) M-CSFR protein levels based on immunoblot of primary bone marrow-derived macrophages from C57BL/6 mice cultured under 21% O_2 or 0.5% O_2 and in the presence of either DMSO or ACF. (B) Proliferation of either control or ARNT-deficient macrophages under 21% or 3% O_2 over a four day period. (C) Normoxic and hypoxic migration of BL6, $Arnt^{\Delta/+}$, or $Arnt^{\Delta/\Delta}$ mice macrophages treated with DMSO or ACF. Shown as percent total. (mean ± SEM, PBS: $n = 3$, ACF: $n = 3$ # $p > 0.05$, * $p < 0.05$**, $p < 0.01$, *** $p < 0.001$)
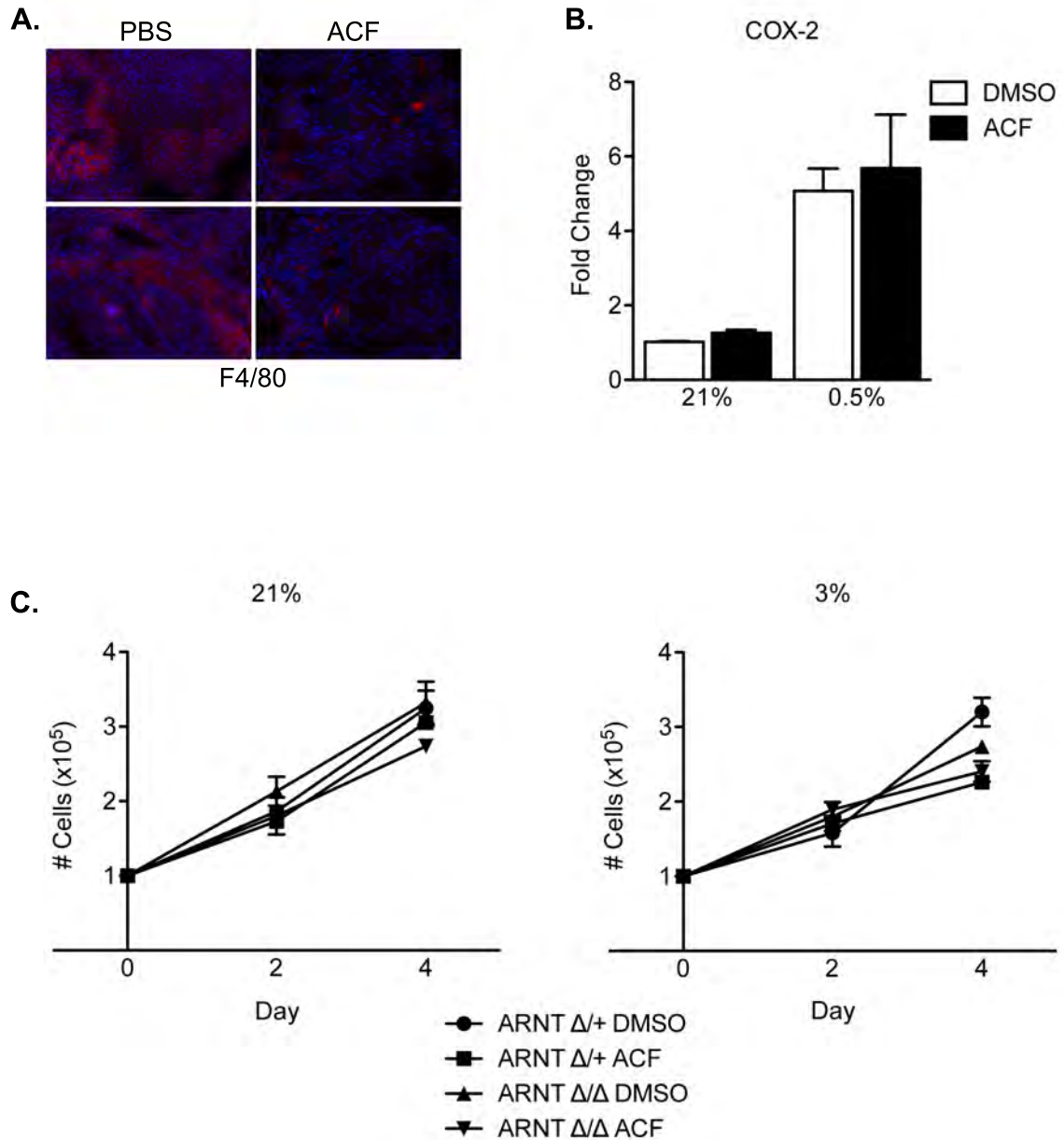
Figure 17. Macrophage recruitment and proliferation under hypoxia *in vivo* and *in vitro*. (A) F4/80 immunofluorescence of frozen sections. (B) *Cox2* expression levels after acriflavine treatment in macrophages. (C) Proliferation of either control or ARNT-deficient macrophages under 21% or 3% O_2 over a four day period. (mean ± SEM, PBS: *n* = 3, ACF: *n* = 3)

macrophage recruitment and signaling through HIF-α specific mechanisms, with little to no impact on the NF-κB pathway.

HIF signaling is inhibited by acriflavine treatment in murine colorectal cells. In addition to its effects on macrophages, ACF treatment likely inhibits CAC progression by inhibiting HIF responses in transformed colonic epithelial cells. To address this hypothesis, we analyzed ACF effects on murine CT26 cells (derived from Balb/C colon carcinoma) and CMT93 cells (derived from C57BL/6 polyploid carcinoma of the rectum). As expected, ACF administration did not impact HIF-1α stabilization under 0.5% O_2 in either CT26 or CMT93 cells (Figure 22A), consistent with its proposed role in blocking HIF-α/ARNT dimerization rather than α subunit accumulation (Lee et al. 2009). Furthermore, ACF treatment had no effect on *Hif1α* or *Arnt* transcript levels. Importantly, ACF treatment instead limits nuclear localization of HIF-1α in both CT26 and CMT93 cells under 0.5% O_2 (Figure 18A-B) with AKT and HDAC-1 immunoblotting demonstrating cytosolic and nuclear fractions, respectively. In contrast, ACF administration had no effect on the cellular localization of NF-κB (Figure 18A-B), or expression of *Cox-2* (Figure 22C), indicating that ACF is unlikely to be affecting the NF-κB pathway. As noted for macrophages, *Cox-2* transcription is likely regulated by multiple hypoxia-dependent, HIF-independent factors and does not appear to be impacted by ACF treatment. Instead, HIF targets *Vegf* and phosphoglycerate kinase 1 (*Pgk1*) were markedly reduced in both CT26 and CMT93 cells upon ACF exposure (Figure 20A-B). Additionally, ACF administration resulted in decreased HRE-driven luciferase reporter gene expression in both CT26 and CMT93 cells under hypoxia (Figure 20A), demonstrating decreased HIF transcriptional activity. Unlike that observed with HRE-driven luciferase assays, ACF had no effect (hypoxic or otherwise) on an NF-

A.

CT26

B.
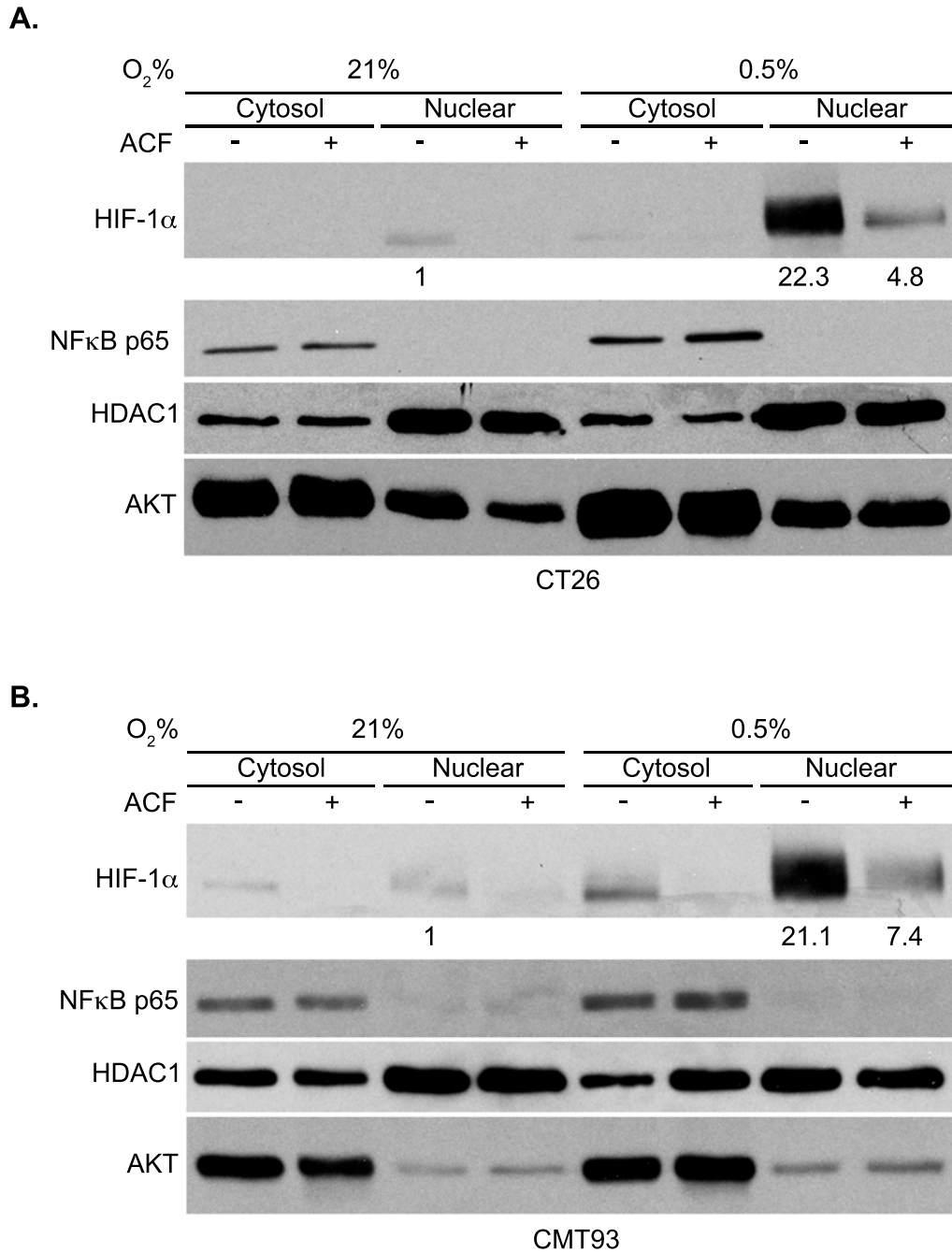
CMT93

Figure 18. Decreased nuclear localization of HIF-1α in presence of ACF. Cytoplasmic nuclear fractionation of (A) CT26 and (B) CMT93 lysates under normoxia (21% O_2) and hypoxia (0.5% O_2) with or without ACF treatment, and immunoblotting for HIF-1α, NFκB p65, HDAC1, and AKT. Representative images shown.

A.

CT26

B.
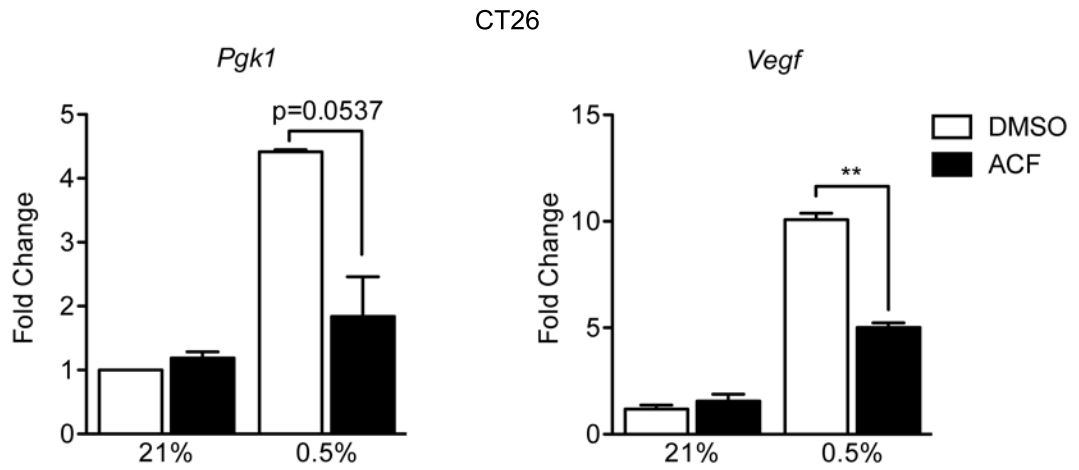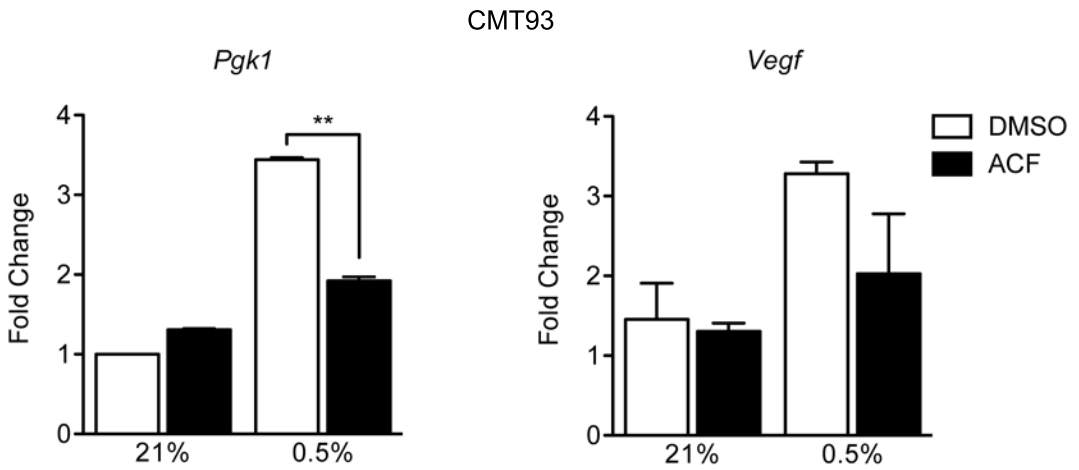
CMT93

Figure 19. ACF inhibits HIF transcription. (A) Expression of HIF targets *Pgk1* and *Vegf* under hypoxia in both CT26 and (B) CMT93 cell lines upon treatment with DMSO or ACF. (mean ± SEM, PBS: n = 3, ACF: n = 3, # p > 0.05, * p < 0.05, ** p < 0.01)

A.

B.
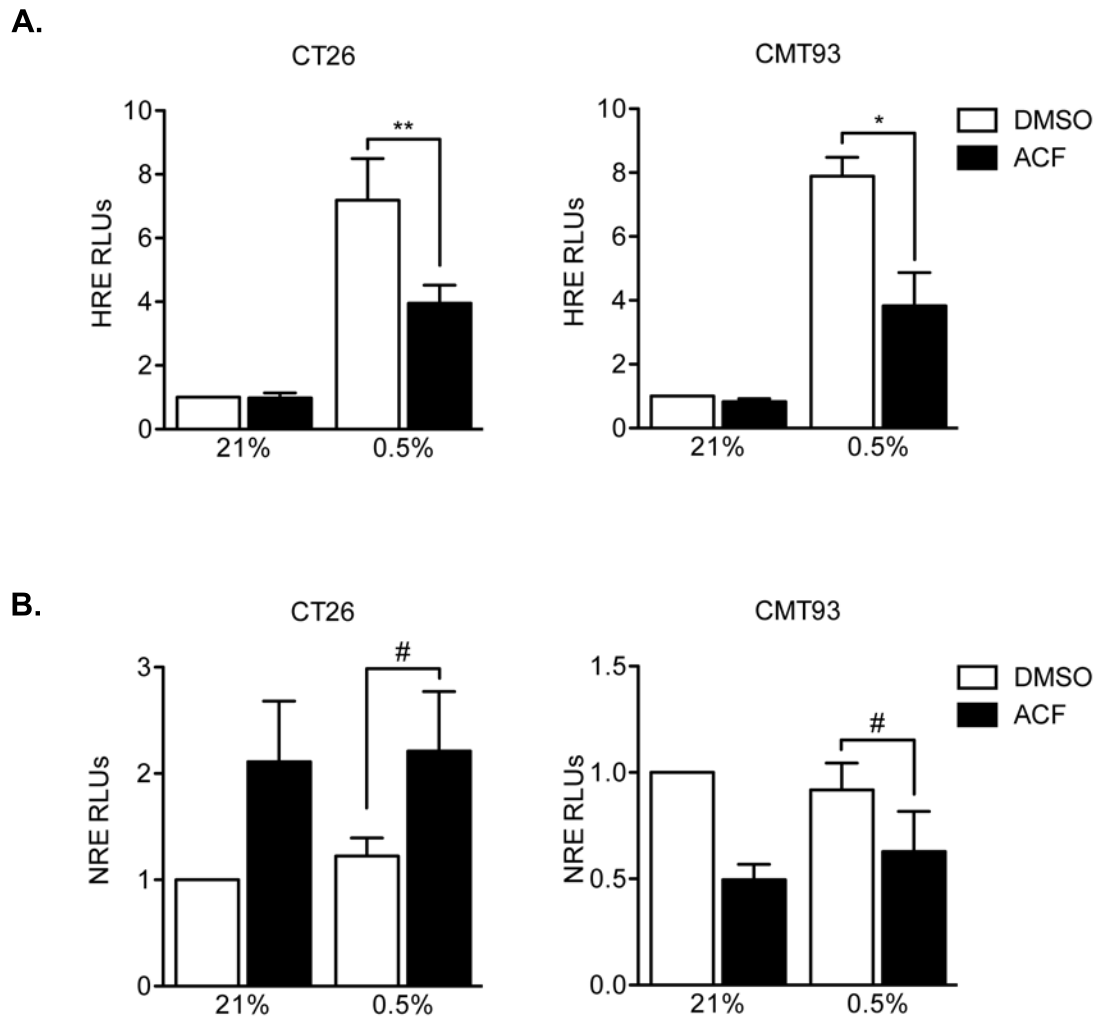
Figure 20. HREs but not NREs exhibited decreased activity with ACF treatment. (A) CT26 and CMT93 cells were transfected with a plasmid containing firefly luciferase under control of the *PGK* promoter (with three consecutive HRE sequences) and cultured under normoxia (21% O_2) or hypoxia (0.5% O_2) in the presence or absence of ACF. (B) CT26 and CMT93 cells were transfected with a plasmid containing firefly luciferase under control of an NRE and cultured under normoxia (21% O_2) or hypoxia (0.5% O_2) in the presence or absence of ACF. (mean ± SEM, PBS: $n = 3$, ACF: $n = 3$, # $p > 0.05$, * $p < 0.05$, ** $p < 0.01$)

A.

Pgk1

B.
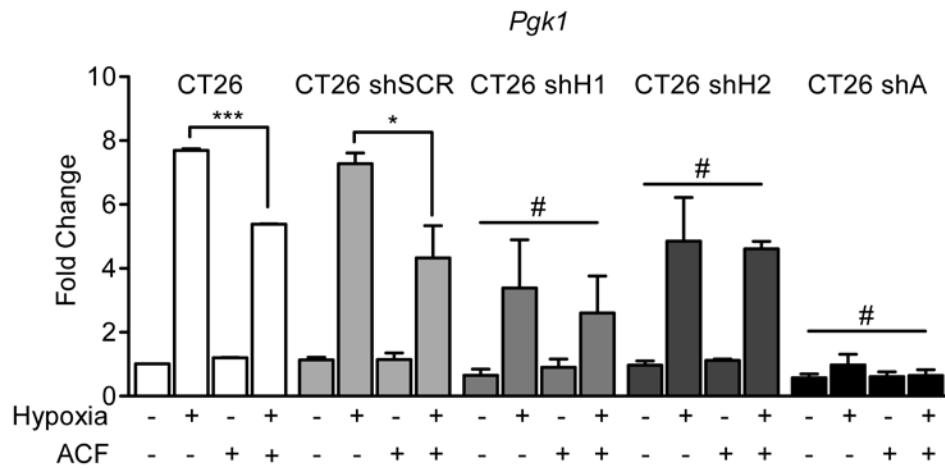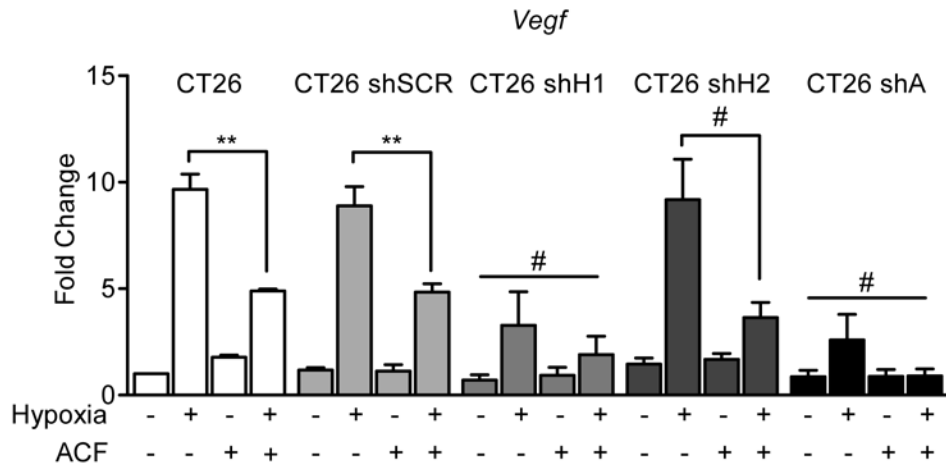
Vegf

Figure 21. Genetic HIF inhibition limits ACF efficacy. (A) *Pgk1* and (B) *Vegf* expression levels in control, shSCR, shHIF-1α, shHIF-2α, or shARNT CT26 cells when cultured under normoxia or hypoxia in the presence or absence of ACF. (mean ± SEM, PBS: $n = 3$, ACF: $n = 3$, # $p > 0.05$, * $p < 0.05$, ** $p < 0.01$)
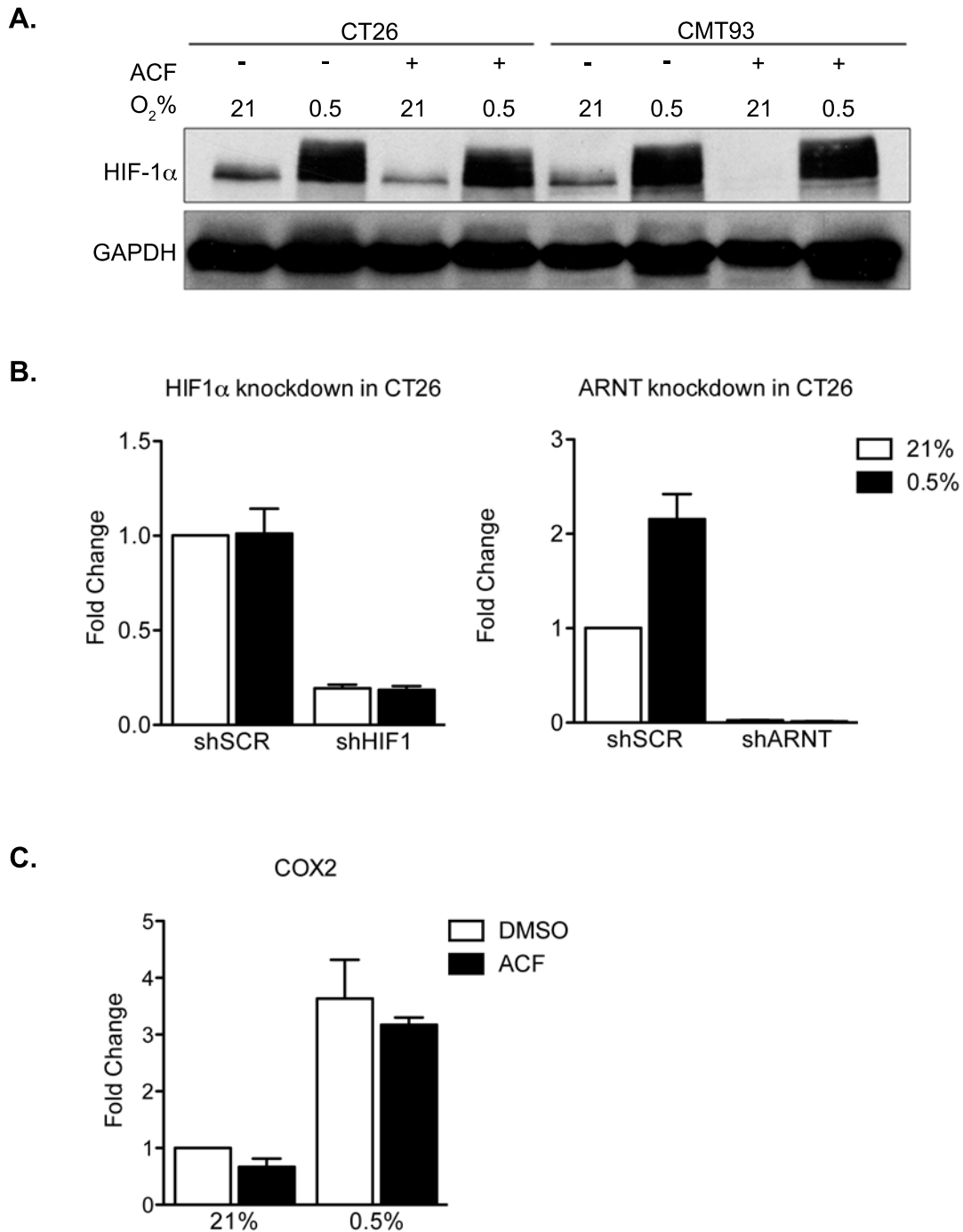
Figure 22. Total HIF levels are unchanged with ACF treatment. (A) Immunoblot for HIF-1α in both CMT93 and CT26 cells under normoxia (21% O_2) and hypoxia (0.5% O_2) with or without ACF treatment. (B) Expression of *Hif1α* or *Arnt* in respective shRNA knockdowns in CT26 cell line. (C) *Cox2* RNA expression under normoxia and hypoxia with addition of acriflavine. (mean ± SEM, PBS: n = 3, ACF: n = 3, # p > 0.05, * p < 0.05, ** p < 0.01)

κB response element (NRE) luciferase reporter assay in either cell line (Figure 20B). Importantly, in the absence of HIF-1α or HIF-2α in CT26 cells, ACF treatment resulted in a modest decrease in *Vegf* or *Pgk1* expression, while in the absence of ARNT, no further reduction in HIF target gene expression was observed (Figure 21A-B, Figure 22B). These findings reinforce the notion that ACF is acting through the HIF transcriptional pathway.

Acriflavine slows allograft tumor growth, dependent on HIF-α/ARNT activity. To further investigate the HIF specificity of ACF treatment, we employed a lentiviral shRNA construct to inhibit ARNT expression, and thus HIF-1α– and HIF-2α–mediated responses, in CT26 cells (Figure 23A). GFP-expressing CT26 cells transduced with either control (shSCR) or ARNT-specific (shARNT) lentiviruses were injected into the left and right flank, respectively, of syngeneic Balb/C mice (Figure 25B). Mice then received daily i.p. injection of PBS or ACF for three weeks. Over the course of treatment, shSCR tumors in mice receiving PBS grew significantly larger than shSCR tumors in mice administered ACF (Figure 23B). Interestingly, there was minimal effect on growth rate in shARNT tumors as a result of ACF treatment, and shSCR tumors in mice treated with ACF grew at a similar rate as the shARNT tumors. The fact that shARNT tumors in mice receiving ACF were nearly identical in size to shARNT tumors in PBS-treated mice, strongly suggests that ACF is primarily targeting the HIF pathway. Moreover, an appreciable decrease in tumor weight was observed in ACF-treated shSCR tumors; however, there was no difference in the weight of shARNT tumors (Figure 23C). These observations indicate that a majority of the anti-tumorigenic effects of ACF are directly related to HIF inhibition. RNA analysis of FACS-sorted tumor cells (based on GFP+ staining) confirmed that suppression of ARNT expression was
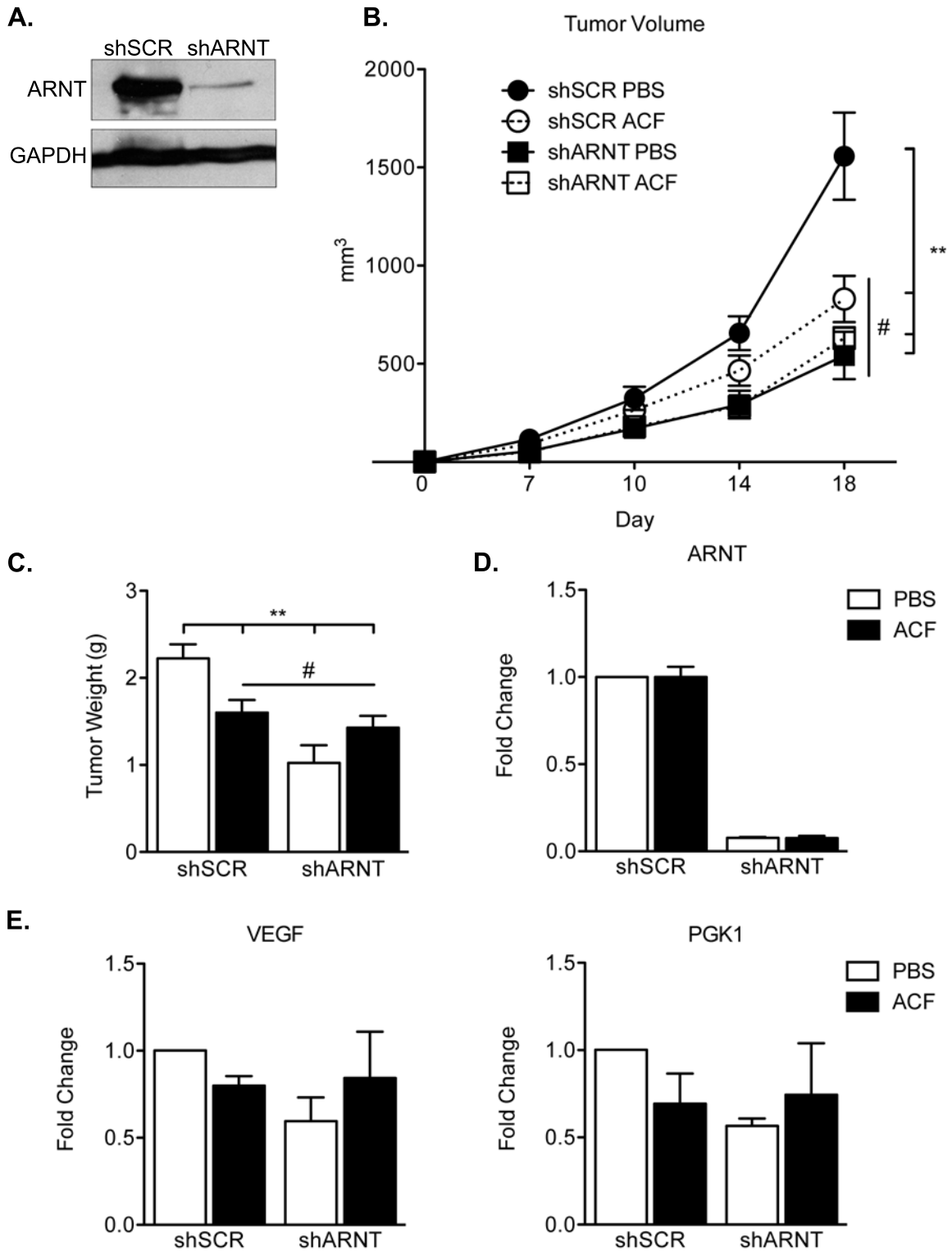
Figure 23. Acriflavine slows allograft tumor growth, dependent on HIF-α/ARNT activity. (A) Immunoblot of ARNT protein in shSCR and shARNT cells respectively. (B) Tumor volume changes in each cohort of mice over time – measured with calipers every three days. Two mice in the PBS cohort had to be euthanized on day 18 due to advanced disease. (C) Average tumor weight of each cohort at culmination of experiment. (D) Relative ARNT expression in FACS sorted CT26 cells. (E) *Vegf* and *Pgk1* RNA expression in isolated tumor cells from each treatment cohort. (mean ± SEM, n = 5 for each group, * p < 0.05, ** p < 0.01)

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A.
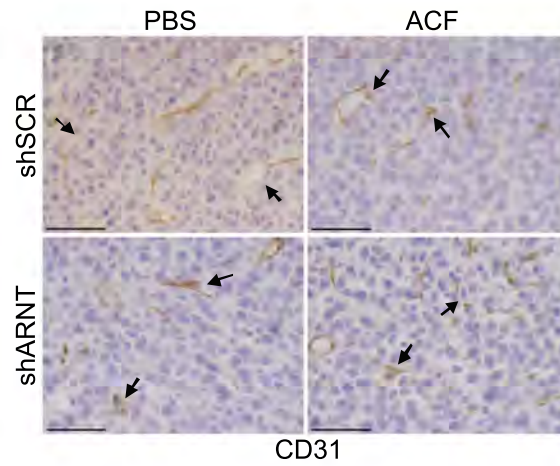
B.
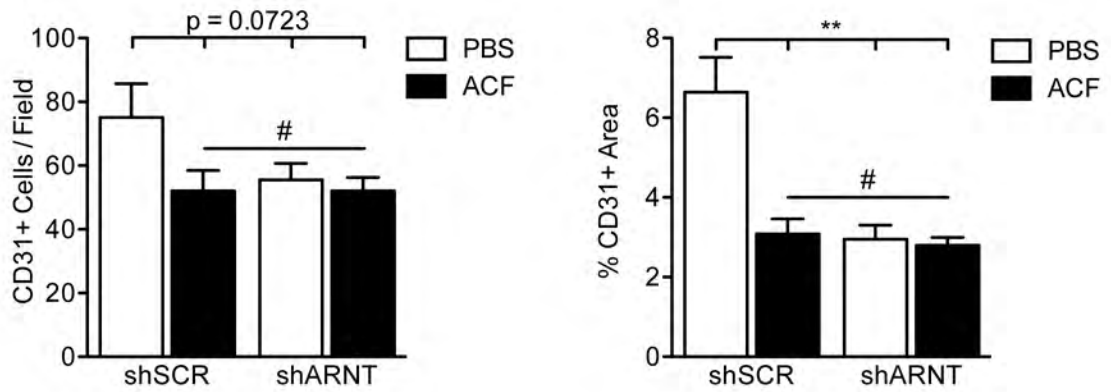
Figure 24. Allograft tumors treated with ACF exhibit decreased vascularity. (A) CD31 staining of shSCR and shARNT tumors treated with PBS and ACF respectively with (B) quantification of vessel number and size of. (mean ± SEM, n = 5 for each group, * p < 0.05, ** p < 0.01)

maintained in shARNT cells throughout the experiment (Figure 23D). Similarly, the expression of canonical HIF targets *Vegf* and *Pgk* was reduced in shSCR tumors from ACF-treated mice compared to controls (although they do not achieve statistical significance), and no additional decrease was observed in shARNT tumors treated with ACF (Figure 23E). Immunohistochemical staining of subcutaneous tumors revealed little inflammatory infiltration as evidenced by a lack of CD68 staining (data not shown), demonstrating that the bulk of each tumor was composed of CT26 cells, as suggested by relatively prolific GFP staining (Figure 25B). Of note, reduced CD31+ positive cells were detected in sections from ACF-treated shSCR tumors, similar to values in PBS- and ACF-treated shARNT tumors (Figure 24A-B). The decrease in vascularity was not as pronounced as in the autochthonous CAC model, which exhibited dramatically higher numbers of infiltrating macrophages. TAMs are known to influence tumor angiogenesis (Crowther et al. 2001; Dirkx et al. 2006; Murdoch et al. 2008). As such, although ACF treatment has clear HIF-dependent effects in transformed colorectal cell lines, its anti-tumorigenic properties may be magnified by the changes in TAM activity in the setting of an inflammation-driven tumor model.

DISCUSSION

HIFs are important transcription factors involved in cellular adaptation to low O_2, a common feature of solid tumors, and thus represent attractive potential therapeutic targets (Semenza 2007; Onnis et al. 2009). Additionally, many tumors exhibit extensive leukocytic infiltration – especially those occurring as a result of chronic inflammation (Balkwill et al. 2001). Therefore, therapies designed to target specific features of the tumor microenvironment may be impacted by naturally occurring O_2 gradients, as well as hypoxic adaptations in both tumor parenchyma and stroma, including recruited

A.
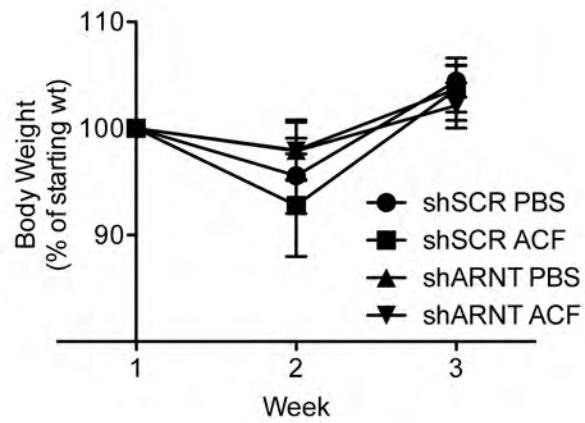
B.
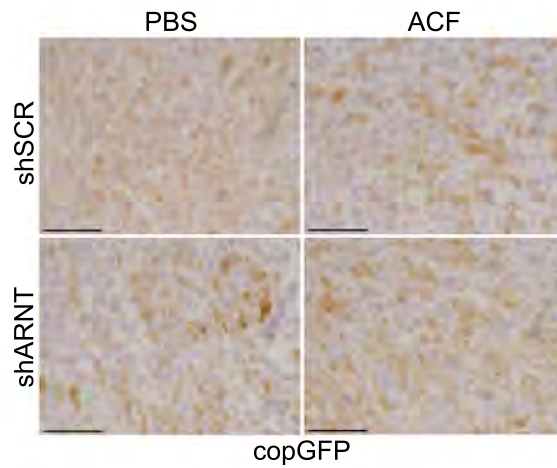
Figure 25. Additional analysis of allograft tumors. (A) Change in weight over time with ACF treatment or PBS treatment in mice with subcutaneous shSCR or shARNT syngeneic tumors. (B) copGFP immunostaining in subcutaneous tumors resulting from shSCR or shARNT CT26 cell injection in mice treated with either PBS or ACF – images at 400x. (mean ± SEM, *n* = 5 for each group)

inflammatory cells. The HIFs function in hypoxic responses of both tumor compartments, making HIF inhibition in tumor cells, TAMs, or both likely to mitigate tumor progression. One known HIF inhibitor, digoxin, is currently in phase II clinical trials for breast cancer (http://clinicaltrials.gov/). Importantly, acriflavine (ACF) is an example of a HIF inhibitor that has already been proven safe in patients for up to 5 months, with very few side effects (Wainwright 2001). Although previous reports demonstrated HIF inhibition by ACF in xenograft and orthotopic models (Lee et al. 2009; Wong et al. 2012), both used severe combined immunodeficiency (SCID) mice. This study represents the first time the effects of ACF-mediated HIF inhibition have been explored in fully immunocompetent mice, mimicking the complexity of HIF activity in tumor microenvironments within an autochthonous setting. Despite the utility of xenograft (or allograft) tumor models, they typically cannot recapitulate the cellular complexity and natural history of autochthonous tumors in immunocompetent hosts, and treatments that eradicate xenograft tumors have often proved ineffective in patients. Similarly, the use of tissue- or cell type-specific genetic deletion can provide important insights into the role of specific genes in tumor initiation and progression, but may be formally distinct from using pharmacological compounds to target a particular molecular target that is expressed in extant tumors.

The work described here investigates ACF treatment of autochthonous tumors, and suggests that pharmacological HIF inhibition in multiple cell types, including epithelial and innate immune cells, reduces tumor growth and progression. Mice treated with ACF consistently developed fewer and smaller colonic lesions with a marked decrease in vascularity and number of recruited macrophages. We demonstrated that ACF acts largely on HIF-dependent responses in macrophages, without effect on the NF-κB pathway. Because HIFs have been shown to be important in multiple components of the tumor microenvironment, inhibiting HIF activity in any single cell population may effect tumor progression with increased efficacy observed when multiple

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compartments are targeted simultaneously (Keith et al. 2011). Previous studies have demonstrated the importance of HIFs in TAMs. We have now shown that ACF limits macrophage infiltration and signaling in the tumor microenvironment in a HIF-dependent manner. A likely mechanism for the reduced macrophage infiltration detected in ACF-treated mice is decreased hypoxic induction of M-CSFR expression – a finding similar to loss of HIF-2α in these cells (Imtiyaz et al. 2010). There may also be HIF-dependent effects on resident macrophages of the colon as a result of ACF-treatment, contributing to reduced tumor progression. ACF exhibits clear effects on multiple colorectal cancer cell lines *in vitro* and *in vivo* in HIF-dependent mechanisms, and is very likely acting on the tumor parenchyma. In future work, it will be important to employ Cre-mediated recombination to delete ARNT, and thereby both HIF-1α and HIF-2α activity, in both colonic epithelial cells and macrophages, to assess the effects of pan-HIF ablation during tumor initiation and progression.

These observations are clinically relevant, as increasingly specific HIF inhibitors will likely have a more significant effect on tumor progression. Whereas our work has focused extensively on the HIF-dependent effects of ACF treatment on the tumor microenvironment, it is possible that ACF has effects that are partially independent of HIF transcriptional activity (Wainwright 2001; Hassan et al. 2011; Lim et al. 2012). However, as transcription factors are effectively targeted for cancer therapeutics in the future, HIF inhibition in the tumor microenvironment by a safe, naturally occurring compound, in the setting of inflammation-driven cancer, represents an important finding. Targeting HIFs may be a viable therapeutic strategy in a myriad of cancers, as the data collectively indicate HIF inhibition can slow advancement of established tumors. Finally, the observations of HIF inhibition in both colorectal cancer cells and recruited macrophages provide insight into the usefulness of future genetic models for studying effects of HIF activity in the setting of inflammation-driven.

Chapter Three

Complete loss of hypoxic response in macrophages alters in vivo response to acute, chronic, and tumor-associated inflammation

SUMMARY

Inflammatory lesions and solid tumors contain regions of varying oxygen (O_2) levels and are comprised of complex, highly heterogeneous cell populations. Infiltrating leukocytes can be identified within hypoxic regions of both solid tumors and sites of inflammation and are known to influence disease progression. In particular, tumor-associated macrophages (TAMs) have been implicated in promoting tumorigenesis, often as a result of chronic inflammation. Hypoxia-driven inflammatory signaling and macrophage infiltration clearly enhance tumor progression. Several studies have implicated the oxygen-sensitive Hypoxia Inducible Factor (HIF) transcriptional regulators in controlling TAM gene transcription in hypoxic tumors, with different adaptive functions regulated by the closely related subunits HIF-1α and HIF-2α. We show, for the first time, complete loss of HIF activity, through a myeloid specific deletion (LysMCre) of the HIF constitutive binding partner ARNT, results in severe macrophage defects.

Efficient ARNT deletion results in significant down-regulation of multiple canonical HIF targets, with no impact on the aryl-hydrocarbon receptor (AhR) signaling pathway. Interestingly, ARNT-deficient macrophages were less responsive to M1 and M2 polarizing stimuli, particularly when cultured under hypoxic conditions. In an acute model of skin inflammation, *LysMCre;Arnt*$^{fl/fl}$ mice exhibited decreased edema and inflammatory infiltrate compared to littermate controls. Similarly, in a model of colitis-associated colon cancer, *LysMCre;Arnt*$^{fl/fl}$ mice exhibited lower stage disease and

decreased tumor inflammation. These results suggest that loss of all HIF activity significantly alters macrophage phenotypes and activity under hypoxia in the setting of acute, chronic, and tumor-associated inflammation. Lastly, as pharmacologic HIF inhibitors progress, targeting the hypoxic response in macrophages may prove beneficial in a variety of inflammation-associated diseases.

INTRODUCTION

Inflammation is a biologic response to harmful stimuli, either infection or tissue injury, in an effort to restore tissue homeostasis (Barton 2008; Medzhitov 2008). Macrophages are members of host innate immunity and are characterized by immense plasticity and diversity in phenotype and function (Lewis et al. 2006). Of note, macrophages found within regions of intense inflammation as well as the tumor microenvironment are subjected to levels of low oxygen (O_2) known as hypoxia (Lewis et al. 1999; Eltzschig et al. 2011). Hypoxia may result from decreased O_2 availability secondary to decreased perfusion as a result of thrombosis, trauma, and edema. Similarly, increased metabolic activity of both foreign pathogens and recruited leukocytes can also contribute to hypoxic conditions observed within the inflammatory microenvironment (Karhausen et al. 2005; Nizet et al. 2009). As such, the hypoxic response under inflammatory conditions is a necessary step to restore tissue homeostasis. Inappropriate activation of resident and recruited macrophages will influence inflammation resolution and may eventually result in tumor-promoting properties.

Macrophages are known to exhibit adaptive changes in gene expression under low O_2 and do so, in large part, through the Hypoxia Inducible Factors (HIFs) (Cramer et al. 2003; Murdoch et al. 2005; Fang et al. 2009; Imtiyaz et al. 2010). The HIFs are comprised of an oxygen-sensitive alpha subunit and a constitutively expressed beta subunit. Under normal oxygen tensions the alpha subunit is hydroxylated by prolyl hydroxylases (PHDs) and targeted for proteasomal degradation by the E3 ubiquitin ligase von Hippel Lindau factor (VHL) (Keith et al. 2007; Keith et al. 2011). Although there are currently three identified HIF-α subunits (HIF-1α, HIF-2α, and HIF-3α), HIF-1α and HIF-2α are the most well characterized (Majmundar et al. 2010). Whereas HIF-1α is

expressed ubiquitously, HIF-2α has a far more restricted tissue expression profile (Mucaj et al. 2012; Shay et al. 2012). However, both are expressed – with partially overlapping and partially distinct roles – in macrophages (Burke et al. 2002; Burke et al. 2003; Cramer et al. 2003; Murdoch et al. 2005; Fang et al. 2009; Mastrogiannaki et al. 2009; Doedens et al. 2010; Imtiyaz et al. 2010; Takeda et al. 2010). Investigating the macrophage response to hypoxia is therefore a complex but necessary undertaking to better understand physiologic and pathologic consequences of macrophage HIF activity.

Although they exhibit any number of intermediate phenotypes *in vivo*, *in vitro* extremes in macrophage polarization can be characterized by M1 (classical) activation or M2 (alternative) activation. Whereas M1 activation corresponds with inflammation and infection, M2 activation is more closely linked with wound healing and tissue repair. As such, these two extremes in macrophage polarization have been adopted to study macrophage responses *in vitro*. HIF-1α has been previously identified as particularly important in macrophage roles in acute inflammation, glycolytic adaptations to hypoxia and M1 stimuli, and suppressing T cell activation (Cramer et al. 2003; Peyssonnaux et al. 2007; Fang et al. 2009; Doedens et al. 2010; Takeda et al. 2010). Indeed, macrophages that lack HIF-1α expression have decreased cellular ATP. Recently the importance of HIF-1α signaling in controlling metabolic flux of macrophages has come to light. Lipopolysaccharide (LPS), a characteristic feature of gram-negative bacteria, strongly polarizes macrophages towards an M1 phenotype. A consequence of this reaction is an increase in glycolysis, an accumulation of the TCA intermediate succinate, and stabilization of HIF-1α leading to increased secretion of IL-1β (Tannahill et al. 2013).

HIF-2α has also been implicated in acute inflammation, however there is no known role in hypoxia-mediated metabolic changes (Imtiyaz et al. 2010). Whereas HIF-1α expression appears to be upregulated in macrophages stimulated by classic

inflammatory signals, HIF-2α may play a larger role in the immunosuppressive effects of TAMs (Takeda et al. 2010). Although both HIF-1α and HIF-2α are expressed in macrophages, elevated expression of HIF-2α in TAMs corresponds directly with clinical severity of many different human cancers (Talks et al. 2000; Hu et al. 2003; Hu et al. 2007; Qing et al. 2009). HIF-2α has also been shown to be instrumental in M-CSF-mediated macrophage recruitment and appears to be a major player in expression changes that occur in tumor-associated macrophages – especially those found in inflammation driven cancers.

Until now, both HIF-1α and HIF-2α have been studied in isolation in the setting of myeloid responses to hypoxia. Although of obvious importance, studying the effects of the loss of a single HIF-α subunit will not, on its own, elucidate the major role of hypoxia and HIF-mediated signaling in macrophages. We have employed Cre-lox technology and have taken advantage of their common binding partner HIF-1β/ARNT to limit HIF transcriptional effects in macrophages. Here we efficiently inhibit ARNT expression in macrophages and severely limit HIF transcriptional roles in macrophages to better understand the complex role of the response to hypoxia in settings of acute, chronic, and tumor-associated inflammation.

<div align="center">MATERIALS AND METHODS</div>

Autochthonous colorectal cancer model

Generation of *LysMCre* and *Arnt*^{fl/fl} mice has been previously discussed (Tomita et al. 2000; Stadtfeld et al. 2005). *LysMCre;Arnt* mice were created by crossing *LysMCre* mice (obtained from Jackson Laboratories) to *Arnt*^{fl/fl} mice on a mostly C57BL/6 background. For induction of autochthonous colorectal tumors, mice received a single intraperitoneal (i.p.) injection of 12.5mg/kg azoxymethane (AOM) at 8 weeks of age

followed by 3 cycles of 1.8-2% dextran sulfate sodium (DSS) in their drinking water (cycle 1: 1.8% DSS for 5 days, cycle 2: 2% DSS for 7 days, cycle 3: 2% DSS 5 days) with two weeks of regular water between each cycle for autochthonous induction of colitis-associated colon cancer. The laboratory animal program is accredited by the American Association for Accreditation of Laboratory Animal Care. Animal health, well-being, and comfort were monitored constantly by certified veterinary staff. Every effort to minimize discomfort, stress, pain, and injury to the mice and the mice was maintained in accordance with the Animal Welfare Act and the DHHS Guide for the care and use of laboratory animals. These procedures were performed according to the protocols reviewed by the Institutional Animal Care and Use Committee (IACUC).

TPA Model of Acute Inflammation

8-10 week-old male mice were anesthetized with isoflurane according to protocol and received 10μl of acetone on either side of left ear (20μl total) and 10μl of 0.125 μg/μl phorbol 12-myristate 13-acetate (TPA) on either side of right ear (20μl total). 24 hours later, mice were euthanized. Ear thickness was measured using calipers, punch biopsy performed, and equivalent areas/sizes of tissue weighed. Ear tissue was then fixed in 4% paraformaldehyde and paraffin embedded for H&E and immunohistochemistry.

Bone marrow-derived macrophages

Macrophages were isolated from littermate *LysMCre;Arnt $^{fl/+}$* or *LysMCre;Arnt$^{fl/fl}$* mice by removing the long bones and flushing the marrow followed by red blood cell lysis. BMDMs were cultured in DMEM containing 20% Hyclone serum, 30% LCM, 1% L-glutamine, 1% Anti-Anti, and 0.1% beta-mercaptoethanol and stimulated with 5 ng/ml LPS (Sigma L3024) and 20 ng/ml IFN-γ (R&D 485-MI) for M1 polarization or for M2 polarization IL-4 (R&D Systems 404-ML-010) and IL-13 (R&D Systems 413-ML-005).

For hypoxia induction, BMDMs were cultured under normoxia (21% O_2) or hypoxia (0.5% O_2, 1.5% O_2, or 3% O_2).

RT-qPCR

RNA was isolated from tumor tissue or cells using the RNAeasy minikit (Qiagen #74106). RNA concentration was quantified using the Nanodrop with equal amounts of mRNA used for reverse transcription to cDNA using the High-Capacity RNA-to-cDNA kit (ABI #4387406). Expression was determined by quantitative PCR of synthesized cDNA using the Applied Biosystems 7900HT system and $\Delta\Delta$CT program settings. Target cDNA amplification was measured using the following TaqMan primers: *Vegf* (Mm00437304_m1), *Il1β* (Mm00434228_m1), *Il6* (Mm00446190_m1), *iNos* (Mm00440502_m1), *Glut1* (Mm00441480_m1), *Tnfα* (Mm00443258_m1), *Il12* (Mm00434165_m1), *Tgfβ* (Mm00441724_m1), *Il10* (Mm00439616_m1), *Adm* (Mm00437438_g1), *Arg1* (Mm00475988_m1), *Fizz1* (Mm00445109_m1), *Ym1* (Mm00657889_mH), *Fn1* (Mm01256734_m1), *Cxcl1* (Mm04207460_m1), *Pgk1* (Mm00435617_m1), *Arnt* (Mm00507836_m1) and *Hprt* (Mm01318743_m1). Results were analyzed with HPRT as an endogenous control.

Immunostaining and Imaging

Immunohistochemistry was performed using enzymatic Avidin-Biotin Complex (ABC)-diaminobenzidine (DAB) staining (Vector Labs) with hematoxylin used for counterstaining of nuclei. Stained sections were visualized using an Olympus IX81 microscope. CD68 1:100 (Abcam ab955) used according to instructions (Vector PK-2200), CD31 1:50 (Abcam ab28364), Ki67 (Novocastra NCL-Ki67-MM1) used according to instructions (Vector PK-2200), TUNEL staining done according to instructions

(Millipore ApopTag S7111), and copGFP staining performed using anti-TurboGFP antibody (Evrogen AB514). Staining was quantified using ImageJ software.

Immunoblot assays

Whole cell extracts were isolated in SDS/Tris pH 7.6 lysis buffer. Subcellular fractionation was performed as previously described (Pan et al. 2004). Protein was quantified using BCA and equal protein amounts were run on an 8% or 10% SDS-PAGE gel, transferred to nitrocellulose, and probed with the following antibodies: HIF-1α 1:1000 (Cayman 10006421), ARNT 1:1000 (Cell Signaling #5537), GAPDH 1:1000 (Cell Signaling #2118), NF-κB (Cell Signaling #3034), p-NF-κB (Cell Signaling #3039), IκBα (Cell Signaling #4814) p-IκBα (Cell Signaling #2859), M-CSFR (Cell Signaling #3152). Representative western blots from multiple independent experiments are presented.

Cytokine Array

Cytokine Array analysis was performed on pooled supernatants from 10 separate mice for each group (5 female, 5 male). $1x10^6$ cells/ml were plated in 100μl in a 96 well plate and cultured for 24 hours at 21% or 0.5% O_2 in the presence or absence of stimuli. Pooled supernatants were then incubated with nitrocellulose membranes spotted with individual antibodies specific for a variety of cytokines according to manufacturer instructions (R&D Systems ARY006).

ELISAs

$1x10^6$ cells/ml were plated in 100μl in a 96 well plate and cultured for 24 hours at 21% or 0.5% O_2 in the presence or absence of stimuli. Supernatant was collected and used to measure nitrite levels according to manufacturer instructions (R&D Quanitkine)

VEGF (MMV00), IL-1β (MLB00C), IL-1α (MLA00), CXCL1 (MKC00B), IL-6 (M6000B), IL-10 (M1000), IL-12 (M1270), IFNg (MIF00).

Griess Assay

1×10^6 cells/ml were plated in 100μl in a 96 well plate and cultured for 24 hours at 21% or 0.5% O_2 in the presence or absence of stimuli. Supernatant was collected and used to measure nitrite levels according to manufacturer instructions (Promega G2930). Excess lysate was used in BCA assay to adjust nitrite levels to total protein per sample.

Arginase Activity Assay

3.5×10^6 cells were plated on 10cm dishes and cultured for 24 hours at 21% or 0.5% O_2 in the presence or absence of stimuli. Cell lysates were collected and incubated with a known amount of arginine according to manufacturer instructions (Abnova KA1609). Excess lysate was used in BCA assay to adjust arginase activity units to total protein per sample.

ATP Production

1×10^6 cells/ml were plated in 100μl in a 96 well plate and cultured for 24 hours at 21% or 0.5% O_2 in the presence or absence of stimuli. ATP levels were then measured according to manufacturer protocol (Perkin Elmer ATPlite™ Luminescence Assay Kit #6016941). Luminescence was measured on a plate reader. Cells were either counted in parallel or excess lysate was used in BCA assay to adjust ATP levels to total protein per sample.

Flow Cytometry and Sorting

Hip and long bones were isolated from mice. Bones were grossly dissected, crushed using a mortar and pestle, and run through a 70μm cell-strainer to generate a single cell suspension. Live cells were run on a BD LSR II. For progenitor studies, cells were stained with DAPI, FITC-CD3, FITC-CD4, FITC-CD8, FITC-B220, FITC-Ter-119, FITC-CD19, FITC-IgM, FITC-IL7Rα, FITC-Gr1, FITC-Sca-1 (Ly6A/E), PE-Cy7-c-kit, PE-CD34, APC-Cy7-FcγII/III (CD16/32). For cell surface markers, cells were stained with FITC-MHCII and APC-CD86 and run on a BD FACSCalibur. Acquired data was analyzed using FlowJo software.

Statistical Analysis

Unless otherwise indicated, data is shown as mean ± SEM. GraphPad Prism software was used to conduct statistical analyses and graph data. Unless otherwise indicated, unpaired 2-tailed Student's *t* test was performed to evaluate statistical differences between control and experimental groups. In situations where more than two groups were compared, a one-way Anova was used followed by post-test Tukey analysis. Significance is demonstrated by "#" indicating $p > 0.05$, "*" representing $p < 0.05$, "**" representing $0.001 < p < 0.01$, and "***" representing $p < 0.001$.

RESULTS

Generation of myeloid-specific ARNT-null mice. To facilitate characterization of complete loss of HIF activity through loss of the constitutive binding partner ARNT, we crossed mice carrying the floxed *Arnt* allele to LysM-Cre mice (Tomita et al. 2000; Stadtfeld et al. 2005). Myeloid-derived cells isolated from mice bearing the mutant genotype (*LysMCre;Arnt^{fl/fl}*) were designated *Arnt^{Δ/Δ}*, whereas myeloid-derived cells isolated from mice bearing the control genotype (*LysMCre;Arnt^{fl/+}*) were designated

A.

ARNT Δ/+   ARNT Δ/Δ

ARNT

GAPDH

B.

21%   0.5%

+/+   Δ/+   Δ/Δ   +/+   Δ/+   Δ/Δ

ARNT

GAPDH

C.

LysMCre;Arnt^fl/+   *LysMCre;Arnt^fl/fl*

T MΦ T MΦ   T MΦ T MΦ

2-lox
WT

1-lox

D.

LysMCre;Arnt^fl/+   *LysMCre;Arnt^fl/fl*

c-kit

Comp-FITC-A, Comp-PE-Cy7-A subset
1.94%

Lin/Sca-1/IL-7Rα

c-kit

Comp-FITC-A, Comp-PE-Cy7-A subset
1.83%

Lin/Sca-1/IL-7Rα

FcγII/III

cd34+-fcghi
33.0%
cd34+-fcgrlo
19.1%
cd34- fcgrlo
35.4%

CD34

FcγII/III

cd34+-fcghi
34.2%
cd34+-fcgrlo
17.0%
cd34- fcgrlo
34.5%

CD34

Myeloid Progenitors

% Total Viable Cells

0.8
0.6
0.4
0.2
0.0

GMP   CMP

□ *LysMCre;Arnt^fl/+*
■ *LysMCre;Arnt^fl/fl*

Figure 26. Efficient ARNT deletion without an impact on myeloid development. (A) Immunoblot of lysates generated from BMDMs derived from *LysMCre;Arnt^fl/+* or *LysMCre;Arnt^fl/fl* mice respectively. Littermates are shown with GAPDH as loading control. (B) Immunoblot of lysates generated from wildtype, *LysMCre;Arnt^fl/+*, or *LysMCre;Arnt^fl/fl* mice with GAPDH as a loading control. (C) PCR based genotyping of tail (T) or macrophage (MΦ) DNA from *LysMCre;Arnt^fl/+*, or *LysMCre;Arnt^fl/fl* mice. 1 lox indicates recombination. (D) Representative FACS plot of progenitor markers with quantification to the right.

$Arnt^{\Delta/+}$. Littermates were used for all experiments. Immunoblotting of lysates obtained from bone marrow-derived macrophages (BMDMs) confirms efficient loss of ARNT (Figure 26A). When $Arnt^{\Delta/+}$ and $Arnt^{\Delta/\Delta}$ macrophages are directly compared to $Arnt^{+/+}$ macrophages, there is an apparent intermediate phenotype observed in $Arnt^{\Delta/+}$ macrophages (Figure 26B). As such, $Arnt^{\Delta/+}$ macrophages appear to express less of the ARNT protein than do wild-type counterparts and likely exhibit an intermediate phenotype between $Arnt^{+/+}$ and $Arnt^{\Delta/\Delta}$ macrophages. This intermediate phenotype likely minimizes differences between control and experimental groups however we decided the most appropriate controls were heterozygous littermate mice. Furthermore, genotyping of both tail and macrophage DNA reveals formation of a 1-lox band in macrophages indicating successful cre-driven recombination (Figure 26C).

Loss of ARNT does not impact myeloid development. Because ARNT is a ubiquitously and constitutively expressed protein, loss of expression could impact maturation or differentiation, especially in hematopoietic cells (Krock et al. 2013). Although loss of ARNT has been previously implicated in stem cell differentiation, the Lysozyme M promoter controlling cre recombinase expression is most highly expressed in mature macrophages (Cross et al. 1988; Clausen et al. 1999). To investigate the effect of ARNT deficiency on myeloid differentiation, bone marrow from $LysMCre;Arnt^{fl/fl}$ and $LysMCre;Arnt^{fl/+}$ mice (littermate controls) was isolated and subjected to FACS analysis of known progenitor and lineage markers (Figure 26D). We investigated multiple populations including the common myeloid progenitor (CMP) and granulocyte/macrophage progenitor (GMP) based on known cell surface markers (Akashi et al. 2000). Loss of ARNT had no impact on percent progenitors of either CMP (0.237% ± 0.007% for $LysMCre;Arnt^{fl/+}$ and 0.216% ± 0.023% for $LysMCre;Arnt^{fl/fl}$ mice) or GMP (0.559% ± 0.039% for $LysMCre;Arnt^{fl/+}$ and 0.451% ± 0.063% for

LysMCre;Arnt^{fl/fl} mice) groups based on FACS analysis (Figure 26D). The effects of ARNT on myeloid function are thus largely confined to mature cell types.

Little change in ARNT expression observed in neutrophils or dendritic cells isolated from *LysMCre;Arnt^{fl/fl}* mice. Lysozyme M Cre has been previously shown to have high recombination efficiency in granulocytes and limited recombination in dendritic cells (Cross et al. 1988; Clausen et al. 1999). Because of this fact, it was imperative to determine the effect of ARNT loss on other members of the myeloid lineage – namely granulocytes and dendritic cells. Importantly, HIF-1α and HIF-2α have been implicated in physiologic responses of neutrophils and dendritic cells experiencing hypoxia (Kohler et al. 2012; Thompson et al. 2013).

To investigate ARNT loss in granulocytes, Cd11b+Gr1+ cells were purified from bone marrow from *LysMCre;Arnt^{fl/fl}* and *LysMCre;Arnt^{fl/+}* mice. Based on FACS analysis, the Gr1+ population was relatively pure for both cohorts (90.4% ± 1.64% for *LysMCre;Arnt^{fl/+}* mice and 91.6% ± 0.79% for *LysMCre;Arnt^{fl/fl}* mice) (Figure 27A). After Gr1+ and dendritic cells were isolated from the bone marrow and spleen respectively, RNA was isolated and expression of various transcripts analyzed. Interestingly, although both neutrophils (Gr1+) and dendritic cells express *Arnt* at greater levels than macrophages from the same mouse, there is no statistically significant loss of expression in *LysMCre;Arnt^{fl/fl}* mice (Figure 27B). Additionally, downstream targets *Vegf* and *Pgk1* were expressed at similar levels in neutrophils and dendritic cells isolated from *LysMCre;Arnt^{fl/fl}* and *LysMCre;Arnt^{fl/+}* mice (Figure 27C). These findings indicate that neither neutrophils nor dendritic cells derived from *LysMCre;Arnt^{fl/fl}* mice exhibit significant defects in the HIF signaling pathway. *Arnt* may be expressed at high enough levels in neutrophils and dendritic cells that even partial cre recombination may have only minimal effects on total *Arnt* levels and downstream HIF targets.
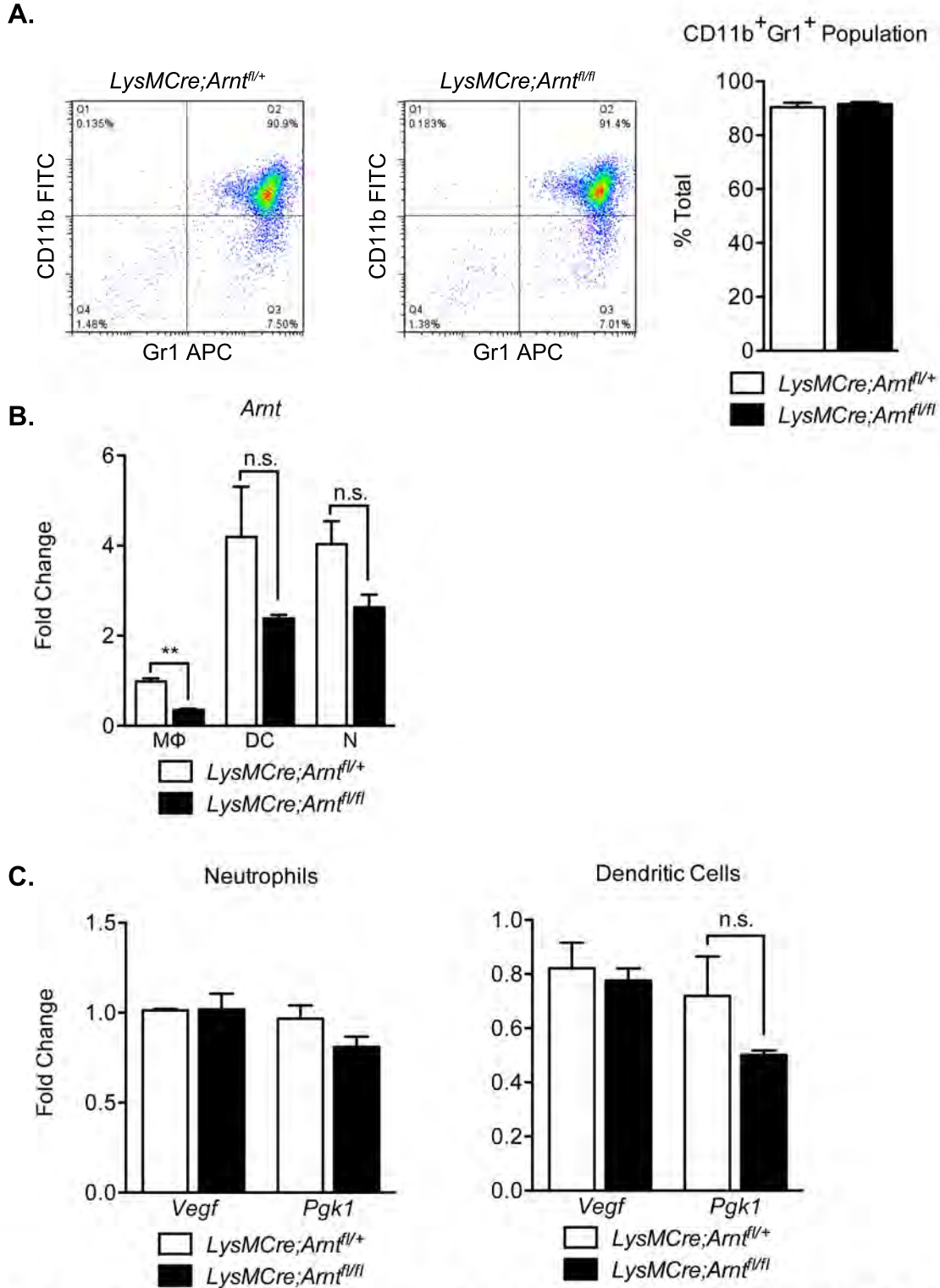
Figure 27. *Arnt* expression is unaffected in neutrophils and dendritic cells. (A) Representative FACS plots and quantification of Cd11b⁺Gr1⁺ populations. (B) Relative *Arnt* expression in macrophages (MΦ), dendritic cells (DC), and neutrophils (N) isolated from *LysMCre;Arnt^{fl/+}* or *LysMCre;Arnt^{fl/fl}* mice. (C) Relative *Pgk1* and *Vegf* expression in neutrophils and dendritic cells isolated from *LysMCre;Arnt^{fl/+}* or *LysMCre;Arnt^{fl/fl}* mice. (mean ± SEM, *n* = 5)

Loss of ARNT corresponds with decreased proliferative capacity in bone marrow-derived macrophages. Loss of HIF-1α has previously been implicated as a major influencing factor in macrophage ATP production (Cramer et al. 2003). As such, loss of ARNT is expected to have a similar impact on ATP generation and macrophage proliferation. *Arnt*$^{\Delta/+}$ and *Arnt*$^{\Delta/\Delta}$ macrophages were seeded and cultured under 21%, 3% or 1.5% O_2 levels for 8 days (Figure 28A-C). No difference was noted at 21% or 3% O_2 (Figure 28A-B) however by day 8, under 1.5% O_2, *Arnt*$^{\Delta/\Delta}$ macrophages had plateaued whereas *Arnt*$^{\Delta/+}$ macrophages had not, indicating an oxygen-dependent proliferative defect when complete loss of ARNT occurs (Figure 28C). Furthermore, when cultured under 0.5% O_2 for 24 hours, *Arnt*$^{\Delta/\Delta}$ macrophages exhibit a 57% decrease in ATP production when compared to littermate *Arnt*$^{\Delta/+}$ macrophages (Figure 28D). Based on our observations, it appears that under various levels of oxygen deprivation, macrophages with complete loss of ARNT expression exhibit moderate to severe defects in proliferative and ATP-producing capacity. These results are not surprising as loss of ARNT mitigates the adaptive changes in metabolism and proliferation that are a direct result of HIF-1α signaling in macrophages.

Canonical HIF targets are expressed at greatly diminished levels in ARNT deficient macrophages. To determine transcriptional effects of ARNT loss in BMDMs, macrophages were cultured for 18 hours under normoxia (21% O_2) or hypoxia (0.5% O_2) in the presence or absence of M1 or M2 polarizing stimuli. Canonical HIF targets *Vegf* (vascular endothelial growth factor), *Pgk1* (phosphoglycerate kinase 1), and *Glut1* (glucose transporter 1) were expressed at greatly diminished levels in *Arnt*$^{\Delta/\Delta}$ macrophages when compared to littermate *Arnt*$^{\Delta/+}$ macrophages (Figure 29A-C). This effect was observed under M1 and M2 polarizing conditions implying HIF transcriptional effects are a large mediator of these phenotypes. Furthermore, VEGF secretion was also diminished in ARNT deficient macrophages (Figure 29D). Interestingly, with loss of
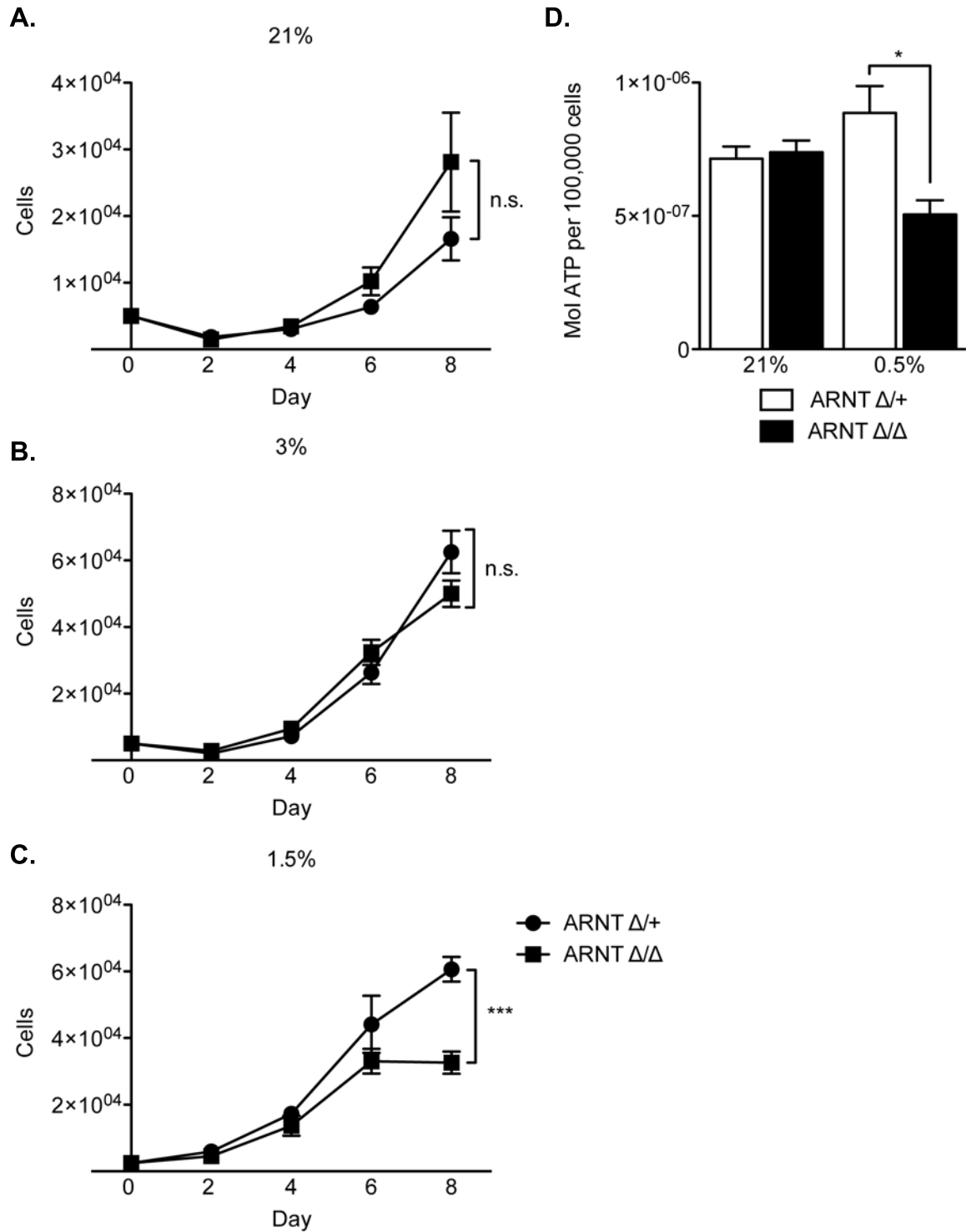
Figure 28. Loss of ARNT results in decreased proliferation and ATP production under hypoxia. (A) Proliferation of $Arnt^{\Delta/\Delta}$ or $Arnt^{\Delta/+}$ macrophages under 21% O_2 (B) 3% O_2 or (C) 1.5% O_2. (D) Relative ATP production from $Arnt^{\Delta/\Delta}$ or $Arnt^{\Delta/+}$ macrophages under 21% O_2 or 0.5% O_2 after 24 hours. (mean ± SEM, n = 5, * $p < 0.05$, *** $p < 0.001$)
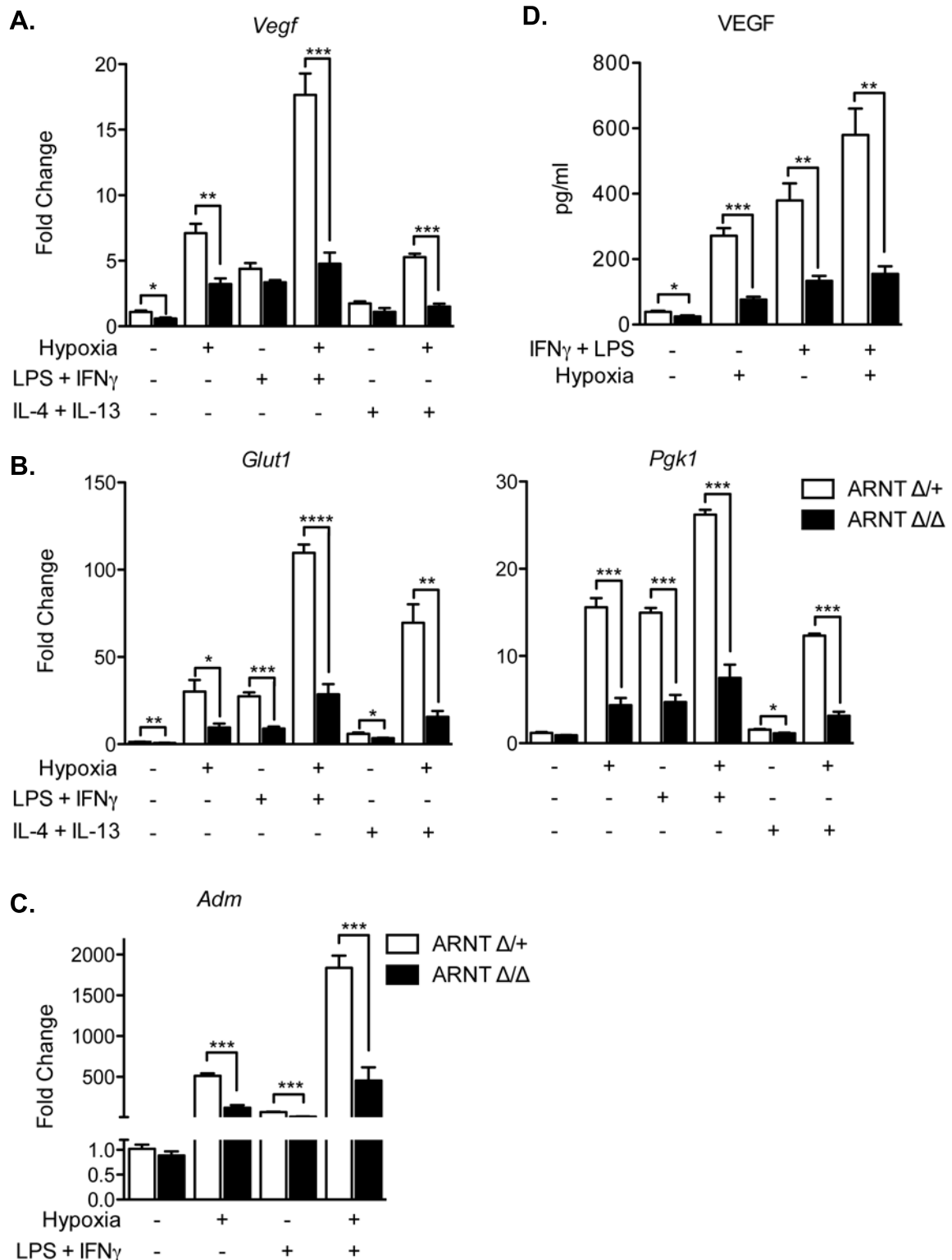
Figure 29. Canonical HIF targets are decreased in _Arnt_$^{\Delta/\Delta}$ macrophages. (A) Relative _Vegf_ (B) _Pgk1_ and _Glut1_ or (C) _Adm_ expression in _Arnt_$^{\Delta/\Delta}$ or _Arnt_$^{\Delta/+}$ macrophages cultured under M1 (LPS + IFNγ) or M2 (IL-4 + IL-13) polarizing conditions in the presence of 21% O_2 or 0.5% O_2. (D) Relative VEGF secretion in _Arnt_$^{\Delta/\Delta}$ or _Arnt_$^{\Delta/+}$ macrophages cultured under M1 (LPS + IFNγ) polarizing conditions in the presence of 21% O_2 or 0.5% O_2. (mean ± SEM, _n_ = 4, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001)

HIF-2α alone, no effect was observed on transcripts encoding glycolytic enzymes (Imtiyaz et al. 2010). These observations are not surprising as ARNT is the binding partner for both HIF-1α and HIF-2α and loss of ARNT should impact both alpha subunits equally.

Loss of ARNT corresponds with varying effects on classical M1 targets. Further analysis of classical M1 transcripts revealed multiple genes such as *iNos* (nitric oxide synthase), *Il1β* (interleukin 1β), *Il10* (interleukin 10), *Adm* (adrenomedullin), and *Tgfβ* (transforming growth factor β) to exhibit decreased transcription with the loss of ARNT (Figure 30A-D). Additionally, decreased *iNos* expression corresponds with decreased nitrite in BMDM-conditioned media as determined by Griess assay (Figure 30E). Interestingly, certain M1 targets increase in the loss of HIF transcriptional regulation. - namely *Il6*, *Tnfα*, and *Il12* (Figure 31A-C). Although counterintuitive, these results may be explained by the opposing roles HIF-1α and HIF-2α appear to have on Th1 and Th2 driven T cell maturation and NO regulation (Doedens et al. 2010; Takeda et al. 2010).

Loss of ARNT results in decreased M2 polarization *in vitro*. Similar to results seen with M1 targets, ARNT deficient macrophages appear to express decreased levels of multiple M2 transcripts including *Arg1* (arginase 1), *Fizz1* (resistin-like molecule alpha), and *Ym1* (chitinase-3 like-3) (Figure 32A-C). Fibronectin 1, a previously identified HIF-2α target, is also decreased in ARNT deficient macrophages under hypoxia alone and in the presence of M2 stimuli (IL-4 and IL-13) (Figure 32D). To test arginase activity in BMDMs, *Arnt*$^{\Delta/+}$ and *Arnt*$^{\Delta/\Delta}$ macrophages were cultured under 21% or 0.5% O2 in the presence or absence of M2 stimuli. Lysates were then collected and incubated with a known amount of arginine. Arginase conversion of arginine to urea and ornithine was measured and quanitified using a colorimetric assay. *Arnt*$^{\Delta/\Delta}$ macrophages exhibited
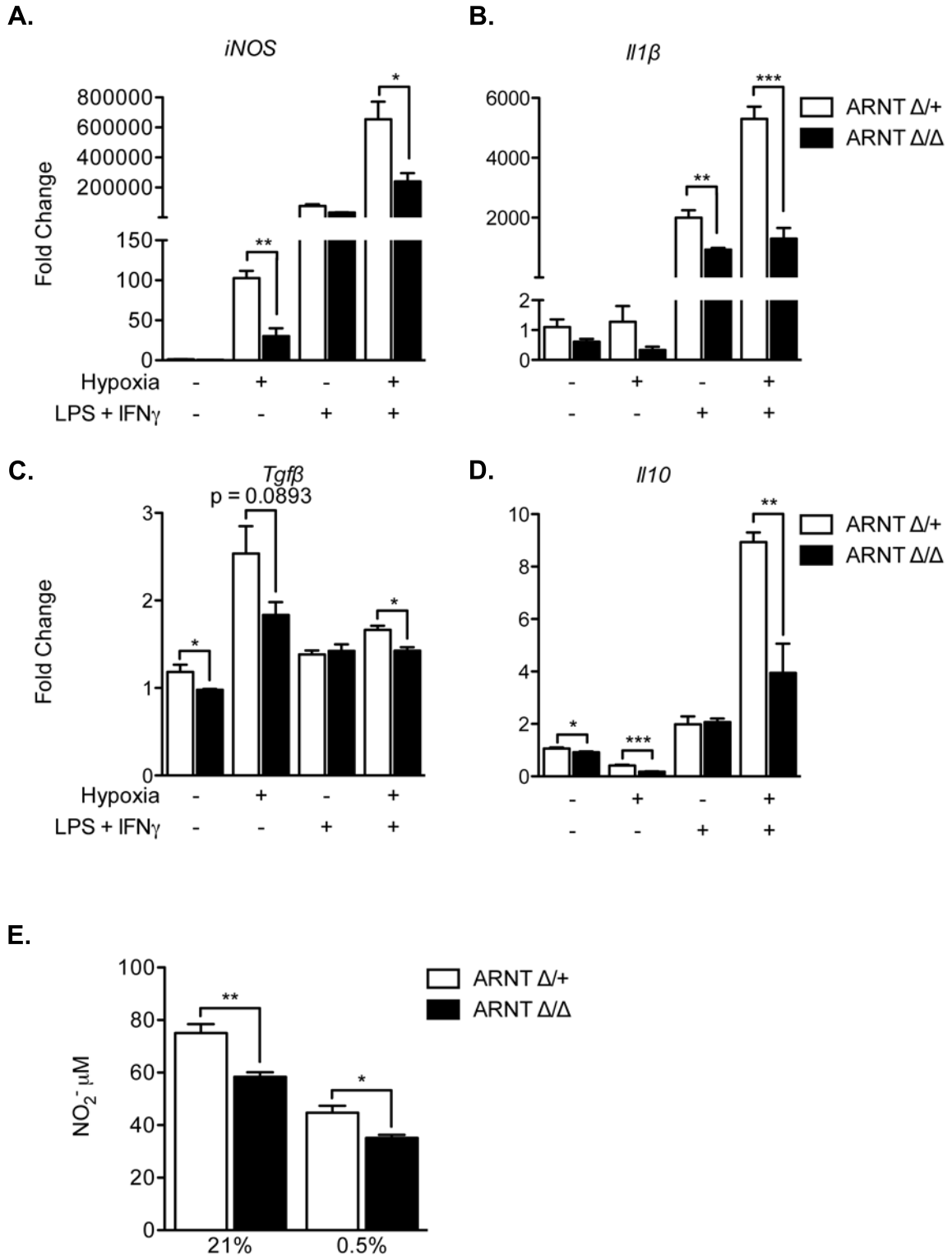
Figure 30. Loss of ARNT corresponds with decreases in multiple classical M1 targets. (A) Relative *iNos* (B) *Il1β* (C) *Tgfβ* or (D) *Il10* expression in $Arnt^{\Delta/\Delta}$ or $Arnt^{\Delta/+}$ macrophages cultured under M1 (LPS + IFNγ) polarizing conditions in the presence of 21% O_2 or 0.5% O_2. (E) Relative NO production from $Arnt^{\Delta/\Delta}$ or $Arnt^{\Delta/+}$ macrophages cultured under M1 (LPS + IFNγ) polarizing conditions in the presence of 21% O_2 or 0.5% O_2 as determined by Griess assay measuring NO_2^- concentration. (mean ± SEM, $n = 4$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)
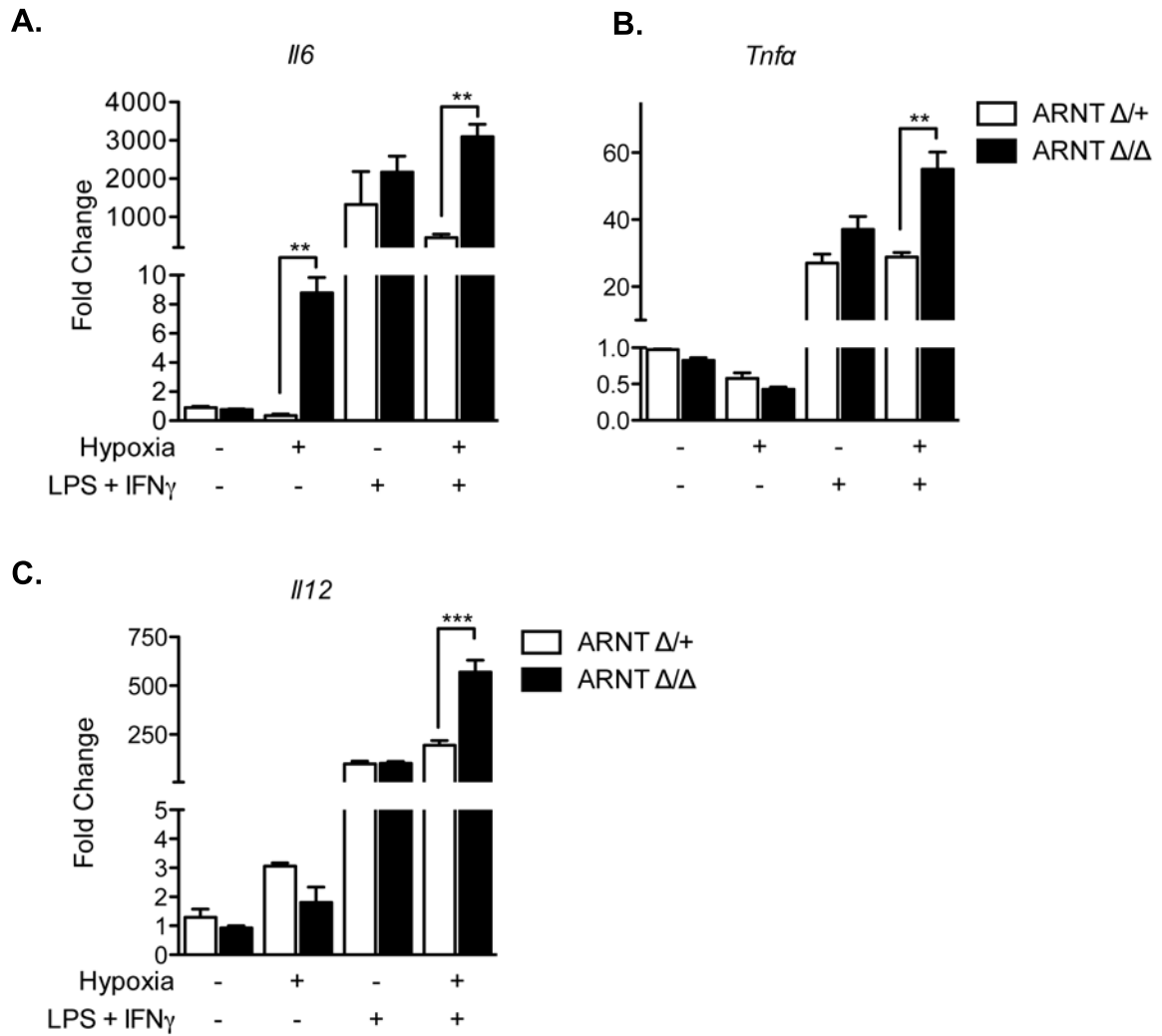
Figure 31. Paradoxical increase in certain pro-inflammatory cytokines in ARNT deficient macrophages. (A) Relative *Il6* (B) *Tnfα* and (C) *Il12* expression in $Arnt^{\Delta/\Delta}$ or $Arnt^{\Delta/+}$ macrophages cultured under M1 (LPS + IFNγ) polarizing conditions in the presence of 21% O_2 or 0.5% O_2. (mean ± SEM, n = 4, ** p < 0.01, *** p < 0.001)
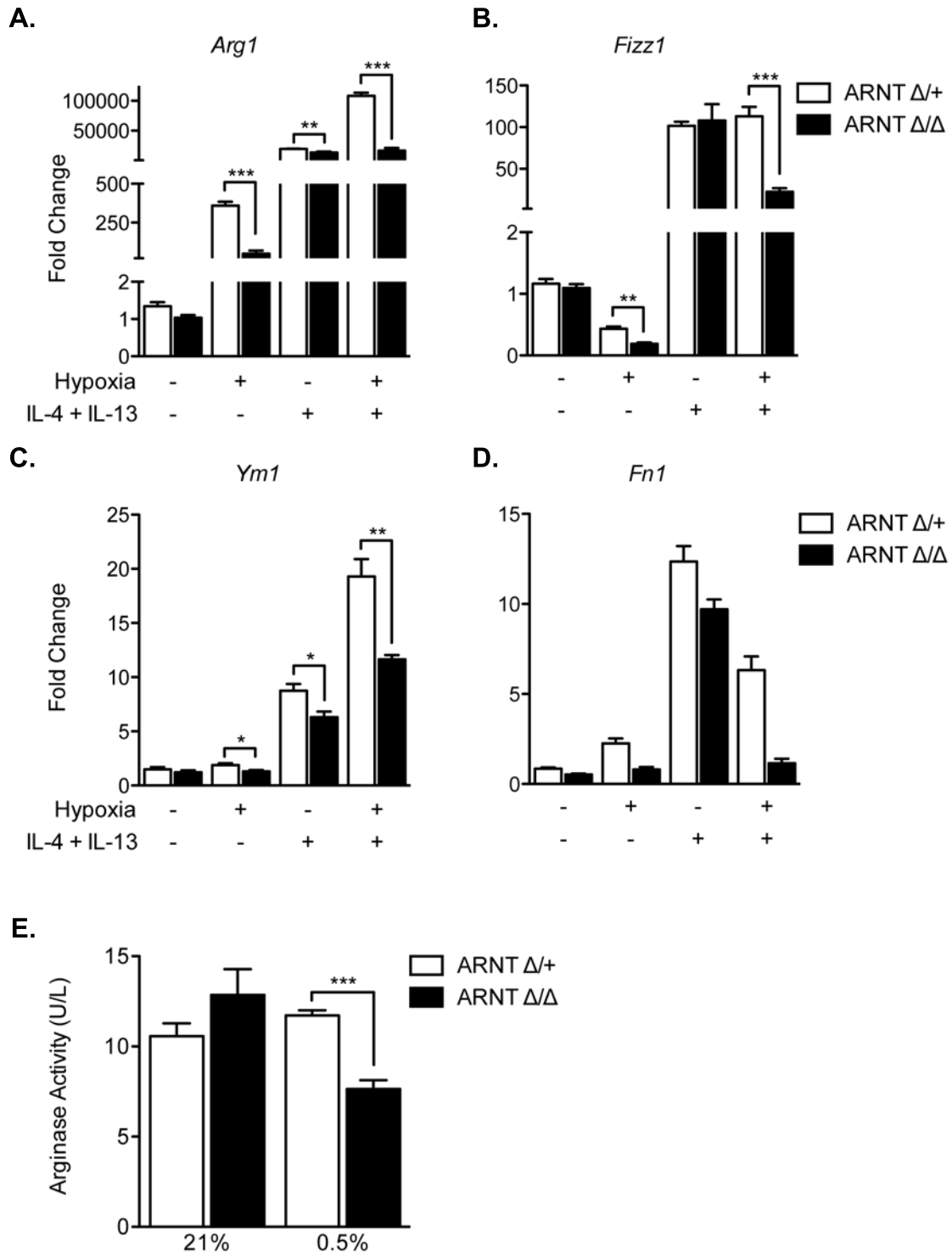
Figure 32. Decreased *in vitro* M2 polarization of ARNT deficient macrophages. (A) Relative *Arg1* (B) *Fizz1* (C) *Ym1* or (D) *Fn1* expression in $Arnt^{\Delta/\Delta}$ or $Arnt^{\Delta/+}$ macrophages cultured under M2 (IL-4 + IL-13) polarizing conditions in the presence of 21% O_2 or 0.5% O_2. (E) Relative arginase activity of $Arnt^{\Delta/\Delta}$ or $Arnt^{\Delta/+}$ macrophages cultured under M2 polarizing conditions in the presence of 21% O_2 or 0.5% O_2 as determined by Arginase activity assay measuring urea production from arginine. (mean ± SEM, *n* = 4, * p < 0.05, ** p < 0.01, *** p < 0.001)

decreased arginase activity when compared to littermate $Arnt^{\Delta/+}$ macrophages (Figure 32D).

Further identification of HIF/ARNT regulated cytokines. In order to identify additional ARNT/HIF-regulated cytokines, we performed a cytokine array. Supernatants from 10 different macrophages (half male half female) were pooled for either $Arnt^{\Delta/\Delta}$ or $Arnt^{\Delta/+}$ macrophages and incubated with nitrocellulose membrane spotted with antibodies for a variety of secreted cytokines (Figure 33A). Under hypoxia, $Arnt^{\Delta/\Delta}$ macrophages secreted/expressed decreased levels of a number of different cytokines and other signaling factors. Of those, some, like VEGF and IL-1β, had been previously identified through Q-PCR and ELISA analysis of macrophage samples. As such, the cytokine array agrees with previously identified targets. Interestingly, IL-1α and CXCL1 were both identified. While both factors have been previously identified by microarray analysis (Fang et al. 2009), we have confirmed both to be regulated at the RNA and protein level by ARNT/HIF transcriptional activity. Further quantitative analysis of IL-1α, IL-1β, and CXCL1 was performed (Figure 33B-C).

Loss of ARNT does not impact Aryl-hydrocarbon receptor (AhR) transcripts. ARNT was first identified as the nuclear transporter for AhR. Loss of ARNT would thus be expected to have some impact on AhR signaling. This is particularly relevant as AhR has recently been identified as a main regulator of intraepithelial lymphocytes in the intestine and mediates interaction with exogenous stimuli (Li et al. 2011). To investigate this, we analyzed macrophage expression of AhR specific targets *Cyp1a1* and *Ugt1a1* (Beischlag et al. 2008). Whereas *Cyp1a1* was not expressed to any degree in BMDMs, *Ugt1a1* was expressed to a modest a degree and exhibited some hypoxic upregulation (Figure 34A-B). Importantly, neither AhR target gene was
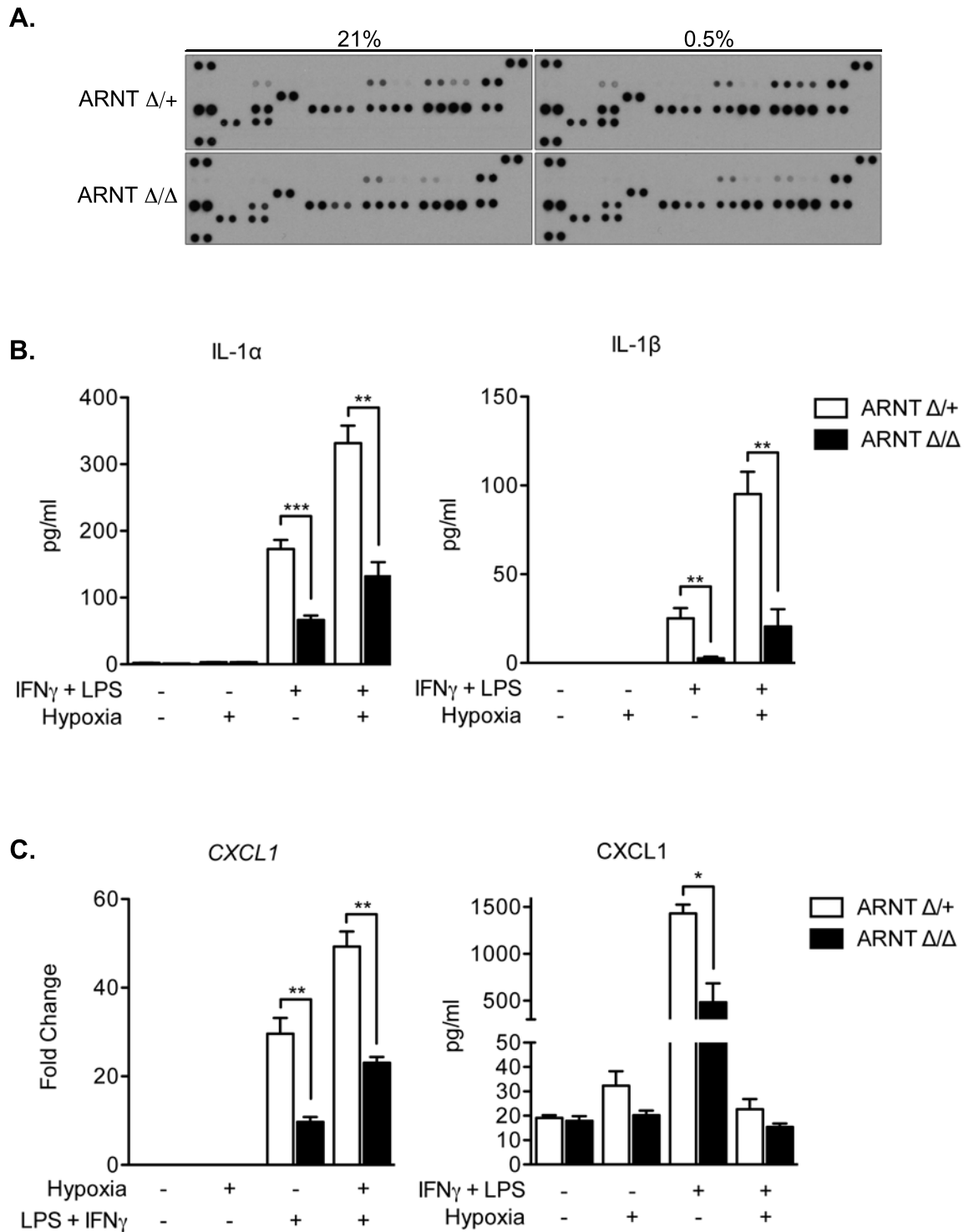
Figure 33. Identification of additional HIF targets through cytokine array. (A) Nitrocellulose-based cytokine array. Pooled supernatants from M1 polarized $Arnt^{\Delta/\Delta}$ or $Arnt^{\Delta/+}$ macrophages in the presence of 21% O_2 or 0.5% O_2. (B) Confirmation of identified targets such as IL-1α or IL-1β by ELISA. (C) Relative expression and secretion of CXCL1 from M1 polarized $Arnt^{\Delta/\Delta}$ or $Arnt^{\Delta/+}$ macrophages in the presence of 21% O_2 or 0.5% O_2. (mean ± SEM, $n = 4$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

A.

Cyp1a1

| IFNγ + LPS | - | - | + | + |
| Hypoxia | - | + | - | + |

B.

Ugt1a1

☐ ARNT Δ/+
■ ARNT Δ/Δ

| IFNγ + LPS | - | - | + | + |
| Hypoxia | - | + | - | + |

Figure 34. ARNT loss does not impact AhR signaling. (A) Relative expression of *Cyp1a1* and (B) *Ugt1a1* in *Arnt*$^{\Delta/\Delta}$ or *Arnt*$^{\Delta/+}$ macrophages cultured under M1 (LPS + IFNγ) polarizing conditions in the presence of 21% O_2 or 0.5% O_2. (mean ± SEM, *n* = 4)

differentially expressed in $Arnt^{\Delta/\Delta}$ or $Arnt^{\Delta/+}$ macrophages implying that the major role for ARNT in these cells is through the HIF signaling pathway and not through AhR signaling.

Decreased invasion of ARNT deficient macrophages towards M-CSF. Previous work has demonstrated the effect of HIF-2α activity on the hypoxic induction of M-CSFR expression in BMDMs (Imtiyaz et al. 2010). Similarly, loss of ARNT results in modest decrease in macrophage recruitment towards M-CSF under low O_2 as demonstrated by M-CSF-mediated invasion of seeded macrophages in a modified Boyden chamber (Figure 35A-B). Because proliferative defects were not observed until day 8 under 1.5% O_2, it is unlikely that ARNT dependent invasion under 0.5% O_2 is secondary to any defects in ATP production or proliferation. Furthermore, this observation lends credence to the notion that HIF-2α signaling is being impacted to a similar degree as HIF-1α.
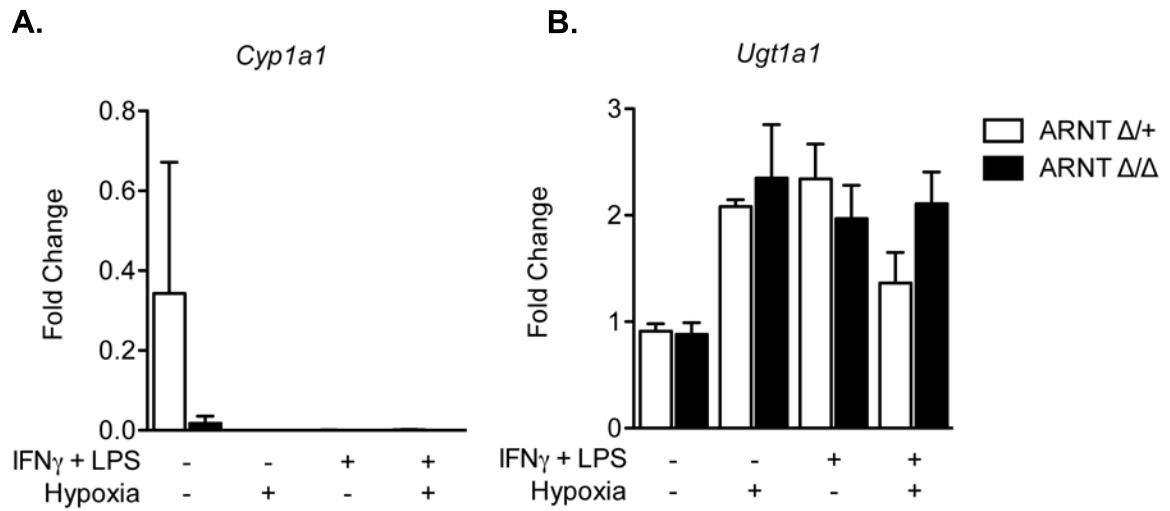
Increased expression of cell surface markers corresponding to antigen presentation in ARNT deficient macrophages. Complete loss of HIF signaling (through loss of ARNT) in macrophages in the presence of M1 stimuli and hypoxic conditions resulted in a counterintuitive change in macrophage expression (based on Q-PCR analysis) of a number of pro-inflammatory cytokines. In order to further investigate any changes in M1 polarization in either $Arnt^{\Delta/\Delta}$ or $Arnt^{\Delta/+}$ macrophages, cell surface expression of markers CD86 and MHC II were analyzed by flow cytometry. Briefly, macrophages were cultured in 21% or 0.5% O_2 in the presence of LPS and IFNγ or left unstimulated. FACS analysis revealed a strong increase in CD86 expression in $Arnt^{\Delta/\Delta}$ macrophages that was not observed in $Arnt^{\Delta/+}$ macrophages (Figure 36A-B). Indeed, $Arnt^{\Delta/\Delta}$ macrophages exhibit a 3-fold increase in CD86 positive cells when compared to littermate controls. Interestingly, the increase does not appear to be altered under hypoxia and no such difference was observed for MHC II expression. These results are

99

A.

B.
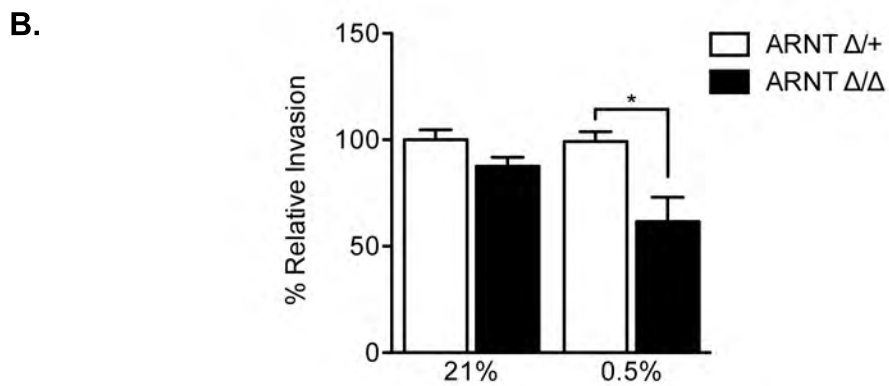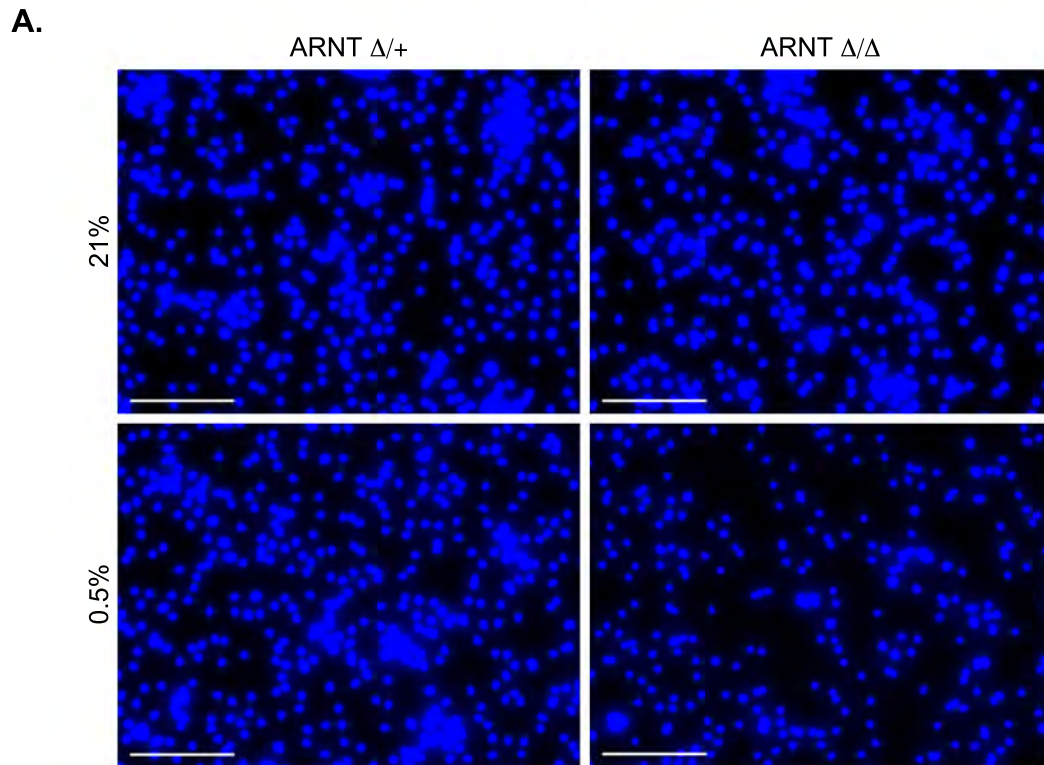
Figure 35. Limited invasion of ARNT deficient macrophages towards chemokine M-CSF. (A) Representative images and (B) quantification of $Arnt^{\Delta/\Delta}$ or $Arnt^{\Delta/+}$ macrophage invasion towards M-CSF under 21% O_2 or 0.5% O_2. (mean ± SEM, $n = 4$, * $p < 0.05$)

A.

21%   0.5%

Count

CD86 APC

MHC II FITC

··········· ARNT Δ/+, LPS + IFNγ
────── ARNT Δ/Δ, LPS + IFNγ
────── ARNT Δ/+, untreated
− − − − ARNT Δ/Δ, untreated

B.

CD86

MHC II

% Parent Population

Hypoxia   −   +   −   +
LPS + IFNγ   −   −   +   +

□ ARNT Δ/+
■ ARNT Δ/Δ

Figure 36. Loss of ARNT impacts expression of specific cell surface markers. (A) Representative FACS plots and (B) quantification of CD86 and MHCII cell surface expression in M1 polarized *Arnt*$^{Δ/Δ}$ or *Arnt*$^{Δ/+}$ macrophages under 21% O_2 or 0.5% O_2. (mean ± SEM, *n* = 4, ** p < 0.01, *** p < 0.001)

consistent however, with the previously identified upregulation of certain pro-inflammatory cytokines that may act to stimulate T cells.

Decreased inflammatory infiltrate in TPA ear model of acute inflammation observed in *LysMCre;Arnt^{fl/fl}* mice. Previous works have implicated both HIF-1α and HIF-2α in acute inflammation (Cramer et al. 2003; Imtiyaz et al. 2010). To model acute inflammation, we employed the well-known TPA model of ear inflammation. Administration of 2-O-tetradecanoylphorbol-13-acetate (TPA) in acetone to the ear of the mice results in an acute inflammatory infiltrate and edema in 24 hours. After 24 hours, mice were euthanized and ear skin was analyzed for edema and leukocytic infiltration. Ear skin that did not receive TPA was used as a control for each mouse. *LysMCre;Arnt^{fl/fl}* mice exhibited far less edema in TPA-treated ears (based on ear thickness measurements from calipers and weighing tissue sections) than did littermate controls. Similarly, based on H&E staining, there was far less leukocytic infiltration in TPA trea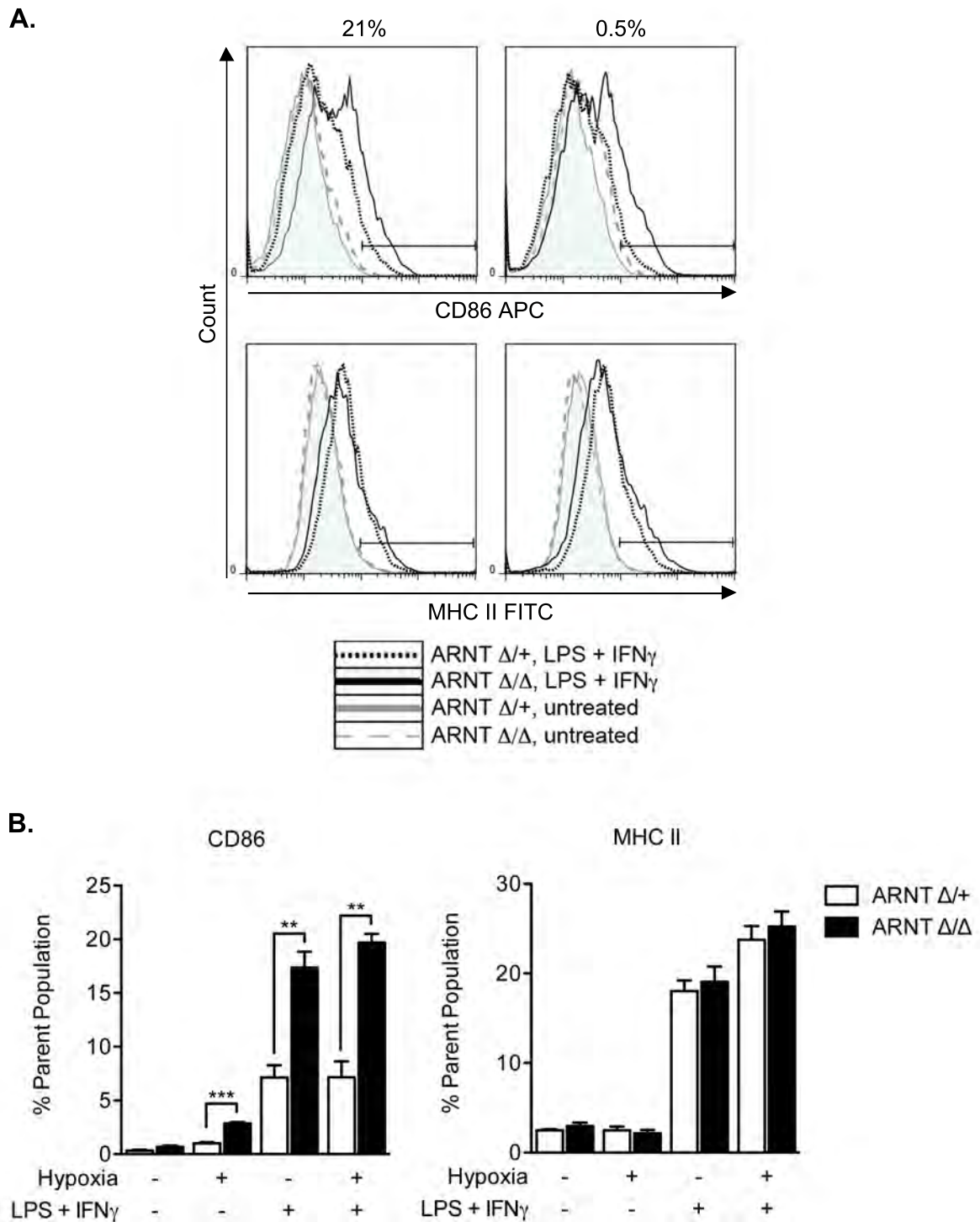ted sections obtained from *LysMCre;Arnt^{fl/fl}* mice than did *LysMCre;Arnt^{fl/+}* littermate controls (Figure 37A-B). Immunohistochemistry staining with a neutrophil-specific antibody revealed that most of the inflammatory infiltrate in control mice are neutrophils however, there are far fewer infiltrating neutrophils in TPA-treated ears from *LysMCre;Arnt^{fl/fl}* mice (Figure 38A-B).

Although LysMCre has previously demonstrated extremely high recombination efficiency in granulocytes, we have not observed a noticeable decrease in ARNT or HIF-target expression in granulocytes isolated from *LysMCre;Arnt^{fl/fl}* mice. As such the decreased neutrophil recruitment seen in the TPA-treated ears of *LysMCre;Arnt^{fl/fl}* mice may be secondary to changes in resident macrophages such as decreased CXCL1 secretion. Resident mast cells and macrophages have been previously shown to be responsible for secreting CXCL1 and recruiting neutrophils in settings of acute inflammation (De Filippo et al. 2013).

A.

B.
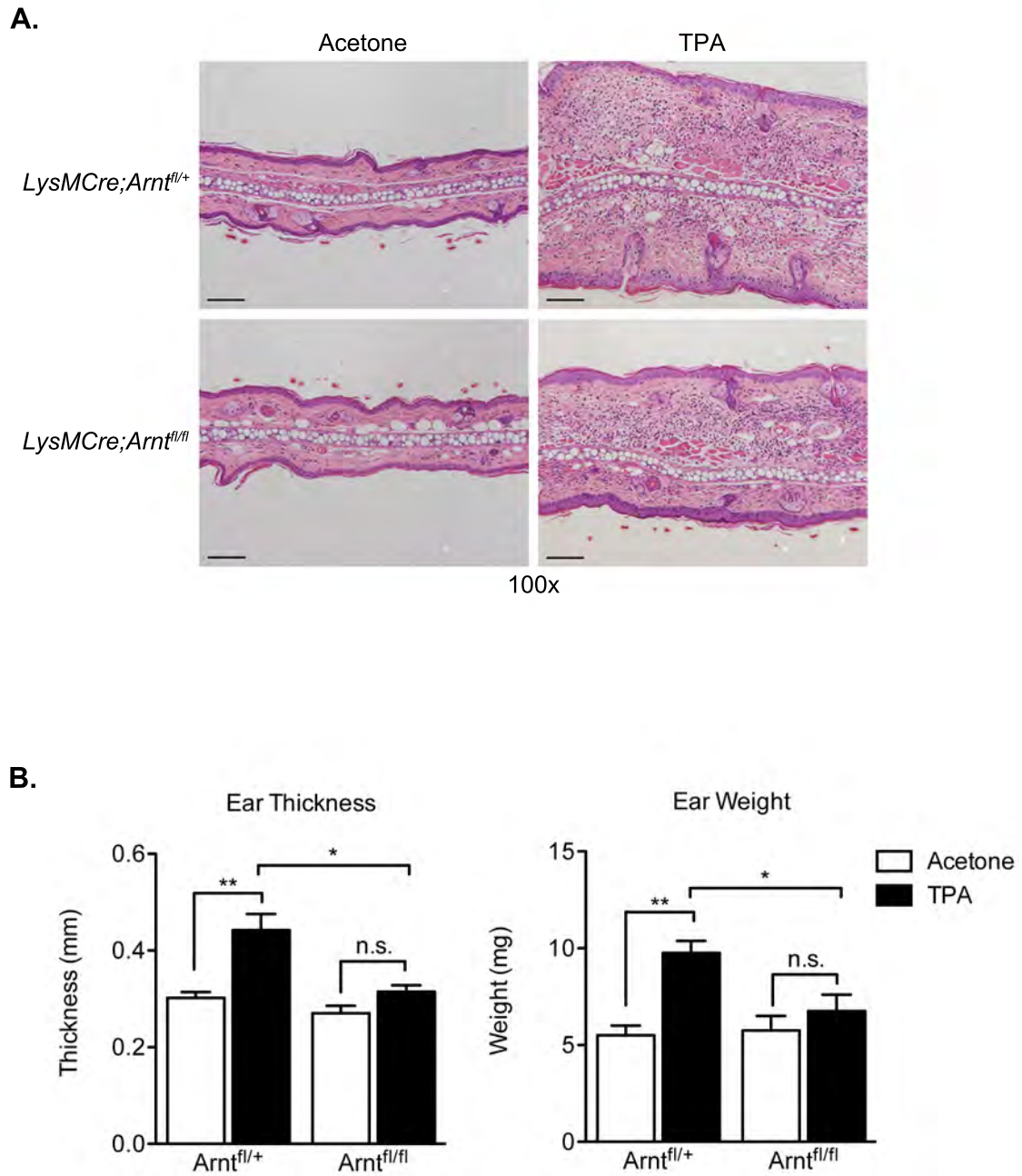
Figure 37. Acute skin inflammation model reveals decreased leukocyte infiltration in ***LysMCre;Arnt*** *fl/fl* **mice.** (A) Representative H&E images of acetone treated and TPA treated ears from *LysMCre;Arnt* *fl/+* or *LysMCre;Arnt* *fl/fl* mice. (B) Quantification of edema through caliper measurement of ear thickness and ear weight. (mean ± SEM, *n* = 4, * p < 0.05, ** p < 0.01)

A.

B.
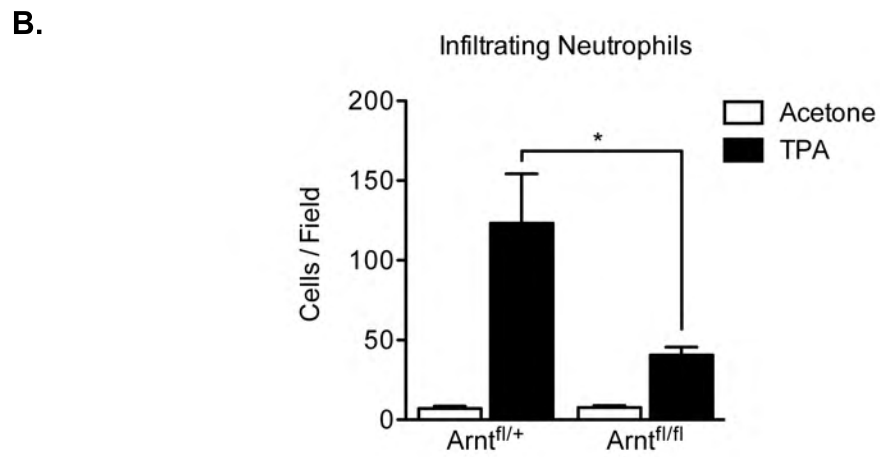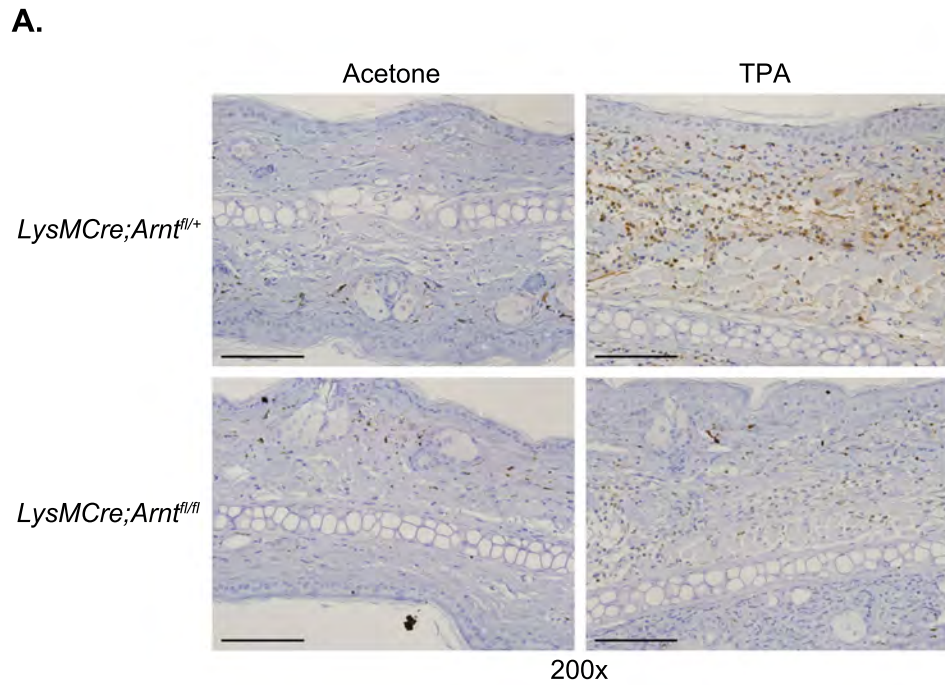
Figure 38. Decreased neutrophil infiltration in TPA treated ears of *LysMCre;Arnt*$^{fl/fl}$ mice. (A) Representative images and (B) quantification of neutrophil infiltration in acetone treated and TPA treated ears from *LysMCre;Arnt*$^{fl/+}$ or *LysMCre;Arnt*$^{fl/fl}$ mice. (mean ± SEM, n = 4, * p < 0.05)

Chronic colitis-induced colon cancer model. To evaluate the importance of macrophage ARNT expression in the setting of chronic inflammation, eight-to-ten week-old *LysMCre;Arnt*^{fl/+} or *LysMCre;Arnt*^{fl/fl} mice were subjected to a single intraperitoneal (i.p.) injection of the pro-carcinogen azoxymethane (AOM), followed by repeated treatments of 1.8-2% dextran sulfate sodium (DSS) to induce autochthonous colitis-associated colon cancer (CAC) (Okayasu et al. 1990; Okayasu et al. 1996; Okayasu et al. 2002) (Figure 39). Surprisingly, at the culmination of the experiment, no difference in gross tumor number was observed between the cohorts (Figure 40A-B). However, after tumor staging was performed on H&E sections, it became apparent that *LysMCre;Arnt*^{fl/fl} mice had a lower percentage of high-grade lesions compared to littermate controls and a trend towards fewer mitotic figures (Figure 41A-B). Similarly, tumor and colon inflammation was also less severe in *LysMCre;Arnt*^{fl/fl} mice than in their *LysMCre;Arnt*^{fl/+} counterparts (Figure 41C). Although these results are surprising, it may be that loss of HIF-1α and HIF-2α signaling pathways results in altered phenotypes of resident macrophages that may, in turn, have unexpected interactions with other arms of the immune system. This could be explained, in part, by the unexpected observations of M1 polarized ARNT-deficient macrophages cultured under 0.5% O_2 *in vitro*.

DISCUSSION

Myeloid cells adapt to low O_2 in a multitude of ways. This study extends findings from works looking at the individual and differential effects of HIF-1α and HIF-2α on macrophage function in settings of hypoxia, inflammation, and tumorigenesis (Cramer et al. 2003; Fang et al. 2009; Imtiyaz et al. 2010; Takeda et al. 2010). Hypoxia alone, or in conjunction with various stimuli, can elicit striking changes in macrophage biology. With loss of the obligate HIF-α binding partner, HIF-1β/ARNT, we observe defects in
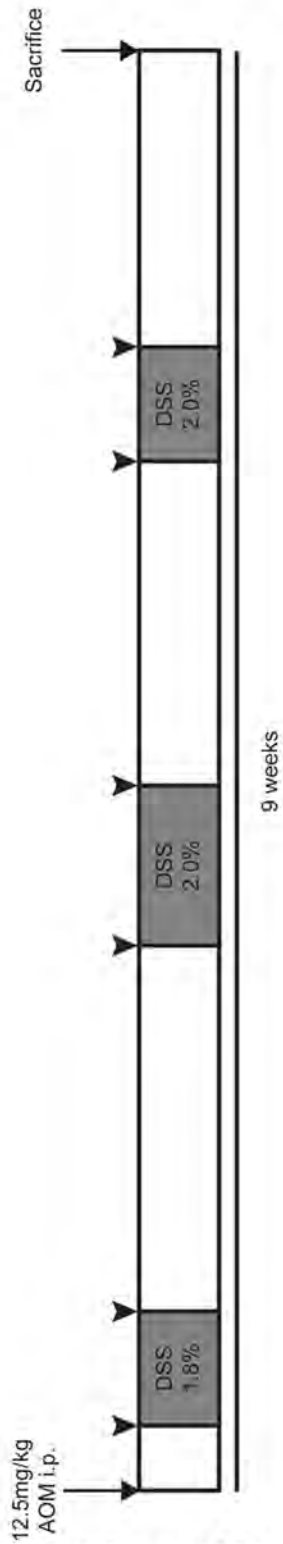
Figure 39. AOM/DSS model of autochthonous CAC development.

A.
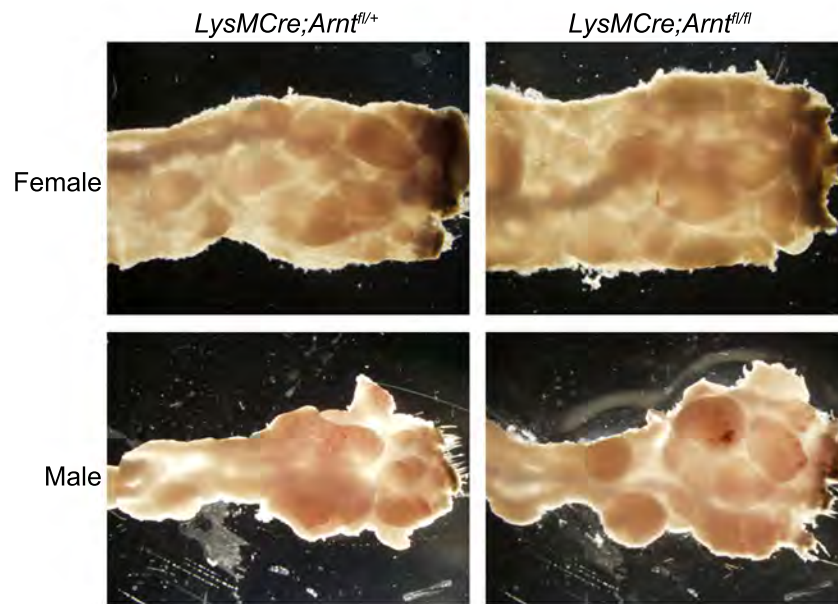
B.
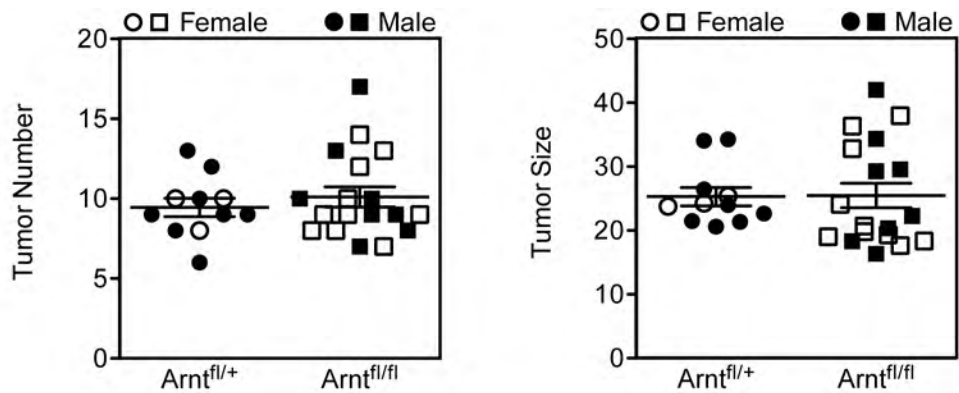
Figure 40. Loss of ARNT expression in macrophages does not impact tumor number or size in CAC model. (A) Representative gross images of female or male *LysMCre;Arnt*^{fl/+} or *LysMCre;Arnt*^{fl/fl} mice with CAC. (B) Tumor number and size measured using calipers. (mean ± SEM, *n* = 11 for *LysMCre;Arnt*^{fl/+}, *n* = 17 for *LysMCre;Arnt*^{fl/fl})

A.

Tumor Stage

B.

C.
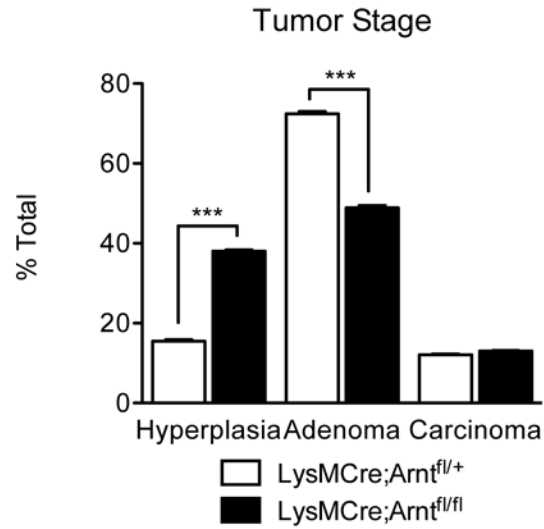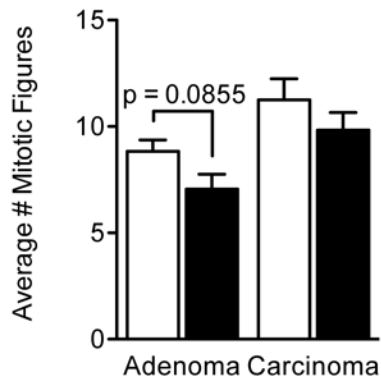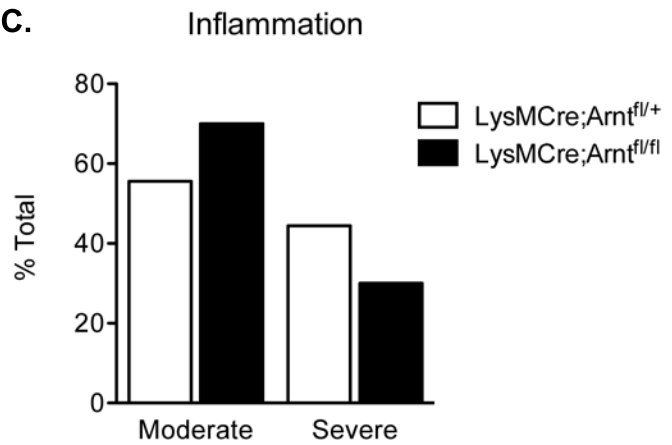
Inflammation

Figure 41. Decreased tumor staging and inflammation in *LysMCre;Arnt*$^{fl/fl}$ mice with CAC. (A) Tumor staging based on H&E slides from each mouse for *LysMCre;Arnt*$^{fl/+}$ or *LysMCre;Arnt*$^{fl/fl}$ cohorts. (B) Average number of mitotic figures for adenomas and carcinomas for either cohort baed on H&E analysis. (C) Relative tumor inflammation scoring for each lesion in either *LysMCre;Arnt*$^{fl/+}$ or *LysMCre;Arnt*$^{fl/fl}$ mice, again based on H&E analysis. (mean ± SEM, *n* = 11 for *LysMCre;Arnt*$^{fl/+}$, *n* = 17 for *LysMCre;Arnt*$^{fl/fl}$)

both traditional HIF-1α and HIF-2α pathways – glycolytic genes that are known HIF-1α targets, such as *Pgk1* and *Glut1*, were severely downregulated in ARNT-deficient macrophages as were more HIF-2α dependent genes such as *Adrenomedullin* (Figure 3A-E). These early findings demonstrate that our novel model of a myeloid-specific ARNT null mouse results in an efficient loss of both HIF-1α and HIF-2α signaling pathways. Additionally, these findings also bring to light the interesting metabolic changes that macrophages likely undergo in the setting of low O_2 and classic inflammatory stimulation. Glycolytic genes such as *Pgk1* and *Glut1* increase drastically under hypoxia alone or under normal oxygen tensions in the presence of LPS and IFNγ. In the setting of combined hypoxia and M1 stimuli – such as that likely encountered in areas of intense inflammation – *Pgk1*, *Glut1*, and *Vegf* transcript levels increase to even greater levels. In certain instances the increase appears almost synergistic as in the case of *Glut1* whereby hypoxia alone results in a 30 fold increase, M1 stimuli results in a 27 fold increase, however the two combined lead to a striking 110 fold increase in transcript levels. These findings also provide evidence that HIFs provide transcriptional regulation under normoxic conditions, especially apparent in the setting of M1 polarization as ARNT deficient macrophages exhibit significant defects in *Pgk1* and *Glut1* transcription under normoxia when stimulated with LPS and IFNγ.

Similar to changes seen in glycolytic enzyme transcription, there are also numerous defects in the transcription of other classic M1 genes in ARNT deficient macrophages (Figure 29). Arginine metabolism is a highly regulated process (Lewis et al. 1999). Under low O_2, arginine can be metabolized with NO produced as a byproduct, through activation of *iNOS*, a downstream target of HIF-1α activation. Alternatively, *Arg1*, a gene regulated by both HIF isoforms, may metabolize arginine to urea and ornithine. Upregulation of either *iNos* or *Arg1* can have important effects on the

inflammatory microenvironment as both deplete L-Arginine from the extracellular space and may therefore limit proliferation of other recruited cells types, including T cells (Bronte et al. 2005). Both enzymes exhibit hypoxic induction that appears to be HIF dependent as under low O2 alone, *iNos* undergoes a 100 fold increase (Figure 4A) and *Arg1* exhibits a nearly 300 fold increase compared to levels under normoxia (Figure 6A). Under M1 polarizing conditions, *iNos* transcript levels also correspond to a net increase in NO production as quantified by Griess assay (Figure 4E). Again, HIF activity appears to not be limited to hypoxia alone as even under 21% O_2, there is a significant decrease in NO production in supernatant isolated from ARNT deficient macrophages. Similarly, under M2 polarizing conditions, *Arg1* transcript levels correspond with Arginase activity as confirmed by a colorimetric assay whereby arginine conversion to urea is quantified (Figure 6E). Again, HIF activity is of obvious importance in arginine metabolism in multiple inflammatory settings.

Interestingly, although many of the observed $Arnt^{\Delta/\Delta}$ phenotypes can be attributed to the loss of HIF-1α, HIF-2α, or both, there were some marked changes in transcription of a number of genes that were unanticipated. The pro-inflammatory signaling cytokines *Il6*, *Tnfα*, and *Il12* were all upregulated in ARNT deficient macrophages cultured under 0.5% O_2 in the presence of LPS and IFNγ (Figure). $Arnt^{\Delta/\Delta}$ macrophages expressed *Il12* and *Tnfα* at 3- and 2-fold higher levels respectively, than did $Arnt^{\Delta/+}$ macrophages. *Il6* is even more striking. Under hypoxia alone, $Arnt^{\Delta/\Delta}$ macrophages expressed *Il6* at 25-fold higher levels than $Arnt^{\Delta/+}$ counterparts. When stimulated with LPS and IFNγ alone, both cohorts exhibit large increases in *Il6* expression. When M1 polarized and cultured at 0.5% O_2, $Arnt^{\Delta/\Delta}$ macrophages expressed *Il6* at nearly 7-fold higher levels than $Arnt^{\Delta/+}$ counterparts. The unexpected dysregulation in certain pro-inflammatory cytokines brings

to light possible counterbalancing and oppositional effects of the individual HIFα subunits.

Acute inflammatory settings are marked first by an infiltration of neutrophils, the "first responders". Neutrophils, like macrophages, are derived from a common myeloid progenitor, and also express the Lysozyme M gene. Certain chemotactic factors are known to recruit neutrophils. One such factor, CXCL1, is upregulated under hypoxia (Fang et al. 2009). Here we show that CXCL1 is HIF/ARNT dependent as loss of ARNT has severe consequences in *Cxcl1* expression and secretion in BMDMs cultured under normoxia or hypoxia in the presence of M1 polarizing stimuli. Recent published work has conclusively demonstrated CXCL1 as a major player in the early stages of neutrophil recruitment towards sites of inflammation (De Filippo et al. 2013). We can then reasonably assume resident macrophages found at sites of low O_2 (such as that found acutely in sites of inflammation and infection), upregulate CXCL1 and facilitate neutrophil recruitment. In this scenario, HIFs act to mediate innate immunity responses to exogenous stimuli, such as low oxygen, found within sites of acute inflammation.

In the absence of HIF-mediated responses, macrophages are likely ineffective at recruiting neutrophils towards sites of acute inflammation. Intriguingly, this corresponds precisely with what we observed in an acute model of ear inflammation. In the setting of TPA-induced inflammation of the ear, *LysMCre;Arnt$^{fl/fl}$* mice exhibited decreased edema and leukocyte infiltration than did corresponding *LysMCre;Arnt$^{fl/+}$* littermates (Figure 13). The vast majority of the infiltrating leukocytes were confirmed, through immunohistochemistry, to be neutrophils (Figure 14). Concomitant with the decrease in edema and leukocyte infiltration, *LysMCre;Arnt$^{fl/fl}$* mice exhibited decreased neutrophil recruitment, based on immunohistochemistry, when compared to littermate controls. Although the regulation of cell recruitment is quite complex, we have identified CXCL1 as a possible chemoattractant that is hypoxia and HIF-regulated and may be responsible

for signaling neutrophil infiltration. In the absence of ARNT/HIF signaling in resident tissue macrophages there is a defective response to hypoxia and inflammation. This is evidenced by a loss of CXCL1 expression and secretion in ARNT deficient macrophages and decreased neutrophil recruitment towards sites of inflammation in *LysMCre;Arnt*^{fl/fl} mice. Although there are likely many other mechanisms involved, low O_2 can result from intense inflammation and the physiologic response to hypoxia, mediated through the HIFs, is a necessary function of the innate immune system to stimulate host immune responses and eventual resolution.

The unexpected changes in cytokine expression observed in M1 polarized *Arnt*^{Δ/Δ} macrophages cultured under 0.5% O_2 may contribute, in part, to the less than striking effect on tumor number and size in the autochthonous model of CAC induction. Previous works have demonstrated decreased tumor number and size in a similar CAC model in *LysMCre;Hif2α*^{fl/fl} mice when compared to littermate controls (Imtiyaz et al. 2010). To date there is no published account of this model using *LysMCre;Hif1α*^{fl/fl} mice however, one may assume a certain amount of redundancy between the two HIFα subunits. Interestingly, in a Polyoma middle T (PyMT) model of breast carcinoma, *LysMCre;Hif1α*^{fl/fl} mice exhibit decreased tumor mass and lower overall stage. Unlike *LysMCre;Hif2α*^{fl/fl} mice however, it was determined that myeloid expression of HIF-1α was tumor-promoting in the PyMT model through T-cell suppression rather than promoting TAM infiltration (Doedens et al. 2010). Therefore, it may be that ARNT-deficient macrophages in the lamina propria of autochthonous CAC lesions exhibit different expression profiles and interact with other arms of the immune system than do *Arnt*^{Δ/+} macrophages in littermate controls.

ARNT deficient macrophages exhibit a variety of expected and unexpected changes in expression and function. Ultimately however, to fully investigate the distinct

differences between macrophage HIF-1α and HIF-2α expression, *Hif1*$\alpha^{\Delta/\Delta}$ macrophages, *Hif2*$\alpha^{\Delta/\Delta}$ macrophages, and *Hif1*$\alpha^{\Delta/\Delta}$*Hif2*$\alpha^{\Delta/\Delta}$ macrophages will need to be compared side-by-side using *in vitro* and *in vivo* assays. These findings identify multiple HIF-mediated pathways in resident and recruited macrophages found within acute and tumor-associated inflammatory states and bring to light the complexity of targeting hypoxic responses in the innate immune system.

Chapter Four

Concluding Remarks

HIFs mediate the hypoxic response in a variety of physiologic and pathologic conditions. As previously described, inflammation often coincides with tissue hypoxia and the innate immune system has evolved to function in a highly specialized manner under low O_2 conditions (Nizet et al. 2009). Hypoxic responses of the innate immune system are a critical element in inflammation. The relationship and crosstalk between HIFs, hypoxia, the innate immune system, and cancer is epitomized in the setting of inflammation-associated cancers. TAMs are recruited to hypoxic regions within the tumor microenvironment where they play a critical role in driving tumor progression. Although there exists a number of redundancies between HIF-1α and HIF-2α, there are also distinct features of each. The overlapping and distinct roles of the HIFα subunits are epitomized in the inflammatory responses of macrophages. This body of work investigates the role of hypoxia and HIF-mediated signaling in both tumor cells and macrophages across the natural history of inflammation-induced cancers. Chapter Two starts at the end and examines the effect of HIF inhibition in tumor parenchyma and stromal cells in extant colitis-associated colon carcinomas. Chapter Three investigates the effect of genetic HIF loss through cre-lox driven ARNT deletion in myeloid derived cells in the earliest stages of acute and chronic inflammatory responses that eventually result in inflammation-associated cancer development. In both cases, it is clear the hypoxic response is necessary for sustained inflammation and tumor progression.

Therapies designed to target specific features of the tumor microenvironment may be impacted by naturally occurring O_2 gradients, as well as hypoxic adaptations in both tumor parenchyma and stroma, including recruited inflammatory cells. The HIFs function in hypoxic responses of both tumor compartments, making HIF inhibition in

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tumor cells, TAMs, or both likely to mitigate tumor progression. Importantly, acriflavine (ACF) is an example of a HIF inhibitor that has already been proven safe in patients for up to 5 months, with very few side effects (Wainwright 2001). The work described in Chapter Two investigates ACF treatment of autochthonous tumors, and suggests that pharmacological HIF inhibition in multiple cell types, including epithelial and innate immune cells, reduces tumor growth and progression. We have now shown that ACF limits macrophage infiltration and signaling in the tumor microenvironment in a HIF-dependent manner. As transcription factors are effectively targeted for cancer therapeutics in the future, HIF inhibition in the tumor microenvironment by a safe, naturally occurring compound, in the setting of inflammation-driven cancer, represents an important finding. Targeting HIFs may be a viable therapeutic strategy in a myriad of cancers, as the data collectively indicate HIF inhibition can slow advancement of established tumors. Finally, the observations of HIF inhibition in both colorectal cancer cells and recruited macrophages provide insight into the usefulness of future genetic models for studying effects of HIF activity in the setting of inflammation-driven.

Myeloid cells adapt to low O_2 in a multitude of ways. The work described in Chapter Three extends findings from works looking at the individual and differential effects of HIF-1α and HIF-2α on macrophage function in settings of hypoxia, inflammation, and tumorigenesis (Cramer et al. 2003; Fang et al. 2009; Imtiyaz et al. 2010; Takeda et al. 2010). Hypoxia alone, or in conjunction with various stimuli, can elicit striking changes in macrophage biology. With loss of the obligate HIF-α binding partner, HIF-1β/ARNT, we observe defects in both traditional HIF-1α and HIF-2α signaling pathways. These early findings demonstrate that our novel model of a myeloid-specific ARNT null mouse results in an efficient loss of both HIF-1α and HIF-2α signaling pathways in cultured macrophages. However, although a slight decrease in ARNT

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expression was observed in ARNT deficient neutrophils and dendritic cells, it was neither statistically significant nor did it result in a decrease in canonical HIF targets such as *Vegf* or *Pgk1*. Because of this, the following will focus primarily on the effects of ARNT and HIF loss in primary macrophages. Interestingly, although many of the observed *Arnt*$^{\Delta/\Delta}$ phenotypes can be attributed to the loss of HIF-1α, HIF-2α, or both, there were some marked changes in transcription of a number of genes that were unanticipated. The unexpected dysregulation in certain pro-inflammatory cytokines brings to light possible counterbalancing and oppositional effects of the individual HIFα subunits.

For the sake of simplicity, macrophages have heretofore been described as occupying one of three states: unpolarized, M1 polarized, and M2 polarized. In reality however, it is likely that resident and recruited macrophages exhibit intermediate phenotypes that comprise various features of these three states. This is particularly relevant when one takes into consideration the complex and varied microenvironment that exists within sites of inflammation and solid tumors.

With this in mind, it is important then that the seemingly disparate roles for HIF-1α and HIF-2α are taken into account. Although previous works neatly describe HIF-1α as the primary hypoxic transcription factor in M1 polarized macrophages and can be influenced by Th1 polarizing cytokines and HIF-2α is the overarching hypoxic regulator in M2 macrophages and can be upregulated in the setting of Th2 polarizing cytokines, it is quite clear that both are important in hypoxic and normoxic responses in macrophages at baseline and under settings of inflammation. Previous works have studied the loss of either HIFα subunit in isolation. It was for this reason we targeted the HIFα binding partner ARNT to study complete loss of HIFα activity in a myeloid-specific mouse model.

Importantly, preliminary *in vitro* results from macrophages isolated from *LysMCre;Hif1$\alpha^{fl/fl}$;Hif2$\alpha^{fl/fl}$* mice (*Hif1$\alpha^{\Delta/\Delta}$Hif2$\alpha^{\Delta/\Delta}$* macrophages - here on referred to as

double knockout or DKO) reinforce our findings from $Arnt^{\Delta/\Delta}$ macrophages. Unsurprisingly, loss of both HIFα subunits results in dramatic decreases in expression of multiple canonical HIF target genes including *Vegf*, *Pgk1*, and *Glut1* (Figure 42A-D). Similarly, nearly identical effects on *iNos* expression and NO production and *Il1β* expression and secretion are observed in DKO macrophages cultured hypoxia in the presence of M1 polarizing stimuli (Figure 43A-D).

Intriguingly, the same paradoxical increase in a variety of Th1 polarizing cytokines observed in ARNT deficient macrophages is also observed in DKO macrophages cultured under 0.5% O_2 in the presence of LPS and IFNγ (Figure 44A-C). Importantly, these changes in gene expression are not observed with loss of either HIF-1α or HIF-2α alone (Peyssonnaux et al. 2007; Imtiyaz et al. 2010). This data is furthered by the observation that CD86 surface markers increase in DKO macrophages cultured under hypoxia in the presence of M1 polarizing stimuli (Figure 45A-B). Together these findings, while counterintuitive, provide the framework for understanding the role of HIF signaling in macrophages in the setting of inflammation.

Both IL-12 and CD86 are upregulated on antigen presenting cells (APCs) and help to prime T cells towards a Th1 phenotype. Inappropriate T cell activation may inhibit resolution of inflammation. As hypoxia is a major component of inflamed tissues, the hypoxic response of innate immune cells, via HIF stabilization, may regulate activation of adaptive immunity and eventual resolution. Thus, while HIF activity may stimulate initial immune responses, HIF-1α and HIF-2α-mediated responses may also function to limit excess inflammation and leukocyte recruitment. Since the process of inflammation, infection containment, and resolution is one whereby tissue damage is a necessary yet unfortunate consequence, it is not surprising then, that multiple mechanisms may be in place to limit unnecessary immune activation and enact a negative feedback mechanism
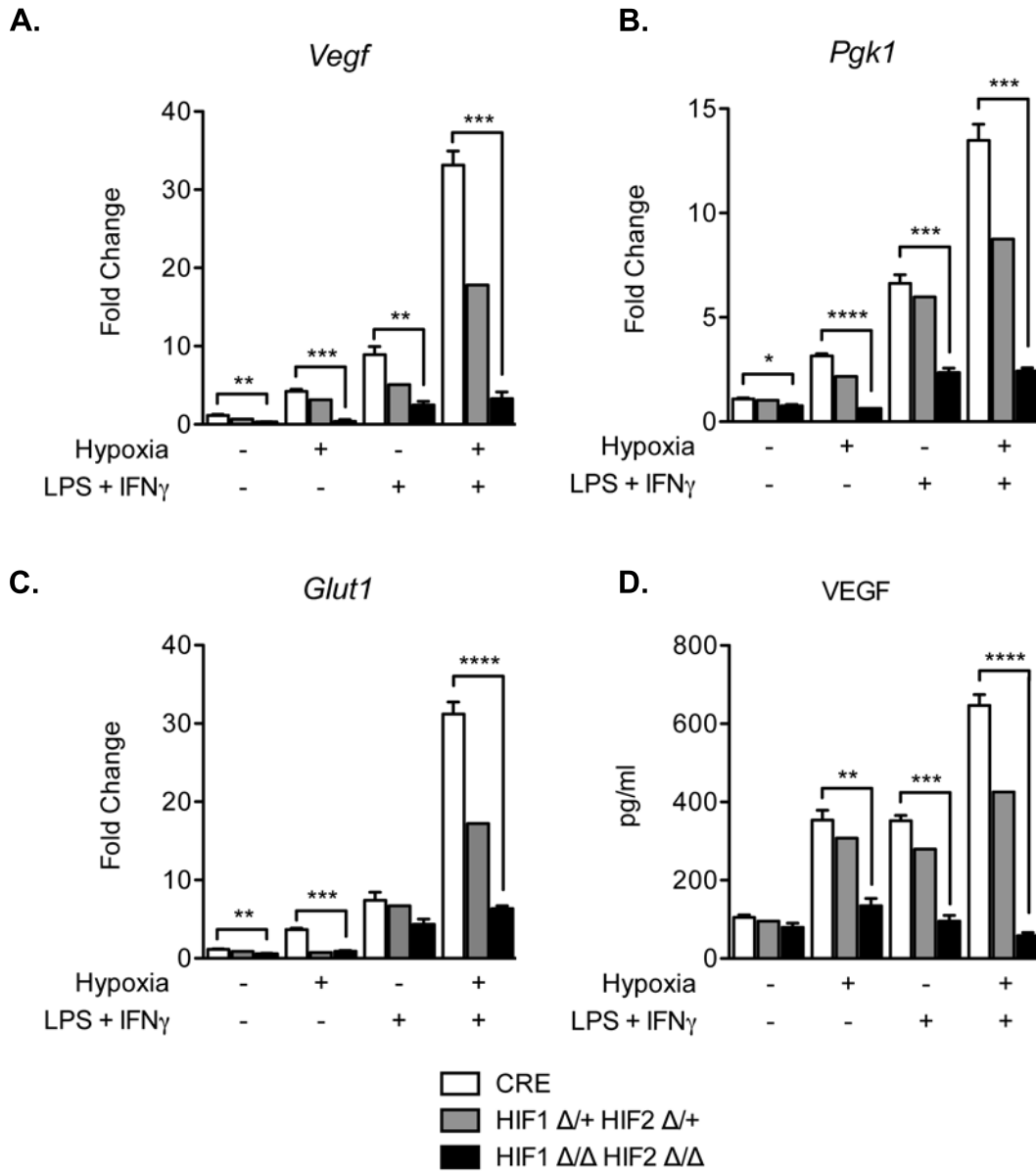
Figure 42. Canonical HIF targets are decreased in DKO macrophages. (A) Relative *Vegf* (B) *Pgk1* and (C) *Glut1* expression in DKO, heterozygous, or control macrophages cultured under M1 (LPS + IFNγ) polarizing conditions in the presence of 21% O_2 or 0.5% O_2. (D) Relative VEGF secretion in DKO, heterozygous, or control macrophages cultured under M1 (LPS + IFNγ) polarizing conditions in the presence of 21% O_2 or 0.5% O_2. (mean ± SEM, $n = 4$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)
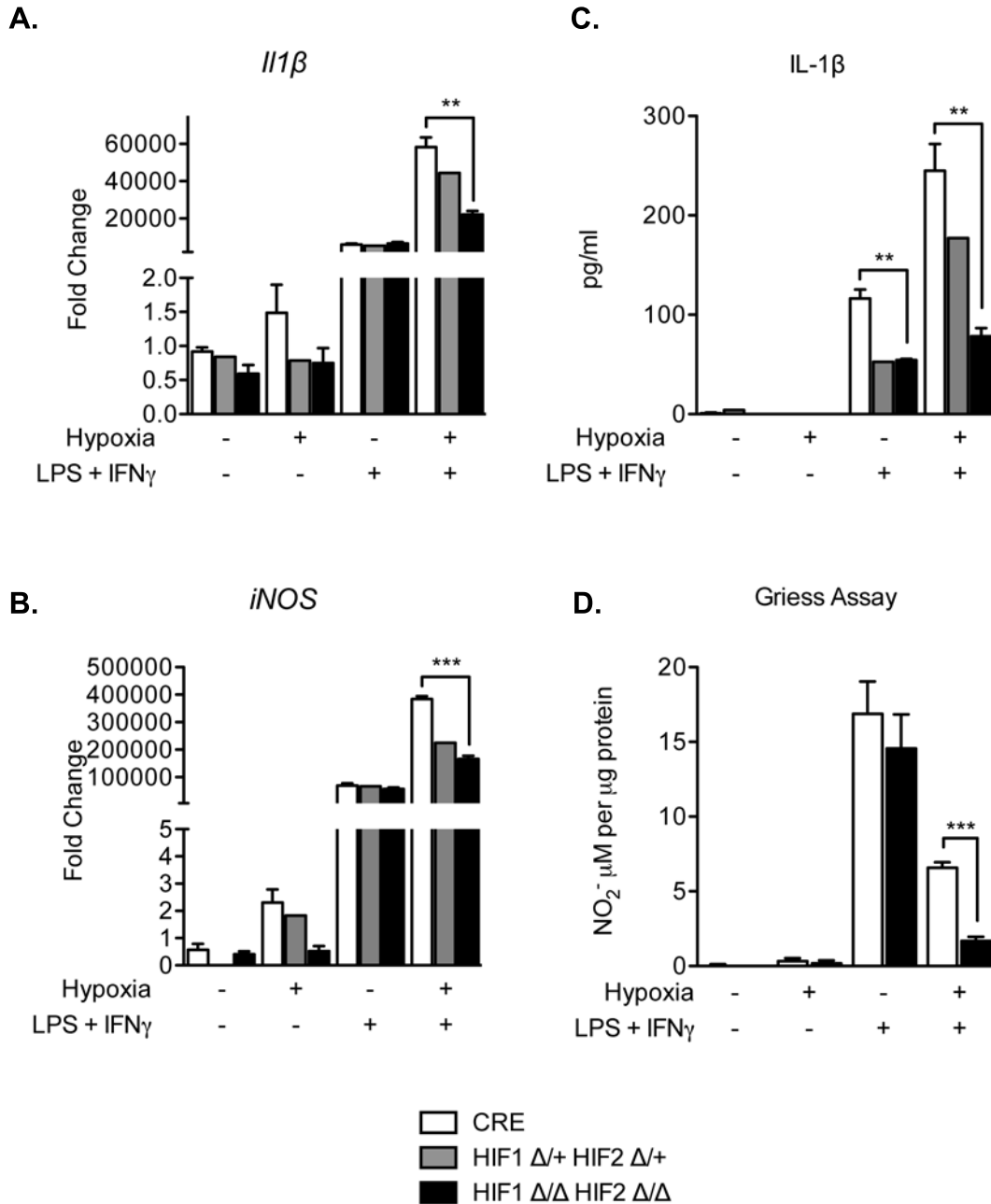
Figure 43. DKO macrophages exhibit decreases in multiple classical M1 targets. (A) Relative *Il1β* (B) *iNos* expression or (C) IL-1β secretion in DKO, heterozygous, or control macrophages cultured under M1 (LPS + IFNγ) polarizing conditions in the presence of 21% O_2 or 0.5% O_2. (D) Relative NO production from DKO or control macrophages cultured under M1 (LPS + IFNγ) polarizing conditions in the presence of 21% O_2 or 0.5% O_2 as determined by Griess assay measuring NO_2^- concentration. (mean ± SEM, n = 4, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)
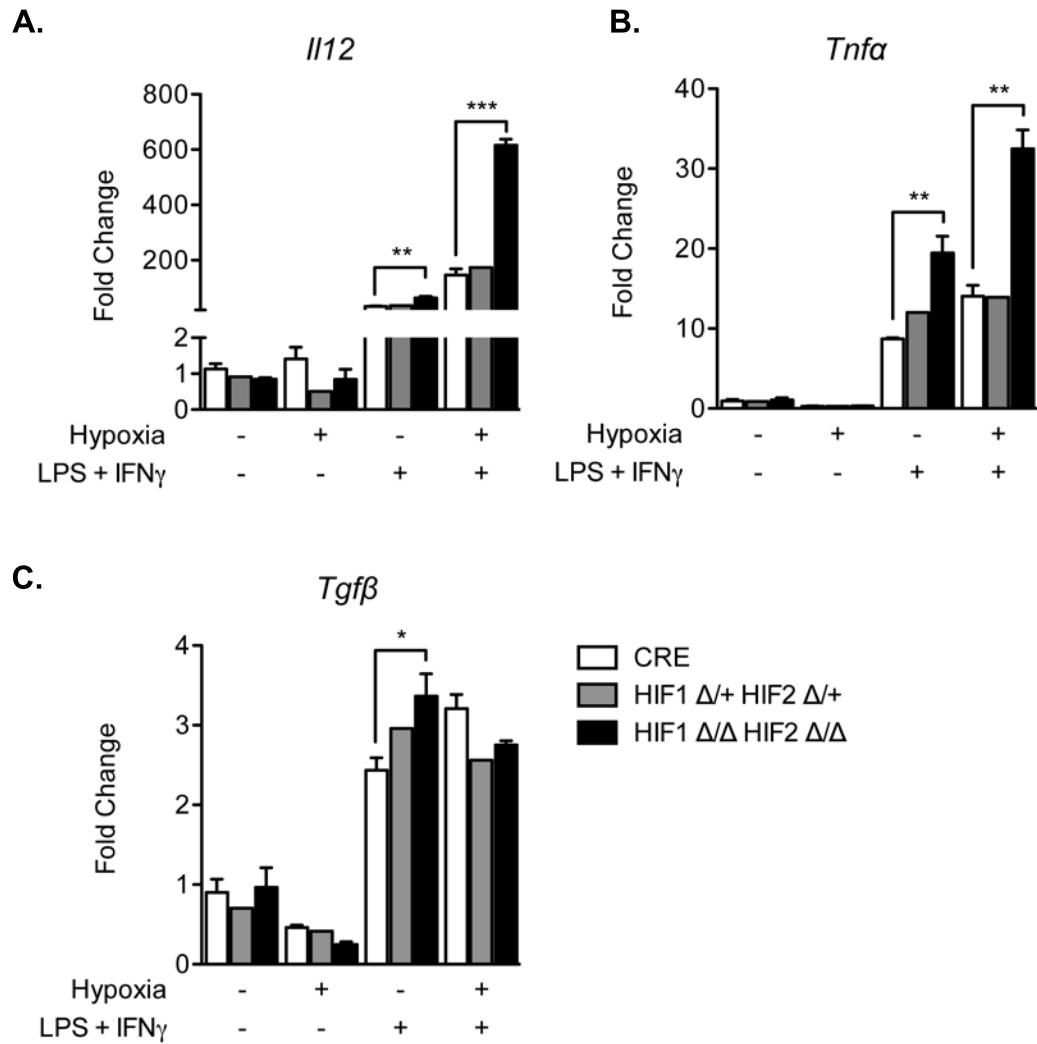
Figure 44. Paradoxical increase in expression of certain cytokines in DKO macrophages.
(A) Relative *Il12* (B) *Tnfα* and (C) *Tnfα* expression in DKO or control macrophages cultured under M1 (LPS + IFNγ) polarizing conditions in the presence of 21% O_2 or 0.5% O_2. (mean ± SEM, *n* = 4, * p < 0.05, ** p < 0.01, *** p < 0.001)

A.
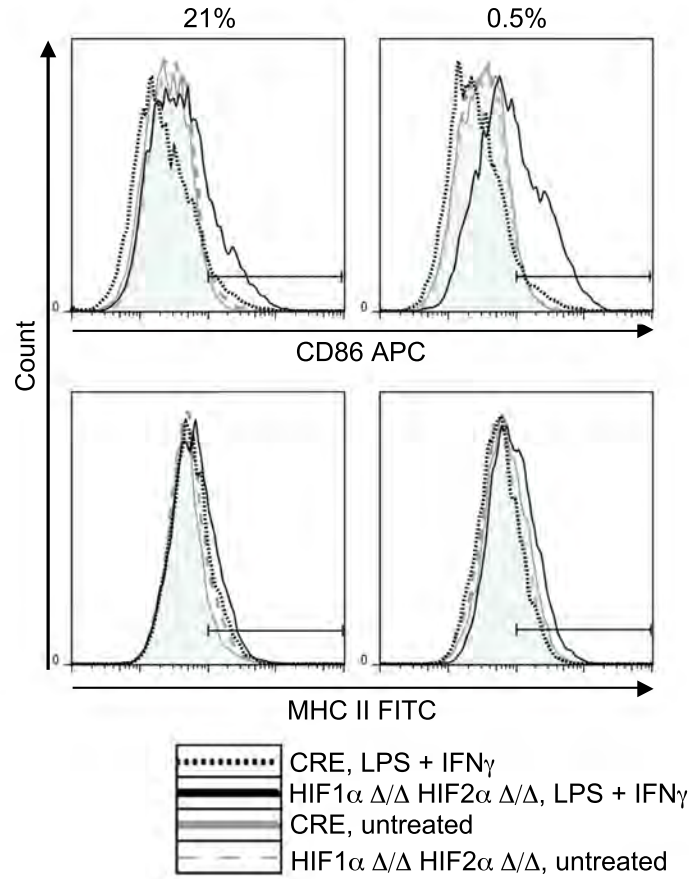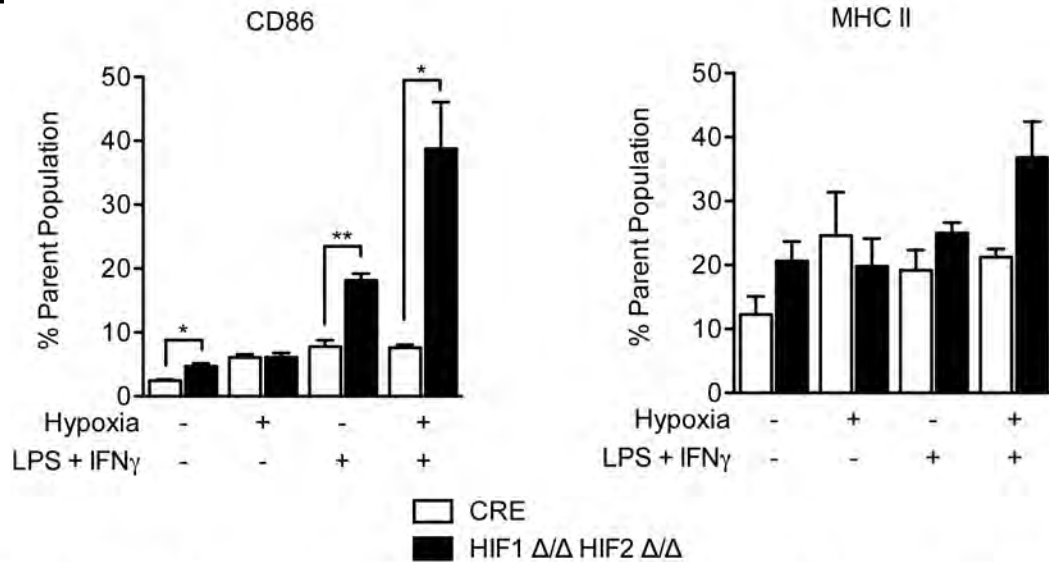
Figure 45. DKO macrophages exhibit increased expression of specific cell surface markers. (A) Representative FACS plots and (B) quantification of CD86 and MHCII cell surface expression in M1 polarized DKO or control macrophages under 21% O_2 or 0.5% O_2. (mean ± SEM, $n = 4$, * $p < 0.05$, ** $p < 0.01$)

to regulate leukocyte recruitment and stimulation once inflammation is underway. HIF activity may result in different macrophage phenotypes based entirely on environmental influences, temporal influences, or a combination of the two.

Future work will need to compare DKO macrophages to single knockout ($Hif1\alpha^{\Delta/\Delta}$ or $Hif2\alpha^{\Delta/\Delta}$ here on referred to as SKO) macrophages in order to clearly identify signaling pathways that require at least one functional HIFα subunit, both HIFα subunits, or instances where HIF-1α signaling opposes or competes with HIF-2α signaling. Exogenous stimuli and temporal influences will need to be carefully controlled so as to understand how the environment may impact macrophage responses. It may prove insightful to perform a series of co-culture experiments – either directly or with macrophage-conditioned supernatant – with naïve T cells to quantify and observe effects of macrophage HIFα activity on T cell polarization. Although multiple cytokines have been implicated in polarizing naïve T cells, a combination of cytokine array, Q-PCR, and co-culture experiments may allow identification of specific macrophage HIFα-dependent cytokines responsible. Once identified, rescue experiments may be performed *in vitro*.

Ultimately however, *in vivo* inflammation models will need to be employed to better understand the complex interaction between resident and recruited macrophages and the surrounding inflammatory environment. The TPA skin inflammation model is an obvious choice to study the effects of resident macrophage signaling on recruiting neutrophils during acute inflammation. Indeed, if macrophage secretion of CXCL1 is a key signaling event of hypoxia and HIF-mediated neutrophil recruitment, experiments may entail intravenous administration of recombinant CXCL1 or a monoclonal CXCL1 antibody to DKO and control mice respectively. Similarly, systemic LPS administration similar to that described by Imtiyaz et al. may, along with monoclonal IL-10 antibody administration, demonstrate the importance of HIF signaling in sepsis as previous work

and current findings show HIF is necessary for *Il10* expression. These findings may prove therapeutically useful as IL-10 is an important protective factor in LPS endotoxemia (Howard et al. 1993).

Other acute and chronic inflammatory models may also prove important in furthering our understanding of how different local environments influence innate immune responses to inflammation. Importantly, HIF-1α has been previously implicated in maintaining intestinal epithelial barrier function in settings of murine experimental colitis (Karhausen et al. 2004). Intestinal epithelial loss of HIF-1α resulted in increased severity of DSS-induced colitis indicating HIF-1α expression in the intestinal epithelium could be considered protective. Conversely, recent work has demonstrated increased HIF-2α expression in colon tissue from UC and CD patients compared to controls and mouse studies indicate HIF-2α expression in intestinal epithelial cells is actually destructive in the setting of DSS-induced colitis (Xue et al. 2013). Although these findings take place in the setting of HIF-2α over expression they suggest endogenous levels may contribute to the pathogenesis of IBD. Although the disparate roles for HIF-1α and HIF-2α in the setting of intestinal epithelial function are initially surprising, these findings are in keeping with multiple previous instances whereby the HIFα subunits exhibit non-overlapping or opposing roles. Investigating HIF responses in the myeloid response in appropriate murine models of acute and chronic colitis may prove equally informative. Ultimately, to most accurately understand the contribution of total and individual HIFα activity in the setting of resident and recruited intestinal macrophages, lamina propria macrophages need to be isolated from DKO and SKO mice and characterized under physiologic and pathologic conditions.

Although this body of work has focused primarily on HIF signaling in macrophages under inflammatory and tumor-promoting conditions within the setting of

inflammation-associated cancers, myeloid-derived cells contribute to multiple aspects of tumorigenesis. In particular, myeloid-derived suppressor cells (MDSCs), have been implicated in driving colitis-associated colon cancer (CAC) (Katoh et al. 2013). MDSCs are suppressive cells of myeloid origin that contribute to negative regulation of the immune system in settings of cancer and inflammation (Gabrilovich et al. 2009). Although multiple cues can recruit and influence MDSCs, expression of *Arg1* and *iNos* are characteristic features (Gabrilovich et al. 2009; Ostrand-Rosenberg et al. 2009). L-arginine metabolism has been shown to inhibit T cell function in the presence of TAMs or MDSCs. Intriguingly, both *Arg1* and *iNos* are HIF targets. HIF transcriptional effects may therefore be important in mediating key features of MDSC activity within the tumor microenvironment. Similarly, in the setting of CAC, MDSCs were shown to be recruited, in part, by chemokines such as CXCL1, CXCL2, and CXCL5 (Katoh et al. 2013). Again, *Cxcl1* and *Cxcl2* are also HIF-regulated genes. Thus, HIF activity may also promote MDSC recruitment by controlling hypoxic release of specific chemokines from a variety of cell types within sites of inflammation or the tumor microenvironment. Response to hypoxia may function through HIF-mediated transcriptional changes that could influence MDSCs metabolic activity through cell intrinsic means or recruitment via extrinsic signaling. Regardless, this is an ongoing area of study and the role of HIF activity in MDSC activity and recruitment remains unclear.

The role of HIF signaling in the setting of inflammation-driven cancers is an ongoing area of research. HIFs mediate changes in transcription and drive hypoxic adaptation in both tumor cells and resident or recruited macrophages and are important at every stage of tumor initiation and progression. Future studies will hopefully expand on the work presented here and investigate new models of inflammation-associated cancers and utilize current mouse models and patient data to study the role of HIF-1α and HIF-2α signaling in interactions between normal tissue and the innate immune

system and subsequently, between the tumor parenchyma and infiltrating myeloid-derived cells. Because HIFs mediate transcriptional changes in a number of different cell types in response to changes in O_2 levels, they may prove to be a desirable therapeutic target in multiple pathologic settings including inflammation and cancer.

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