UC San Diego

UC San Diego Electronic Theses and Dissertations

Title

Role of myeloid Hypoxia-Inducible Factor-1alpha in the tumor microenvironment

Permalink

https://escholarship.org/uc/item/14h1c7m2

Author

Doedens, Andrew L.

Publication Date

2008

Peer reviewed|Thesis/dissertation

eScholarship.org Powered by the California Digital Library
University of California

UNIVERSITY OF CALIFORNIA, SAN DIEGO

Role of Myeloid Hypoxia-Inducible Factor-1alpha in the Tumor Microenvironment

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biology

by

Andrew L. Doedens

Committee in charge:

Professor Randall S. Johnson, Chair
Professor Jeffrey Esko
Professor Stephen Hedrick
Professor Mark Kamps
Professor Cornelis Murre

2008

Copyright

Andrew L. Doedens, 2008

All Rights Reserved.

The dissertation of Andrew L. Doedens is approved, and it is acceptable in quality and form for publication on microfilm:

_____

_____

_____

_____

_____

Chair

University of California, San Diego

2008

TABLE OF CONTENTS

Signature Page..... iii

Table of Contents..... iv

List of Abbreviations..... v

List of Figures..... vi

Acknowledgements..... vii

Vita..... viii

Abstract..... ix

Chapter 1:Introduction to the Mammalian Hypoxic Response.....1

Chapter 2:The role of myeloid cell HIF-1 alpha in the tumor microenvironment.....20

References.....52

LIST OF ABBREVIATIONS

HIF	Hypoxia-Inducible Factor
PyMT	Polyoma Virus Middle T Antigen
MMTV	Murine Mammary Tumor Virus Long Terminal Repeat Promoter
LysM	Myeloid Specific Lysozyme
BMDM	Murine Bone Marrow Derived Macrophages
CFSE	(5 and 6) Carboxyfluorescein, succinimidyl ester
iNOS	Inducible Nitric Oxide Synthase
ArgI	Arginase enzyme, Type I
IFNg	Interferon-gamma
IL-4	Interleukin-4
norNOHA	Nω-hydroxy-nor-l-arginine

LIST OF TABLES AND FIGURES

Chapter 1

Table 1.1: Selected milestones and transgenic mice in 15 years of HIF research.....19

Chapter 2

Figure 2.1: HIF-1alpha, and not HIF-2alpha, controls murine macrophage iNOS and ArgI in response to IFNg or IL-4 in vitro.....42

Figure 2.2: Addition of resident peritoneal macrophages to CD3/28 activated splenocytes 1:10 blocks T cell proliferation as measured by CFSE in a hypoxia/HIF-1alpha/iNOS dependent fashion.....43

Figure 2.3: Myeloid specific HIF-1alpha knockout bred to PyMT model of breast cancer. 44

Figure 2.4: Quantitation of macrophage and T cell infiltration and HIF-1 target enzymatic activity in PyMT WT and myeloid HIF-1alpha KO.....45

Figure 2.5: PyMT T cell responsiveness and arginase I induction in macrophages by diffusible mediator from PyMT cells.....46

Figure 2.6: Injection of Lewis Lung Carcinoma cells into myeloid HIF-1alpha WT and KO mice.....47

Figure 2.7: Model of macrophage HIF-1alpha dependent suppression of T cell activation.....48

Figure 2.8: Perturbation of splenic composition in PyMT tumor bearing mice vs. littermate non-tumor bearing controls.....49

Figure 2.9: Activation and other immunophenotypic markers from splenic T cells of PyMT tumor bearing mice vs. littermate controls.....50

Figure 2.10: Deletion of HIF-1alpha in CD11b+/GR1med and CD11b+/GR1bright, but not in CD11b+/GR1- cells using the LysM-cre recombinase.....51

ACKNOWLEDGEMENTS

I would like to acknowledge many people who have helped me throughout graduate school. My advisor Randall S. Johnson deserves thanks for his guidance and suggestions in developing and furthering the project. Debbie Liao taught me about the PyMT model and aided in palpation during the first weeks of the project, and was party to scientifically productive conversations. Christian Stockmann made important contributions to histological characterization of the tumors.

I would also like to thank Ananda Goldrath and Mark Rubinstein of the Goldrath lab for their expertise in T cell biology, suggestions, supplies, and use of equipment to further this dissertation.

My committee has always been constructive and helpful in both suggestions to help guide the project as well as how to develop as a scientist, and for this I thank them.

I would like to thank my family and my wife for supporting me throughout my education, and without them this would not have been possible.

Chapter 2 is being prepared for publication in a scientific journal as Doedens A, Stockmann C, Rubinstein M, Liao D, Goldrath A, Johnson RS. HIF-1alpha in tumor infiltrating macrophages mediates local T cell immunosuppression. 2008. The dissertation author was the primary investigator and author of this paper.

VITA

1998	Bachelor of Science, Purdue University, West Lafayette, Indiana
1998	Research Internship, University of Edinburgh, Scotland, U.K.
2008	Doctor of Philosophy, University of California, San Diego, California

PUBLICATIONS

Doedens A, Stockmann C, Rubinstein M, Liao D, Goldrath A, Johnson RS. Tumor infiltrating myeloid HIF-1alpha mediates T cell suppression.
In preparation. 2008.

Doedens A, Johnson RS. "Transgenic Models to Understand HIF Function".
Methods in Enzymology, v 435: Oxygen Biology and Hypoxia. Elsevier, 2007

Peyssonnaux C, Cejudo-Martin P, Doedens A, Zinkernagel AS, Johnson RS, Nizet V.
Cutting edge: Essential role of hypoxia inducible factor-1alpha in development of lipopolysaccharide-induced sepsis.
J Immunol. 2007 Jun 15;178(12):7516-9.

Peyssonnaux C, Datta V, Cramer T, Doedens A, Theodorakis EA, Gallo RL, Hurtado-Ziola N, Nizet V, Johnson RS. HIF-1alpha expression regulates the bactericidal capacity of phagocytes.
J Clin Invest. 2005 Jul;115(7):1806-15.

FIELDS OF STUDY

Major Field: Cancer Biology

Studies in Tumor Biology
Professor Randall S. Johnson

ABSTRACT OF THE DISSERTATION

Role of Myeloid Hypoxia-Inducible Factor-1alpha
in the Tumor Microenvironment

by

Andrew L. Doedens

Doctor of Philosophy in Biology

University of California, San Diego, 2008

Professor Randall S. Johnson, Chair

Solid tumors are frequently infiltrated by large numbers of non-cancerous hematopoietic cells, including macrophages. The pro-tumor or anti-tumor role of macrophages in the tumor microenvironment is unclear. Tumors are also characterized by regions of low oxygen tension. Mammalian cells respond transcriptionally to low oxygen tension via the Hypoxia-Inducible Factor-1alpha, which upregulates genes

involved in glycolysis, angiogenesis, and cell survival. Previous work has shown that myeloid cells deficient for HIF-1alpha are impaired in their inflammatory responses. To genetically test the role of the myeloid hypoxic response mediated by HIF-1alpha in the tumor microenvironment, we used the loxP/tissue specifc cre recombianse to generate murine myeloid specific deletion of HIF-1alpha in rodents with an established transgenic model of breast cancer, MMTV-PyMT. Lack of HIF-1alpha in the tumor microenvironment resulted in tumors with less mass, but with increased cell death. Enzyme activities of iNOS and ArgI were reduced in whole tumor lysates. In vitro experiments demonstrated HIF-1 alpha, not HIF-2alpha, control of L-arginine degrading enzymes iNOS and arginase I. Further characterization of the relationship between macrophages and tumor cells using co-culture strategies revealed that tumor cells induce ArgI in a HIF-1alpha and hypoxia dependent fashion at the protein level. iNOS was detected at the RNA level after co-culture with MECs, but was scarcely detectable at the protein level. ArgI and iNOS have been implicated in T cell immunosuppression. PyMT tumor bearing mice displayed evidence of T cell activation, yet T cells isolated from myeloid HIF WT tumors were less responsive than those from myeloid HIF KO tumors after stimulation with CD3/28 *ex vivo*. We propose myeloid HIF-1alpha contributes to local tumor immunosuppression of T cell function. This suggests inhibition of HIF-1 may have beneficial effects not only by blocking tumor growth and survival under hypoxia, but also by relieving myeloid cell mediated immunosuppression in the tumor microenvironment.

Chapter 1

Introduction to the Mammalian Hypoxic Response

Introduction

The Hypoxia Inducible Factor (HIF) is a heterodimeric transcription factor of the basic helix-loop-helix Per-Arnt-Sim (bHLH-PAS) superfamily that regulates over 100 genes involved in glycolysis, metabolism, cell cycle, apoptosis, and angiogenesis (Semenza, 2001). Formation of a heterodimer composed of a HIF α subunit (HIF-1α, HIF-2α, or HIF-3α) and HIF β subunit (typically ARNT1) is required before translocation from the cytoplasm to the nucleus and subsequent binding to Hypoxic Response Elements (HREs) and transcriptional transactivation (Beck et al., 1991; Pugh et al., 1991; Semenza et al., 1994; Semenza and Wang, 1992; Wang et al., 1995). Although both α and β subunits are constitutively expressed, under normoxic conditions one or more prolyl hydrozylase domain containing enzymes (PHDs) modify the α subunit, which allows it to then be bound and polyubiquitinated by the E3 ubiquitin ligase complex VHL, which is made up of pVHL, Cullin2, Elongin B, Elongin C, and Rbx1 (Kaelin, 2002). Polyubiquitinated α subunits are quickly degraded by the cellular proteasome apparatus, thus blocking HIF transcription under normoxia in most cell types. During cellular exposure to hypoxia, low oxygen tensions limit the activity of the PHD enzymes, leaving α subunits unhydroxylated and unrecognizable by the VHL complex. Accumulation of α subunits in the cytoplasm allows for the aforementioned heterodimeric nuclear translocation and transactivation (Bruick and McKnight, 2001; Epstein et al., 2001; Ivan et al., 2001; Jaakkola et al., 2001). A further level of regulation of the HIF transcriptional machinery is exerted by the Factor Inhibiting HIF (FIH). FIH hydroxylates HIF α subunits under normoxia and mild hypoxia, but not in more

pronounced hypoxia. Hydroxylation by FIH inhibits the binding of transcriptional coactivator p300/CBP at the HRE, which limits HIF transactivation potential (Hewitson et al., 2002; Mahon et al., 2001). In this way, the HIF system can modulate transcription from HRE containing genes sensitively over a wide range of oxygen tensions, from mild hypoxia to severe hypoxia.

Transgenic mice (Table 1.1) have been used to unequivocally determine the requirement of a gene for development, as well as to test the role of a gene in all the complexity of an *in vivo* system - both in normal physiology as well as tumor biology. This review aims to sum up the current state of knowledge of the requirement of HIF and related genes for development, their role in physiology as determined *in vivo* by transgenic models, and to discuss selected publications demonstrating the role of HIF family proteins in *in vivo* tumor biology.

Hypoxia Response Pathway Genes and Development

Knockout strategies have been employed in the mouse to determine the role of many of the proteins involved in orchestrating the hypoxic response. Homologous recombination strategies which remove an essential part of the gene or the entire gene (i.e. straight knockout or global knockout) have revealed that many of these proteins are essential for development and thus viability.

HIF α Subunits

HIF-1α is the most widely expressed α subunit and is expressed in nearly all tissues (Wiener et al., 1996). Genetic ablation of HIF-1α in the mouse results in embryonic lethality at mid-gestation. Embryos exhibited a lack of vascularization, reduction in number of somites, neural fold defects, decreased size, and increased tissue

hypoxia (Iyer et al., 1998; Ryan et al., 1998). Further characterization of HIF-1α knockout mice using whole embryo culture revealed defects in ventricle development, pharyngeal arch growth, and cephalic vascularization. Hyperoxia extended survival, but did not result in full rescue (Compernolle et al., 2003).

HIF-2α displays a somewhat more restricted expression pattern, appearing in endothelium, kidney, liver, lung and brain (Tian et al., 1997). HIF-2α has been germline deleted by several independent groups. Unlike HIF-1α, HIF-2α seems to be sensitive to the presence of modifier alleles in the distinct mouse backgrounds used to generate the knockouts. As a result, different phenotypes have been reported by the independent groups. Given that manipulating of the strain background of HIF-2α null mice can even lead to viable mice [(Scortegagna et al., 2003a) see *Hematopoiesis* later in this review], perhaps it is not surprising that different strain backgrounds can result in unique developmental failures in HIF-2α null mice.

The first group to report on deletion of HIF-2α observed embryonic lethality at mid-gestation due to bradycardia as a result of a defect in catecholamine synthesis. Viable mice could be generated by administering pregnant females a catecholamine precursor, DOPS (Tian et al., 1998). HIF-2α targeting by an independent group (Peng et al., 2000), resulted in embryonic lethality between E9.5 and 13.5. However, significant changes in heart rate or catecholamine levels between genotypes were not observed. Although vasculogenesis took place, postvasculogenesis changes required for the formation of the adult vasculature did not take place in the null. The authors also discussed important differences in the mouse background of the mice generated in their

lab versus the null mice generated by Tian et al, 1998, and suggest that strain differences are behind what would appear to be divergent results.

A third group has reported on the role of HIF-2α and the control it exerts over VEGF in lung development (Compernolle et al., 2002). Although half of HIF-2α null mice died in *in utero* after E13.5, surviving mice died within 2-3 days after birth of respiratory distress syndrome caused by failure of pneumocytes to produce surfactant. Mice could be rescued by stimulation of VEGFR2 by antibody or VEGF itself, which restored the surfactant production.

HIF β/ARNT Subunits

Another approach to studying HIF function is to genetically manipulate the obligate partners of the α subunits, the ARNT proteins. HIF-1β/ARNT1 null embryos do not survive past 10.5 days of gestation, with embryos smaller than wild type exhibiting defective angiogenesis in the yolk sac and branchial arches (Maltepe et al., 1997). Another team of investigators reported similar embryonic lethality, along with neural tube closure defects, placental hemorrhage, stunted forebrain growth, and visceral arch anomalies. This team faulted embryonic lethality on the failure to vascularize the placenta and the form the labyrinthine spongiotrophoblast, and found no defects in yolk sac circulation (Kozak et al., 1997).

Although ARNT1 is the most widely expressed and prototypical binding partner of HIF α subunits (Wiener et al., 1996), ARNT2, which is expressed in the brain, liver, and kidney can also form functional heterodimers with the α subunits and thus may be important to hypoxic response in these organs and in CNS in general (Maltepe et al., 2000). Genetic ablation in the mouse results in perinatal lethality with impaired

hypothalamic development. The authors noted the similarity between this phenotype and the knockout of Sim1 (Single Minded 1), a bHLH-PAS protein involved in neuronal development that binds with ARNT proteins to form active transcriptional complexes (Keith et al., 2001). This finding highlights the importance of considering all bHLH-PAS family members that rely on ARNT dimerization when interpreting experimental results using ARNT family knockouts. Depending on cell type, these partners potentially include hypoxia response HIF α subunits, the xenobiotic sensitive Aryl Hydrocarbon Receptor (AhR), proteins involved in the circadian rhythm such as CLOCK and NPAS2, and the above-mentioned Sim1.

Other ARNT proteins (ARNT3 or 4) could potentially bind HIF α subunits, but have not been reported to be important regulators of the hypoxic response *in vivo*. ARNT3/MOP3/bMAL1/ARNTL1 is thought not to participate in the hypoxic response (Cowden and Simon, 2002), despite the fact that it can bind HIF α subunits in yeast two hybrid assays (Hogenesch et al., 1998). Rather, both ARNT3 and ARNT4/bMAL2/ARNTL2 are thought to be critical to circadian rhythm regulation in neuronal tissues as binding partners of CLOCK and NPAS2 (Bunger et al., 2000).

<u>pVHL</u>

The negative regulator of the HIF α subunits, pVHL, which as a member of the von Hipppel-Lindau protein complex recognizes hydroxylated HIF α subunits, is also essential for embryonic viability. Ablation of the pVHL component of the complex by homologous recombination results in embryonic lethality between day 10.5 and 12.5 as a result of placental dysgenesis and hemorrhage (Gnarra et al., 1997). The authors also noted that there were no abnormalities in embryonic development detected until

approximately day 9.5, and that heterozygous null mice were phenotypically normal. Deletion of pVHL in endothelial cells using pVHL floxed mice crossed to Tie2-cre recombinase also results in embryonic lethality similar to the global pVHL knockout. Histological staining revealed a defect in fibronectin deposition, suggesting the importance of this molecule in successful vasculogenesis in the developing embryo (Ohh et al., 1998; Tang et al., 2006)

PHDs

The Prolyl Hydroxylase Domain (PHD) containing enzymes play a key role in HIF regulation by modification of α subunits when oxygen is available, leading to recognition by VHL, polyubiquitination, and proteasomal degradation. Interfering with PHD activity is often used to chemically induce HIF activity *in vitro*. There are three PHD enzymes, PHD1, 2, and 3; however, the relative importance and redundancy of the enzymes in the context of HIF regulation is an important question. Genetic ablation of each of these enzymes has been carried out in mice (Takeda et al., 2006). The team found that PHD1-/- and PHD3-/- null mice were viable and appeared healthy. PHD2-/- embryos died *in utero* between E12.5 and 14.5. Defects in heart and placental development were observed. Placental defects included a reduction in labyrinthine branching morphogenesis, population of the labyrinth with spongiotrophoblasts, and aberrant localization of trophoblast giant cells. Although the placental defects were related to HIF α subunit levels, PHD2-/- mice did not show an increase of HIF α subunits in the heart. As the authors point out, the PHD enzymes likely have other roles *in vivo* apart from HIF α regulation, and these roles may be responsible for the observed defects in the heart.

VEGF

The vascular endothelial growth factor A, VEGF-A (here referred to as VEGF), is an important target of HIF in development and cancer. In development, VEGF is essential, as even a heterozygous null is embryonic lethal at E11-12 (Carmeliet et al., 1996; Ferrara et al., 1996). Further studies using conditional knockouts and an engineered VEGF receptor protein that binds free VEGF in young mice showed VEGF to be important in viability, growth, and development of organs, especially the liver. VEGF continued to be important until about 4 weeks or age, when the animals lost their dependence on VEGF (Gerber et al., 1999).

HIF in Physiology

HIF-1α Heterozygotes

Although VEGF heterozygous knockouts are embryonic lethal, heterozygous HIF-1α and HIF-2α null mice are viable and appear normal. One approach to studying HIF function involves comparing heterozygous global knockout mice with WT controls. Several groups have used this approach to study the effects of intermittent and long-term hypoxia on whole animal physiology (Brusselmans et al., 2003; Peng et al., 2006; Semenza, 2006; Yu et al., 1999), as well as to study cerebral hypoxia in a model of stroke [see *Brain* later in this review]. Along with pulmonary vascular remodeling, rodents exposed to chronic hypoxia are known to develop pulmonary hypertension, and right ventricular hypertrophy, the combination of which can lead to death. Heterozygosity of HIF-1α delayed pulmonary hypertension in mice exposed to chronic hypoxia (Yu et al., 1999). However, heterozygosity of HIF-2α eliminated pulmonary hypertension and ventricular hypertrophy (Brusselmans et al., 2003). The authors found increases in

endothelin-1 and plasma catecholamine levels in WT vs. HIF-2α heterozygotes, which suggest they may be key players in HIF dependent hypoxia induced pulmonary remodeling.

Conditional Knockouts using the loxP/cre recombinase system

Although regulated HIF function is clearly required for embryonic development, the advent of conditional knockouts has allowed the study of function of hypoxia response genes in adult mice. As a first step towards a conditional knockout approach, typically investigators "flox" essential exons and/or the promoters of target genes by inserting loxP recombination sites in flanking introns or non-coding regions. Mice with "floxed" genes, that is, genes with essential sequences flanked by loxP sites, are normal and display no phenotypes as compared to wild type controls. When floxed mice are genetically crossed with transgenic mice carrying one copy of the cre recombinase driven by a tissue specific promoter, the enzyme can act upon the flox sites and excise the target sequence, thus generating a knockout of the target gene, but only after the tissue specific cre recombinase has been expressed (Orban et al., 1992; Sauer, 1998). Using this approach, many HIF family members and related proteins have been studied in viable adult mice to determine their role in mammalian physiology.

Liver

pVHL ablation via liver specific Albumin-cre recombinase results in hepatocellular steatosis along with vascular tumors and local angiogenesis (Haase et al., 2001). Glycogen granules were also noted in VHL null livers, and the mechanism appears to be HIF dependent upregulation of liver glucose uptake coupled with impaired ability to metabolize the increased sugar (Park et al., 2007). Another study conducted

with HIF-1α and 2α transgenes that could not be recognized by VHL has recapitulated the major findings from the Albumin-cre study, as well as noting increased hepatocyte prolferation. These findings underscore the role of HIF-1α and HIF-2α as the responsible genes for the phenotypes observed in previous liver pVHL knockout studies (Kim et al., 2006). This study also found that, in the liver, although HIF-1α and 2α shared many targets, the target genes upregulated by the two α factors were not identical, whereas in the skin expression of a HIF-2α subunit that cannot be recognized by VHL essentially phenocopied VHL loss.

Myeloid Lineage

Deletion of HIF-1α in myeloid cells led to an unexpected suppression of macrophage and neutrophil function, including reduced ATP levels, cytokine production, inflammatory response, and ability to kill phagocytosed bacteria (Cramer et al., 2003). Myeloid cell development and cell count was unaffected by loss of HIF-1α. In addition, deletion of VHL in myeloid cells had the opposite effect, potentiating the inflammatory capacity of myeloid cells. The relevance of these *in vitro* effects were confirmed using *in vivo* mouse models of acute inflammation and arthritis. Further studies of the role of HIF-1α in bacterial killing detailed the lack of key bactericidal products in HIF null cells, including granule proteases, antimicrobial peptides, and nitric oxide produced by HIF target iNOS. *In vivo* studies demonstrated the null mice were unable to prevent the spread of a bacterial infection (Peyssonnaux et al., 2005).

Lymphocytes

Using a T cell specific cre recombinase, Lck-cre, VHL loss in thymocytes resulted in an atrophic, hemorrhagic thymus. Increased caspase-8 dependent apoptosis

was noted *in vivo* and *in vitro,* and double knockout studies showed the effect to be HIF-1α dependent and Bcl-2 and Bcl-xL independent (Biju et al., 2004). Further characterization of this mutant pinned the T cell activation defect on the overexpression of the HIF regulated calcium transporter SERCA2, which decreased cytoplasmic Ca^{2+} concentrations after T cell receptor ligation, thus blunting TCR signaling (Neumann et al., 2005).

Hematopoiesis

As previously mentioned, HIF-2α null mice have been reported to be embryonic/perinatal lethal, however crossing the null heterozygous alleles on the two inbred mouse backgrounds of 129 x C57 results in about 20% survival (Scortegagna et al., 2003b). Characterization of this surviving portion revealed cardiac hypertrophy, hepatomegaly, increased superoxide levels, and a deficiency in hematopoiesis. These mice could be partially resuced by administration of a superoxide dismutase mimetic, MnTBAP (Scortegagna et al., 2005; Scortegagna et al., 2003b). Deletion of HIF-2α approximately one week after birth using a tamoxifen inducible cre system results in anemic mice, while deletion of HIF-1α does not affect hematocrit levels (Gruber et al., 2007).

Skeletal Muscle

During exercise, skeletal muscle experiences dramatic changes in oxygen tension (Richardson et al., 1995). Conditional deletion of HIF-1α in skeletal muscle resulted in mice that had increased exercise endurance, but at the cost of increased muscle damage after repeated exercise bouts. The increased endurance was a result of increased

mitochondrial activity and decreased lactate production in HIF-1α null animals, both of which point towards increased fatty acid oxidation in the knockout (Mason et al., 2004).

Mammary Gland

The microenvironment of the mammary gland undergoes rapid and drastic change during pregnancy and lactation. Proliferation of the epithelium and later secretion of milk could place excessive demands on the vascular bed that was established during relative quiescence of the resting virgin mammary gland. Pregnancy and lactation studies of HIF-1α conditional knockouts in the mammary epithelium of mice demonstrated failure of differentiation and lipid secretion, which resulted in abnormal milk composition. The vascular density between wild type and null mammary glands was unchanged. Apart from demonstrating the requirement of HIF-1α function in lactation, these results taken together with results from other experiments (Ryan et al., 2000) highlight the complexity of HIF function above and beyond partial control of VEGF and angiogenesis (Seagroves et al., 2003).

Colon

Investigators studying hapten induced colitis noted hypoxia and stabilization of HIF-1α in the colonic epithelium. To test the role of HIF in colitis, they then used an intestinal epithelial specific cre to delete either HIF-1α or pVHL from this tissue. After induction of colitis in these models, the investigators observed a protective function of increased HIF levels, from decreased mortality to diminished weight loss. Mechanistically, decreased barrier function as result of downregulation of a variety of genes including CD73 was noted in HIF-1α null animals, whereas increased barrier function was observed in the pVHL nulls (Karhausen et al., 2004).

Chondrocytes

Chondrocytes, found in growth plates of developing bones, exist in an avascular zone of low oxygen tension, and thus could be expected to rely on hypoxia dependent pathways. Conditional deletion of HIF-1α in this tissue released a hypoxia induced cell cycle arrest, and as a result cells in the hypoxic central region of the growth plate proceeded into untenable cell division, which led to apoptosis. Also noted was a HIF-1α independent upregulation of VEGF resulting in angiogenesis in the hypoxic region of the plate (Schipani et al., 2001).

Skin

Constitutive activation of HIF-1α in the epidermis resulted in drastic increases in dermal capillary density and VEGF expression (Elson et al., 2001). Despite these increases the authors noted no inflammation, edema, or vascular leakage – an unexpected result given the role of VEGF in increasing vascular permeability. Indeed, in an earlier study with VEGF-A 120 kD isoform coupled to a K6 promoter, some progeny died exhibiting increased vascularization and edema which disrupted the skin architecture (Larcher et al., 1998). The loss of HIF-1α in the epidermis results in normal mice when left unchallenged (Unpublished, Boutin and Johnson). Loss of pVHL, similar to the HIF-1α constitutively active model, leads to robust expansion both in vessel size and vascular density, however in this transgenic system, the effect is coupled with increased vascular permeability (Submitted, Boutin & Johnson 2007). Epidermal pVHL null mice or stable HIF-2α expressing mice also exhibit runting, partial alopecia, and erythema (Kim et al., 2006).

Motor Neurons and Brain

Genetic ablation of the HRE sequences from the VEGF promoter resulted in motor neuron degeneration in mice similar to human ALS (Oosthuyse et al., 2001). Noteworthy in the transgenic mice was the lack of effect on baseline VEGF production from muscle, heart, and fibroblasts, but a 40% reduction from neurons. Mice born with homozygous deletion of the HRE in the VEGF promoter suffered 60% mortality perinataly. The surviving fraction was normal until about 5 months of age, when they developed motor neuron dysfunction as demonstrated by decreased grooming, ability to turn over, and behavior when picked up by the tail. Histological characterization of the muscle revealed atrophy, which appeared to be the result of denervation. The investigators proposed that decreased neural perfusion in the transgenics, or perhaps a neuroprotective effect of VEGF-165 binding to KDR as likely mechanisms behind the phenotype.

In order to study HIF-1α function in whole body hypoxia and stroke, a brain specific knockout of HIF-1α was generated by crossing HIF floxed mice to the calcium/calmodulin-dependent kinase cre or by using mice heterozygous for the global knockout of HIF-1α. Neuronal hypoxia was induced by placing mice in a 7% oxygen atmosphere for one hour, and stroke was modeled by occluding one carotid artery for 75 minutes. Subsequent analysis of tissue sections from the brain for apoptosis and necrosis revealed that heterozygous null or neuron specific HIF-1α null mice were protected from hypoxia induced cell death in both neuronal hypoxia and stroke models. The investigators fault HIF-1α dependent upregulation of proapoptotic genes, and conclude that the loss of HIF-1α is neuroprotective in both hypoxic and ischemic episodes (Helton et al., 2005).

HIF Function in Tumor Biology

This portion of the review will focus on murine VHL and HIF α and β transgenics in tumor biology. Given the abundance of reviews available dealing with VEGF function *in vivo*, reports relating to VEGF will be included only in the context of HIF and pVHL models.

First Insights - Xenografts

The ability of HIF to upregulate glycolysis and potentially confer a survival advantage in areas of poor oxygenation coupled with its targeting of angiogenic factors such as VEGF would suggest a protumorigenic role in cancer. Indeed, subcutaneous injection of HIF-1α null embryonic stem cells resulted in tumors with decreased tumor mass, microvessel density, and expression of VEGF (Ryan et al., 1998). An independent group who also generated HIF-1α null ES cells also found changes in vascular density, with EC cords being more dense in HIF null and medium size vessels and vessel beds less dense. This group reported increased tumor mass as a result of decreased apoptosis in HIF-1α null cells (Carmeliet et al., 1998). Further characterization of the role of HIF-1α in tumor growth by injection of mouse embryonic fibroblasts showed a decrease in tumor mass in the null. Interestingly, despite HIF dependent changes in VEGF expression, there were no observed changes in vascular density in these h-ras transformed cell lines (Ryan et al., 2000). Recent siRNA experiments have recapitulated the basic findings already determined genetically related to HIF and VEGF being a pro-tumor factors in most scenarios of carcinogenesis (Detwiller et al., 2005; Jensen et al., 2006; Li et al., 2005; Zhang et al., 2004), although other studies highlight the need to understand how the opposing HIF mediated effects of increased glycolytic energy production and

angiogenesis vs. induction of pro-apoptotic genes and cell cycle inhibitors will balance out in various tumor settings (Acker et al., 2005).

Astrocytoma

Experiments with HIF-1α null cells in an astrocytoma model determined that the site of injection was critical in determining how the WT and HIF-1α null cells behaved. In the poorly vascularized subcutaneous environment, HIF-1α null cells were at a disadvantage; however, the same null cells implanted into the well vascularized brain had a growth advantage (Blouw et al., 2003). Characterization of the vessel density showed the expected decreased vessel density in null subcutaneous tumors. Unexpectedly, the null tumors grown in the brain had increased vascular density. Furthermore, the null cells were more invasive than WT controls. Parallel experiments conducted with VEGF null cells exhibited slower growth in both subcutaneous and brain microenvironments. These results demonstrate how the tumor microenvironment has a powerful role in determining the net effect of HIF-1α on tumor growth, angiogenesis, and invasiveness.

Mammary Carcinogenesis

The viral oncogene polyoma middle T antigen controlled by the murine mammary tumor virus promoter (MMTV-PyMT) is a well characterized and widely used transgenic mouse model of breast cancer and metastasis (Lin et al., 2003). Female mice carrying one copy of the transgene develop mammary gland hyperplasia, carcinoma, and finally pulmonary metastases over the course of approximately six months. Mice carrying this transgene were crossed into HIF WT or mammary epithelium conditional null. HIF-1α mammary null mice displayed increased latency to the first palpable tumor along with decreased vascular density. Pulmonary metastases were halved in a HIF-1α dependent

manner. *In vitro* experiments revealed null cells had impaired random migration and directed chemotaxis versus WT controls when cultured in hypoxia (Liao et al., 2007).

pVHL and von-Hippel Lindau Disease

Silencing or mutating the VHL tumor suppressor predisposes humans to a specific set of cancers including sporadic clear cell renal carcinoma, hemangioblastoma, and pheochromocytoma (Kaelin, 2007). In order to both establish the fidelity of the pVHL null as a mouse model of human von Hippel-Lindau disease and to determine if pVHL deficiency alone can result in symptoms resembling von Hippel-Lindau disease, an important question to ask was whether murine VHL heterozygotes are susceptible to equivalents of the same types of cancer as human VHL disease carriers.

Indeed, mice with only one functional copy of pVHL are prone to developing vascular tumors as they age. The incidence of cavernous hemangiomas was ~50% at 3-12 months of age, increasing to 90% as the mice aged from 12-17 months. In contrast to the high prevalence of vascular tumors, precancerous kidney lesions were rare – only one of thirty heterozygote mice had a renal cyst (Haase et al., 2001). Given the shorter life of mice as compared to humans, perhaps deletion of both pVHL alleles was necessary to observe a higher percentage of renal cysts in the mouse model. Further experiments performed in this area using PEPCK-cre recombinase to knockout pVHL in the kidney and partially in the liver demonstrated, apart from increased EPO production and polycythemia, a higher incidence of macroscopic and microscopic renal cysts that required ARNT but did not require HIF-1α (Rankin et al., 2006).

Another approach to gain insight into pVHL function is the use of xenografts. Deletion of pVHL in fibroblasts and injection subcutaneously into mice yielded

fibrosarcomas with increased vasculature, but with a slower growth rate. Increased levels of the HIF targets cyclin dependent kinase inhibitors p21 and p27 were faulted for the decreased growth (Mack et al., 2005).

Summary

In the 15 years that have elapsed since discovery of the first HRE in the EPO 3' enhancer (Beck et al., 1991; Pugh et al., 1991; Semenza and Wang, 1992), research focusing on the hypoxia response pathway has made significant progress in development, physiology, and cancer biology. In general, genes of the hypoxic response pathway are essential for development, as demonstrated by the requirement for HIF-1α, HIF-2α, ARNT1, ARNT2, pVHL, and VEGF for embryonic viability (Ferrara et al., 1996; Gnarra et al., 1997; Iyer et al., 1998; Keith et al., 2001; Kozak et al., 1997; Ryan et al., 1998; Tian et al., 1998). Hypoxia plays a role in physiology and pathology in adult mammals, from the chondrocytes in the growth plates of developing bone to neurons in stroke and cerebral ischemia to myeloid cells in areas of inflammation (Cramer and Johnson, 2003; Cramer et al., 2003; Helton et al., 2005; Schipani et al., 2001). Indeed, in all of these microenvironments HIF has been found to play a role in physiology and pathogenesis, confirming the relevance of the hypoxic response beyond development. Most reports indicate a protumor role for HIF in the rapidly growing neoplasm, with its requirement for neovascularization and survival under low oxygen tensions (Jensen et al., 2006; Ryan et al., 1998; Ryan et al., 2000; Zhang et al., 2004). That said, many important questions still remain in the relatively new area of investigation of the mammalian hypoxic response *in vivo*.

Table 1.1: Selected milestones and transgenic mice in 15 years of HIF research

1992	*HRE identified and HIF-1a First Described*	(Semenza and Wang, 1992)
1995	*HIF-1a Purified and Characterized*	(Wang et al., 1995; Wang and Semenza, 1995)
1996	VEGF-A Knockout	(Carmeliet et al., 1996; Ferrara et al., 1996)
1997	ARNT1 Knockout	(Kozak et al., 1997; Maltepe et al., 1997)
1997	pVHL Knockout	(Gnarra et al., 1997)
1997	*HIF-2a Published*	(Ema et al., 1997; Flamme et al., 1997; Tian et al., 1997)
1998	*HIF-3a Published*	(Gu et al., 1998)
1998	HIF-1a Knockout	(Iyer et al., 1998; Ryan et al., 1998)
1998	HIF-2a Knockout	(Tian et al., 1998)
2001	ARNT2 Knockout	(Keith et al., 2001)
2001	HIF-1a ODD Mutant in Skin	(Elson et al., 2001)
2001	*FIH Published*	(Mahon et al., 2001)
2001	*HIF Regulation by Prolyl Hydroxylases Published*	(Bruick and McKnight, 2001; Epstein et al., 2001; Ivan et al., 2001; Jaakkola et al., 2001)
2006	ODD-Luciferase Mouse	(Safran et al., 2006)
2006	HIF-1a and HIF-2a Proline Æ Alanine Mutants	(Kim et al., 2006)
2006	PHD1, 2, and 3 Knockouts Described	(Takeda et al., 2006)

Chapter 2

Myeloid HIF-1alpha in the Tumor Microenvironment

Introduction

A clinically significant hallmark of solid tumors is their resistance to immunotherapy. With the exception of melanoma, strategies employing T cells specific to tumor antigens have shown poor efficacy in the clinic despite *in vitro* success (Leen et al., 2007). Recent research has explored how the tumor microenvironment is immunosuppressive to infiltrating T cells (Talmadge, 2007).

The tumor microenvironment exhibits several characteristics not found in normal tissues. Tumors are frequently marked by regions of hypoxia as rapidly dividing tumor cells outstrip the capacity of the established vasculature to deliver oxygen and nutrients and remove waste products (Vaupel et al., 2007; Vaupel and Mayer, 2007). Another aspect of tumor biology is the nearly omnipresent infiltration of hematopoietic cells, including macrophages. It is in precisely this hypoxic, macrophage infiltrated tumor microenvironment that T cells must function if tumor immunotherapy is to work.

The Hypoxia-Inducible Factor-1 alpha (HIF-1alpha) is a constitutively expressed transcription factor found in nearly all mammalian cell types. Normal oxygen tensions result in rapid and specific proline hydroxylation, allowing for recognition by the VHL tumor suppressor complex, which then polyubiquitinates HIF-1alpha targeting it for degradation (Maxwell et al., 1993; Semenza and Wang, 1992; Wang and Semenza, 1993). Lower oxygen levels allow HIF-1alpha to escape hydroxylation, after which it heterodimerizes with HIF-1beta and translocates to the nucleus where it upregulates transcription of genes whose regulatory sequences contain a hypoxic response element (HRE). Initial characterization of the role of HIF-1alpha in myeloid cells demonstrated it

was essential for the capacity to mount a full immune response (Cramer et al., 2003; Peyssonnaux et al., 2005).

There has been much debate about the role of infiltrating macrophages in the tumor microenvironment. Recent research has highlighted the role of macrophages as being pro-tumor rather than anti-tumor as first suspected (Lin and Pollard, 2004). Although macrophages are known to interact with cancer cells, the effect of tumor infiltrating macrophages on T cells in the hypoxic tumor microenvironment is poorly understood.

Myeloid cells are capable of antigen presentation and co-stimulation, and through a variety of mechanisms myeloid cells have the capacity to either promote T cell activation or suppression. Myeloid regulation of T cells is complex, with myeloid cell ligand to T cell co-stimulation receptor interactions, secretion of stimulatory or inhibitory cytokines, production of other diffusible factors, or modification of the amino acid pool in the extracellular environment. Several independent labs have documented the role of myeloid cell L-arginine metabolism in T cell suppression. The effect can be generalized as arginine metabolism because depletion of arginine by arginase I (ArgI) or production of the arginine catabolite nitric oxide by the inducible nitric oxide synthase (iNOS) are both reported mechanisms of T cell suppression. Myeloid cells are capable of a striking upregulation of iNOS and ArgI enzymes dependent on specific signaling events. This upregulation is further potentiated by low oxygen tensions found in cancer and other pathological processes, suggesting a role for the HIF dependent hypoxic regulation of iNOS and ArgI in myeloid cell mediated T cell suppression.

We used *in vitro* and *in vivo* methods to demonstrate the T cell suppressive role of the myeloid cell hypoxic response mediated by HIF-1alpha. Previous work suggests HIF-1 inhibition could have beneficial effects in restricting tumor growth (Ryan et al., 2000). The results of this work in combination with previous results suggest HIF-1 inhibition would both limit tumor growth and potentially enhance anti-tumor adaptive immune capacity. Understanding HIF dependent mechanisms of tumor immunosuppression of T cells may suggest therapeutic strategies which could be coupled with already well developed methods of generating cytotoxic T cells *in vitro* to improve the efficacy of tumor immunotherapeutic approaches.

Materials and Methods

Cell culture and hypoxic incubation

Resident peritoneal macrophages were obtained through peritoneal lavage with 10 mls of cold PBS without Ca2+/Mg2+. Resulting cells were pelleted and resuspended in RPMI 1640/10% FBS/1% PenStrep and plated on 15 cm Petri dishes overnight. Media was then aspirated and plates were washed with DPBS two times before addition of cold PBS + 15 mM EDTA. After incubation for 10-15 minutes, adherent cells were removed by pipetteing, which removed the majority of the cells followed by light scraping to maximize yield. Bone Marrow Derived Macrophages (BMDM) were obtained by incubating the lavage of murine femur and tibia from rodents of the indicated genotype with RPMI 1640/20% FBS/30% L929 Cell Supernatant/1% PenStrep/1% Amphotericin B in two 15 cm Petri dishes. After 6 days in culture, media was aspirated and the dish washed 1X in PBS before harvesting in the same manner as resident peritoneal macrophages detailed above. Cells were then plated in RPMI media as above overnight

before experimental manipulation. Hypoxic incubation was performed in a water jacketed humidified multi-gas tissue culture incubator equipped with nitrogen and carbon dioxide which flushes out oxygen until reaching a final concentration of 1% oxygen/5% carbon dioxide. Parallel normoxic incubation as carried out in standard humidified 5% carbon dioxide TC incubators. Co-culture experiments were carried out in 0.4um pore Transwell Plates (Corning Life Sciences, Lowell, MA) in either the 6-well or 24 well formats following the manufacturers instructions.

T cell macrophage co-culture proliferation assay

Resident peritoneal macrophages were isolated from HIF-1alpha WT or HIF DF/LysM-cre+ littermates as described above. Splenocytes were isolated from a separate WT C57bl/6J mouse by removal of the spleen followed by gentle squeezing between frosted glass slides and ACK hypotonic red blood cell lysis. The resulting single cell suspension lacking platelets and red blood cells was counted and labeled with CFSE to later quantify T cell proliferation. $1x10^5$ CFSE labeled splenocytes were plated in 24 well CD3/28 coated plates to induce T cell proliferation either with or without $1 x10^4$ macrophages, which were added 3 hours after T cell activation (modified from (Atochina et al., 2001). Cells were then incubated in normoxia or hypoxia (1% oxygen) for 60h with or without the iNOS specific inhibitor 1400W at 100μM. Analysis of cells was performed on a FACScalibur flow cytometer after labeling with fluorescent CD8 antibodies and propidium iodide to exclude dead cells. Because cell division results in a decrease in CFSE staining, proliferation was measured with FloJo Analysis software by setting a marker to an arbitrary division and determining the % of cells that make it to this division.

Animal model of mammary carcinogenesis in backcrossed C57bl/6J mice

Backcrossed mice carrying the polyoma middle T oncogene (PyMT) under the control of the Murine Mammary Tumor Virus long terminal repeat (MMTV) (Guy et al., 1992), here referred to simply as PyMT, were originally obtained from the lab of Dr. Leslie Ellies (UCSD Medical Center, La Jolla, CA). Individual transgenic alleles for LysM-cre (Clausen et al., 1999) and HIF-1alpha flox (Ryan et al., 2000) were backcrossed to C57bl/6J to > 99% using Jackson labs speed backcrossing SNP genotyping service. After one further cross to C57bl/6J, mice were crossed to obtain female HIF-1alpha DF/PyMT+/-/LysM-cre+/- (experimental) and female littermate controls HIF-1alpha DF/PyMT+/-/LysM-cre-/-. Mice were palpated weekly to determine time to first tumor and tumor volume. Endpoint was empirically determined to be 20 weeks, at which point mice were sacrificed and endpoints were measured.

Enzyme assays

Tumors were rapidly dissected out of endpoint animals and flash frozen in liquid N_2. A mortar and pestle was used to obtain a powder, and then ~100mg was added to Homogenization buffer from the NOS assay Kit (Cayman Chemical, Ann Arbor, MI). After Polytron homogenization of the sample on ice and pelleting of insoluble material, iNOS activity was determined by measuring the ability of the lysate to hydrolyze ^{14}C labeled L-arginine (GE Healthcare Life Sciences, Piscataway, NJ) into L-citrulline in the absence of calcium. Arginase activity from the same samples was determined by measuring generation of urea from an excess of L-arginine as done by others ((Corraliza et al., 1994). Both iNOS and arginase activities were then normalized to total protein in the lysate as measured by the BCA assay (Thermo Fisher Scientific/Pierce, Rockford,

IL).

Western blotting

Media was aspirated from TC wells and 1ml of PBS was added to wells before removal with a cell scraper. After brief centrifugation, cell pellets were resuspended in RIPA buffer with protease inhibitors. Quantitation of protein was performed using the BCA assay. Twenty micrograms of whole cell extract was analyzed following the manufacturers protocol using the Nupage SDS-PAGE Western blotting system and gel appropriate to resolve the protein of interest (Invitrogen).

Flow cytometry, immunohistochemistry, antibodies, recombinant cytokines, and real-time PCR reagents

Single cell suspensions of tumors were generated by chopping with a razor blade followed by incubation at 300 RPM at 37 degrees for 60 minutes in 5 mls 0.22um filtered RPMI 1640 media (no serum)/0.05-0.1% Collagenase A (Roche Diagnostics, Indianapolis, IN)/DNase I. Resulting suspensions were pelleted, rinsed in PBS, and filtered through a 40μm cell strainer. Hypotonic lysis with ACK buffer was performed before pelleting and resuspending in media. RPMI 1640/10% FBS/25mM HEPES/1% Pen/Strep was used for experiments except where T cells were added, where Beta-mercaptoethanol (Invitrogen) was added.

Cells were typically prepared for cytometry by staining 1 x 10^6 cells with the appropriate 1/200 fluorochrome conjugated aB in staining buffer (PBS no $Ca^{2+/}Mg^{2+}$ /0.2% BSA) on ice for 30 minutes, rinsing in staining buffer, then resuspending in staining buffer with or without propidium iodide and acquiring on a FACSCalibur flow cytometer. All fluorescent antibodies, recombinant mouse IFNg, and Interleukin-4 were

from eBioscience (San Diego, CA) with the exception of F4/80 (AbD Serotec, Raleigh, NC).

For histology, tumors were either frozen in OCT (Sakura Finetek USA, Torrance, CA), fixed in Zinc buffer (BD Biosciences, CA), or fixed in 10% buffered formalin before being cut into 4-10 um sections. ABC Elite HRP or AP kits from Vector Labs (Burlingame, CA) were used for IHC following the manufactures instructions. Biotin anti-CD31 and Biotin anti-PCNA were from BD Pharmingen (La Jolla, CA). Rabbit anti-arginase I was from Santa Cruz (Santa Cruz, CA). Rabbit anti-iNOS was from Calbiochem/EMD Biosciences (San Diego, CA). Colorimetric TUNEL staining was performed using the Promega DEADend kit (Madison, WI).

Gene expression was carried out following the manufactures instructions on approximately 200 ng of total RNA isolated using the Qiagen (Valencia, CA) RNeasy kit with on column DNase treatment and subsequent reverse transcription by the Invitrogen Superscript III first strand kit using random hexamer priming. Real time PCR was performed using an ABI 7700 with either SYBR green or TaqMan fluorescent detection. Fold expression was determined using the ΔΔCT method of target sequence normalized to levels of 18S rRNA. Target and reference real time reactions were run in duplicate and the average used to calculate ΔΔCT. Error bars indicate the SEM of multiple samples, not duplicate reactions.

Results

HIF-1alpha, not HIF-2alpha, controls macrophage ArgI and iNOS

Macrophages are exquisitely sensitive to extracellular signals, including cytokines and oxygen tension (Cramer et al., 2003). The Th1 cytokine IFNg is known to induce

iNOS but not ArgI, whereas the Th2 cytokine IL-4 is has the opposite effect of inducing ArgI but not iNOS (Modolell et al., 1995). Both of these transcripts have been reported to be upregulated under hypoxia (Louis et al., 1998; Melillo et al., 1995). In order to determine the relevance of HIF-1alpha to ArgI and iNOS gene regulation after stimulation by established agonists IFNg or IL-4, in vitro culture of wild type or HIF-1alpha murine BMDM with recombinant cytokines typically produced by Th1 or Th2 cells was performed under ambient oxygen tension or 1% oxygen for 24 hours (Figure 2.1).

As expected, after IFNg stimulation macrophages dramatically upregulated the transcript for iNOS. Hypoxic incubation further potentiated the transcription of iNOS in WT cells by approximately seven fold over the normoxic control. No appreciable induction of iNOS was detected in IL-4 treated BMDM. IL-4 stimulation resulted in induction of the ArgI gene, and hypoxia further increased this expression by approximately nine fold brining total induction over unstimulated controls to nearly 4 log.

HIF-1alpha contributes to iNOS and ArgI gene regulation in both normoxia and hypoxia relative to the knockout. There remains some hypoxic induction of ArgI and iNOS transcripts under hypoxia in the HIF-1 knockout. We decided to test whether this induction was mediated by HIF-2alpha by performing an identical experiment with WT, HIF-1alpha KO, or HIF-1alpha/HIF-2alpha double knockout BMDM. To our surprise, there was no change in hypoxic fold induction of iNOS or ArgI between HIF-1alpha KO and HIF-1alpha/2alpha double knockouts, suggesting another mechanism is responsible for the observed partial HIF-1 independent upregulation of these transcripts under

hypoxia. This is in spite of detection of the HIF-2alpha transcript in the same macrophages (data not shown).

Western blotting of iNOS and ArgI from bone marrow derived macrophage whole cell extracts was performed to verify the HIF-1alpha mediated effects were also reflected at the protein level Strong expression of iNOS and ArgI were detected in WT IFNg or IL4 conditions under hypoxia, respectively. Ablation of HIF-1alpha resulted in a marked decrease of hypoxic iNOS and Arg I protein levels under identical stimulation conditions to the WT.

Macrophages inhibit T cell proliferation in a hypoxia/HIF-1alpha/iNOS dependent fashion

T cell activation and resulting proliferation are essential steps in the adaptive immune response to increase the clonal frequency of an antigen specific T cells as well as to differentiate them into an effector or memory cell. Within hours of activation, T cells upregulate IFNg and secrete it into the extracellular microenvironment. Given the potent upregulation of iNOS in macrophages after IFNg treatment, we wanted to test how T cells would proliferate if activated in close proximity to macrophages either with or without HIF-1alpha (Figure 2.2).

The proliferative capacity of T cells was markedly blunted by co-culture with WT macrophages under hypoxia. Visualization of the number of cell divisions via CFSE labeling and subsequent flow cytometry allowed for quantitation of this effect. T cells are able to proliferate under hypoxia (1% oxygen) after CD3/28 stimulation. However, when macrophages were added to 1/10 of the total cell number, cell cycle progression was blocked in a genotype dependent fashion. Remarkably, macrophages null for HIF-

1alpha showed no such effect, with cell divisions nearly identical to no macrophage added controls. Addition of the specific inhibitor of iNOS 1400W to the WT macrophage cultures restored T cell proliferation to normal levels. Control experiments demonstrated no nonspecific stimulatory or inhibitory effects of 1400W on T cell proliferation.

Myeloid HIF-1alpha in the tumor microenvironment affects tumor mass, TUNEL positivity, ArgI and iNOS activity, but does not affect microvascularity

In order to assess the role of myeloid HIF-1alpha in the tumor microenvironment, we crossed in the myeloid specific deletion of HIF-1alpha (HIF DF/LysM-cre) with the established model mammary carcinogenesis, MMTV-polyoma middle T (Guy et al., 1992; Lin et al., 2003) Because these tumors have a fixed oncogenic stimulus via the transgene yet require further mutations in order to progress to later tumor stages over several months, we felt this model was ideal to test what role macrophage HIF-1alpha would have over an extended time period in the tumor microenvironment, better reflecting human malignancies than xenograft systems which typically take only a few weeks. Furthermore, like many human cancers, the PyMT model exhibits heavy macrophage infiltration after initial tumor formation.

Previous work has shown that macrophages are required for the initial formation of the ductal tree in the murine mammary gland. To ensure similar development of the control and myeloid HIF-1 KO mammary glands, we performed whole mount staining of the epithelial ductal tree. No deficiencies in ductal tree formation were detected between the genotypes, with both displaying branching structures extending through the entire fat pad. Although myeloid cells are required for the initial growth of the ductal tree, there is

scant macrophage infiltration at the earliest stages of carcinogenesis in the PyMT model (data not shown). Therefore, it was not surprising to note no difference in latency, or time to first palpable tumor, between WT or myeloid HIF-1alpha KO mice, given the transgenic oncogene and the lack of myeloid cells to interact with the nascent tumor (Figure 2.3). Ten weeks after detection of the first tumor growth, endpoint tumor mass was taken at 20 weeks of age by dissecting out all 10 glands and taking their mass. Total tumor mass was significantly lower in the HIF-1alpha knockout mice. Histological analysis by Hematoxylin and Eosin revealed striking differences between the tumors, with myeloid KO tumors having a more nodular and cell dense appearance. Analysis of proliferating cells by proliferating cell nuclear antigen (PCNA) demonstrated that it was the solid, nodular masses present in the WT that had the highest amount of proliferating cells.

To our surprise, despite increased proliferation in the myeloid HIF-1alpha WT, TUNEL analysis of the tumors showed an increase in cell death in the myeloid HIF-1alpha KO. This effect occurred in pre-cancerous Ad/MIN and early carcinoma stages. Late carcinoma stages displayed a high level of apoptosis in both WT and KO, likely a result of central necrotic regions typical advanced solid tumors.

A prominent role for the HIF oxygen sensitive transcriptional pathway in development and pathology is angiogenesis. HIF-1 exerts partial control of VEGF transcription, and as such one plausible way that myeloid HIF-1alpha null tumors could be affected is via decreased VEGF expression from macrophages, which could affect tumor vascularization. Despite this regulation, whole tumor VEGF levels were nearly identical as determined by gene expression. Furthermore, CD31 staining of tumor

sections to determine stage dependent vascular density revealed only slight changes in density based on genotype. Myeloid HIF-1alpha KO tumors even displayed a slight increase in CD31 positive cells, which is opposite to what one would expect if macrophage HIF-1alpha were contributing to the tumor vasculature.

As shown previously here, two macrophage HIF-1 dependent enzymes relevant to the tumor microenviroment include ArgI and iNOS. To determine if the tumor demonstrated similar regulation of these enzymes as in vitro experiments, we isolated tumor lysates from myeloid HIF-1alpha WT and KO mice. Analysis of tumor lysates taken from WT and KO tumors displayed differential activity of HIF-1alpha regulated enzymes ArgI and iNOS, with both being higher in the WT tumors (Figure 2.4). Whereas nearly all WT tumors had high ArgI activities, iNOS was high in some samples and lower in others in the WT group. iNOS was uniformly low in the KO group. Restated, iNOS activity levels in WT displayed increased variance relative to KO despite similar infiltration of T cells and macrophages between WT and myeloid KO tumors.

Macrophage HIF-1alpha dependent induction of ArgI after co-culture with PyMT MECs under hypoxia

Although T cells are thought to be the major source of IFNg or IL4 depending on Th1 vs. Th2 polarization, we wanted to know if cancer cells alone combined with hypoxia might induce the iNOS or ArgI genes in macrophages in close proximity. There are reports of induction of ArgI in macrophages based on soluble mediators from cancer cells (Rodriguez et al., 2005), but not under hypoxic conditions. In order to model the tumor microenvironment in vitro, we plated bone marrow derived macrophages on the bottom of a Transwell dish and polyoma middle T mammary epithelial cells on the upper

well. In this culture design, both cells share the same media via a porous membrane on which the PyMT cells were plated. The pores had a diameter of only 0.4 um, which would allow diffusion of soluble factors but not of cells. Incubation of WT or HIF-1alpha null macrophages in normoxia or hypoxia with or without PyMT MEC cells for 24 hours and subsequent western blotting revealed that ArgI is markedly induced by co-culture with macrophages, but only under hypoxia and in macrophages WT for HIF-1alpha.

Macrophage HIF-1alpha makes tumor infiltrating T cells less responsive

HIF-1alpha controlled enzymes ArgI and iNOS are thought to make T cells unresponsive or anergic (Bronte et al., 2003; Kropf et al., 2007; Matlack et al., 2006; Munder et al., 2006). One measurement of cytotoxic T cell responsiveness is the ability to produce interferon-gamma (IFNg) after stimulation. We decided to test tumor T cell responsiveness from myeloid HIF-1alpha WT and KO tumors as by measuring the % of the cells that make IFNg after a strong pan T cell stimulation with anti-CD3/28 antibody stimulation of tumor single cell suspensions in vitro. There was a marked reduction in the % of T cells that made IFNg from myeloid HIF-1 WT PyMT tumors as compared to the myeloid HIF-1alpha null.

Discussion

Tumor immunosuppression of infiltrating T cell anti-tumor activity is a major roadblock to tumor immunotherapy strategies. There have been numerous reported mechanisms of immunusuppression, including local enzymatic activity of iNOS and ArgI (Bronte and Zanovello, 2005; Mazzoni et al., 2002; Munder et al., 2006). Here we show that HIF-1alpha, and not HIF-2alpha, has significant control of both of these

immunosuppressive enzymes and that these enzymes have important effects on T cell proliferative capacity and responsiveness to stimulation.

Macrophage iNOS and ArgI are both inducible enzymes that consume L-arginine. iNOS catabolizes L-arginine into nitric oxide and L-citrulline, while ArgI produces L-ornithine and Urea. iNOS and ArgI have been previously reported to be hypoxia inducible genes (Louis et al., 1998), yet genetic evidence implicating the HIF pathway and the relative contribution of HIF-1 vs. HIF-2 alpha in macrophages has not previously been reported. The lack of HIF-2alpha contribution to ArgI and iNOS hypoxic induction was not expected, as HIF-2alpha has been detected in macrophages (Talks et al., 2000). Indeed, experiments in our lab confirmed HIF-2alpha transcript in murine macrophages, yet regulation of classical target genes was not detected (data not shown). Whereas HIF-1alpha is a functional oxygen responsive transcription factor in nearly all mammalian cell types known, HIF-2alpha forms an active transcription factor in only a subset of cells including the kidney and not, for example, in fibroblasts (Park et al., 2003). We suspect this may be the case in macrophages as well.

The potent anti-proliferative effect on T cells by myeloid HIF-1alpha in the co-culture assay was iNOS and hypoxia dependent. Several previous reports have reported that iNOS can inhibit T cell proliferation (Albina et al., 1991; Atochina et al., 2001; Gallina et al., 2006; Mazzoni et al., 2002), yet none report on whether HIF-1 control of iNOS is sufficient to suppress T cell proliferation. iNOS dependence was clearly established by rescuing the cell cycle suppression with 1400W, a specific iNOS inhibitor. Other experiments with the ArgI inhibitor norNOHA and macrophages from mice deficient in iNOS also showed that, in this assay, the anti-proliferative effect was iNOS

dependent and ArgI independent (data not shown).

The PyMT model of mammary tumorigenesis is a well-characterized, murine transgenic model of human breast cancer (Guy et al., 1992; Lin et al., 2003). Female mice carrying one copy of the transgene are normal compared to littermate controls until 6-8 weeks, when female sex hormones induce the PyMT transgene in the mammary epithelium of the ten murine mammary glands. Despite the activation of the oncogene, there is still an orderly progression of carcinogenesis from hyperplasia, Adenoma/MIN, early carcinoma, and finally late carcinoma. This malignant progression, which depends on mouse inbred strain background, takes months from the first detected hyperplasia in the glands at around 10 weeks of age to the endpoint at 20 weeks of age in the C57bl/6J inbred strain. Although macrophages infiltrate nearly all neoplasms, macrophage infiltration is invariably a salient feature of the PyMT model. We used the PyMT transgenic model with a myeloid specific knockout of HIF-1alpha to determine *in vivo* the role of the myeloid hypoxic response in the tumor microenvironment. Both WT and myeloid HIF-1alpha null animals showed a similar time to first tumor or latency, which is expected since macrophage infiltration is sparse before tumorigenesis. However, at endpoint 10 weeks later, there were marked changes observed in tumor mass and gross histology. The decrease in tumor mass in HIF-1alpha myeloid KO animals at endpoint was accompanied by decreased proliferation and increased apoptosis.

HIF-1 exerts partial control over the hypoxic induction of the angiogenic protein VEGF. It was important to determine if myeloid VEGF under HIF-1 control was controlling tumor microvascularity and resulting in the observed phenotype. To our surprise, we found no change in tumor VEGF or in microvascular density, implying that

partial control of VEGF by HIF-1alpha does not have a role in the observed phenotype.

Upon determining enzymatic activities of iNOS and ArgI were higher in myeloid HIF WT tumors, we initially wondered if cytotoxicity from NO might increase genotoxic stress in the myeloid WT and play a part in the observed phenotype. However, we were unable to detect any increase in nitrated tyrosine residues or the nitrated DNA base 8-nitroguanosine between the genotypes. This led us to look at other ways in which iNOS and ArgI could affect the tumor microenvironment.

Many independent groups have reported the immunosuppressive effects on T cells of both iNOS and ArgI from myeloid cells. We wondered if myeloid HIF-1alpha control of iNOS and ArgI in the hypoxic tumor microenvironment might be modulating the ability of cytotoxic T cells to restrain tumor growth and progression. IHC analysis of tumors detected infiltrating T cells in similar numbers in WT and KO tumors. Further analysis of T cells in tumor bearing PyMT mice versus non tumor bearing controls revealed increased CD62L low T cells along with increased CD25CD44 souble positive cells (Figure 2.8 and 2.9). This immunophenotype is consistent with increased activation of the adaptive immune system in PyMT tumor bearing mice versus controls. Overall, the PyMT results are consistent with lack of immunosuppression of cytotoxic T cells, which were found in both WT and myeloid KO tumors in similar numbers.

Co-culture of PyMT mammary epithelial cells resulted in a robust induction of ArgI protein in macrophages under hypoxia. In this assay, we did not detect iNOS induction at the protein level, although it could be detected at the RNA level only when MECs were present (data not shown). Apart from bacterial and viral components, IFNg from T cells is one of the most potent inducers of iNOS in murine macrophages. This

can be seen in Figure 2.1, with a 4 log induction of the iNOS transcript after IFNg treatment. Tumor cell lines often produce chemokines and cytokines, but IFNg is not usually among them. IFNg is largely produced by T cells when activated or engaged in a cytotoxic response. There were no T cells present in the PyMT-MEC co-culture model. Therefore, the results are consistent with a model having ArgI induced by soluble factors produced by the tumor cells, whereas iNOS is likely induced by other factors in the tumor, likely T cells producing IFNg. Other data in this report detail increased number of T cells that make IFNg after stimulation in the KO. How can this model and the results coexist? One possible scenario consistent with the data and T cell biology requires multiple steps where an initial T cell cytotoxic response and release of IFNg results in neighboring macrophage secretion of iNOS, suppressing T cell proliferation and inducing T cell suppression, reducing the chance that cell will respond by producing IFNg after subsequent antigen exposure. In contrast, in the KO, reduced iNOS activity means cytotoxic T cells can continue to attack tumor cells, reflected by the PyMT histology and increased % of T cells that make IFNg in response to stimulation. ArgI is thought to reduce T cell responsiveness through depletion of local arginine levels, which leads to decreased abundance of the T cell receptor zeta chain CD3ζ (Rodriguez et al., 2004; Rodriguez et al., 2002; Rodriguez et al., 2003). We checked for intracellular CD3ζ expression in T cells isolated from mice WT or KO for HIF-1alpha in myeloid cells, but a significant correlation between genotype and CD3ζ expression was not found. There was significant variation in zeta chain expression, however. The specificity of the CD3ζ staining was an issue, in that cells expected to be negative for staining still displayed significant background fluorescence (data not shown).

Recent characterization of the immunosuppressive enzyme indoleamine 2,3 dioxygenase (IDO) may be relevant to the mechanism of ArgI suppression of T cell activity (Munn et al., 2005). The authors found that suppressive regulatory T cells upregulate their suppressive activity when they have the amino acid sensor GCN2 activated by IDO depletion of tryptophan in the media. GCN2, however, presumably senses deprivation of amino acids other than tryptophan. Therefore, it is tempting to speculate that, in addition to CD3ζ downregulation, ArgI/iNOS activity may deplete arginine levels and activate GCN2 activation in Treg cells in the same way that IDO depletion of tryptophan does.

This work has not established the individual relative contribution of the enzymes iNOS and ArgI to T cell suppression. However, recent reports suggest that it may require reduced activity of both enzymes to result in increased T cell activity. The murine KO of the cationic amino acid transporter-2 (CAT2), essential for myeloid cell production of significant amounts of nitric oxide or ArgI activity (Kakuda et al., 1999; Nicholson et al., 2001), develops spontaneous lung inflammation at 3 weeks of age (Rothenberg et al., 2006). Although spontaneous inflammation has not been detected in myeloid HIF-1alpha cells, increased T cell responsiveness in tumors is consistent with the idea that coordinate reduced ArgI and iNOS activity would partially relieve T cell suppression. iNOS knockout mice are viable and appear normal. Some groups have reported global iNOS deficiency may result in increased T cell activity, although the cell type producing the iNOS was not fully established (Vig et al., 2004). Genetic deletion of ArgI and II results in lethality at less than two weeks after birth. ArgI, while regulated in the macrophage, is essential in the liver in the urea cycle to remove ammonia from the blood

stream. ArgI/II double null mice die of hyperammonemia, and display increased circulating L-arginine along with L-ornithine deficiency (Ohtani et al., 2007). Other authors have drawn attention to the fact that L-arginine metabolism in general results in T cell suppression, again suggesting that a reduction in the activity of both enzymes may be necessary for relief of T cell suppression (Bronte et al., 2003; Bronte and Zanovello, 2005).

We wondered if myeloid ArgI activity might be directly contributing to cancer cell proliferative capacity through production of L-ornithine or polyamines, which could then be used by the cancer cells to sustain proliferation. The products of ArgI include L-ornithine and urea. L-ornithine can be consumed by ornithine decarboxylase (ODC), a rate-limiting enzyme in the synthesis of polyamines including spermine, spermidine, and putrescine . Many authors have drawn attention to the essential nature of polyamines to cell proliferation, and the observation that simple overexpression of ODC can transform cells (Auvinen et al., 1992; Manni et al., 1995; Manni et al., 1997). Because one of the products of ArgI, L-ornithine, feeds into the ODC pathway, there has been speculation about the role of ArgI in polyamine production. We tested this via a co-culture model with resident peritoneal macrophages and MEC cells followed by quantitation of cancer cell cycle by propidium iodide staining and flow cytometry. By using myeloid HIF-1alpha WT and KO cells with or without the ArgI inhibitor norNOHA, we saw no HIF-1 or ArgI dependent effect on cancer cell cycle regulation (data not shown).

Apart from effects detailed here, HIF-1alpha has recently been shown to aid in cell survival and function via suppression of oxidative phosphorylation (Papandreou et al., 2006). Partially blocking oxygen consumption by oxphos raises levels of oxygen in

the cell for other processes while HIF glycolytic upregulation manages ATP production. Maintenance of intracellular oxygen levels has relevance for both the ability myeloid knockout macrophage iNOS to produce nitric oxide as well as for other enzymes that require oxygen. Another myeloid cell enzyme implicated in T cell immunosuppression is indoleamine 2,3 dioxygenase (IDO) (Lob and Konigsrainer, 2007). This enzyme is under investigation for its ability to suppress T cell function through tryptophan catabolism. Given the requirement for oxygen in IDO catalyzed reactions, it may be the case that HIF null myeloid cells would be deficient in IDO mediated suppression under hypoxic conditions, where KO cells would not be able to spare oxygen for IDO use as efficiently as WT cells.

Because of the limited number of animals available as a result of the complexity of the genetic crosses used to generate the PyMT myeloid HIF null animals, we chose an endpoint strategy to characterize the model. It may be the case that other genotype dependent effects could be observed at earlier stages of tumorigenesis. Indeed, given the massive macrophage infiltration of PyMT tumors, it is likely that HIF-1 has a variety of affects on the tumor that we did not examine. Nevertheless, HIF-1alpha control of iNOS and ArgI and their role in T cell responsiveness is important to report given the implications to immunotherapy.

Taken together, these results have important implications for the use of HIF-1alpha inhibitors in anticancer strategies. HIF-1alpha upregulates pro-tumor pathways including glycolytic enzymes and angiogenic factors, while at the same time upregulating cell cycle inhibitors including p21 and the presumably proapoptotic protein bNIP3. Initial excitement over the prospect of targeting HIF-1alpha in cancer was always

tempered with the concern over the potential release from cell cycle inhibition and proapoptotic state that could be the result of HIF-1alpha inhibition in cancer cells. Despite these concerns, the net effect of HIF-1 inhibition appears to be beneficial in animal models. Given the role of myeloid HIF-1 in T cell immunosuppression detailed here, the potential for combining HIF-1 inhibition and immunotherapy presents itself as a therapeutic strategy to target both the tumor and the immunosuppressive microenvironment created by it.

Acknowledgement

We acknowledge the expert assistance and advice of Denis Young of the UCSD Cancer Center Flow Cytometry Core. Additional thanks goes to the Histology core, also of the UCSD Cancer Center.

Chapter 2 is being prepared for publication in a scientific journal as Doedens A, Stockmann C, Rubinstein M, Liao D, Goldrath A, Johnson RS. HIF-1alpha in tumor infiltrating macrophages mediates local T cell immunosuppression. 2008. The dissertation author was the primary investigator and author of this paper.

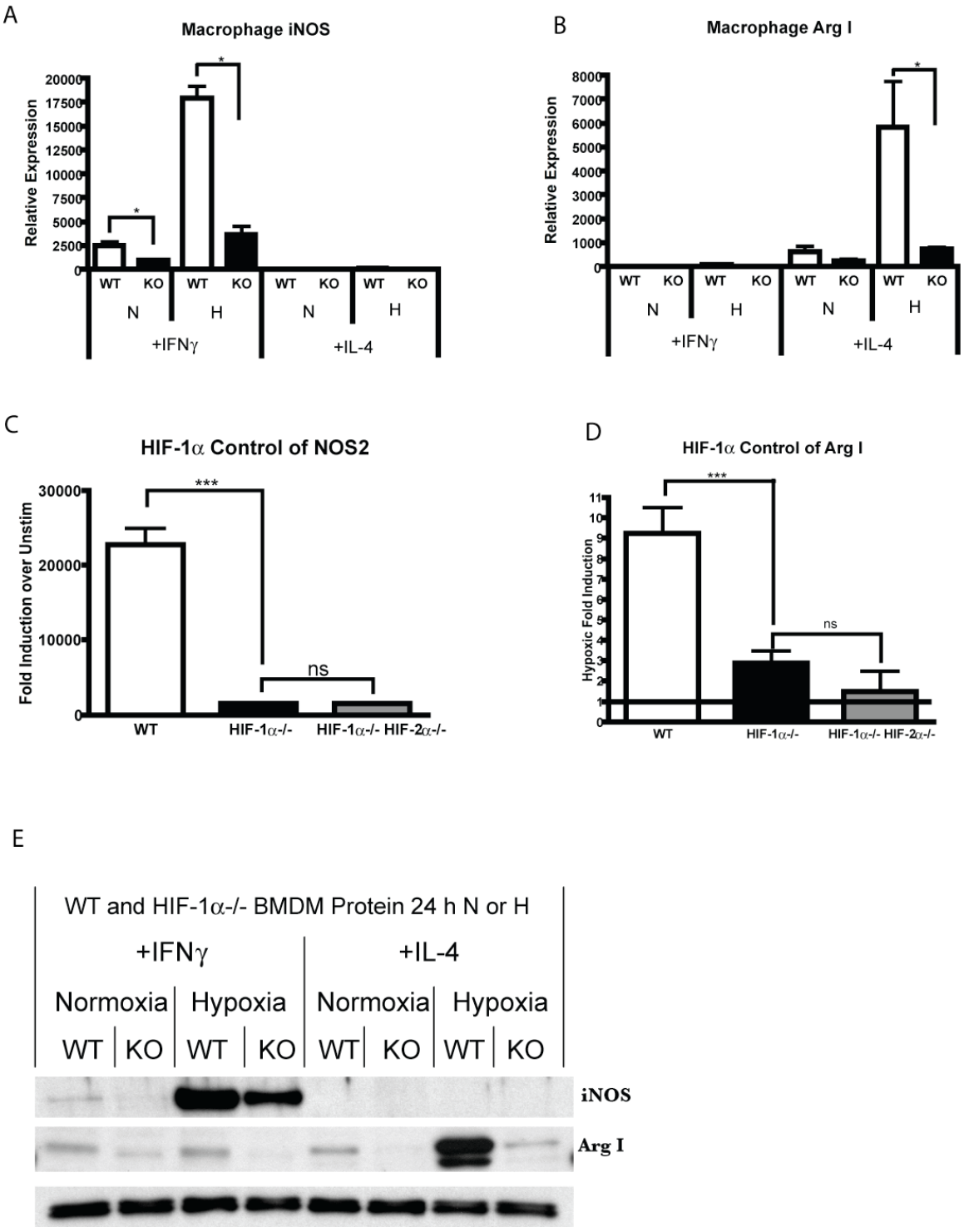


Figure 2.1: (a-d) HIF-1alpha, and not HIF-2alpha, controls murine macrophage iNOS and ArgI in response to IFNg or IL-4 in vitro. (e) This effect is also reflected at the protein level.

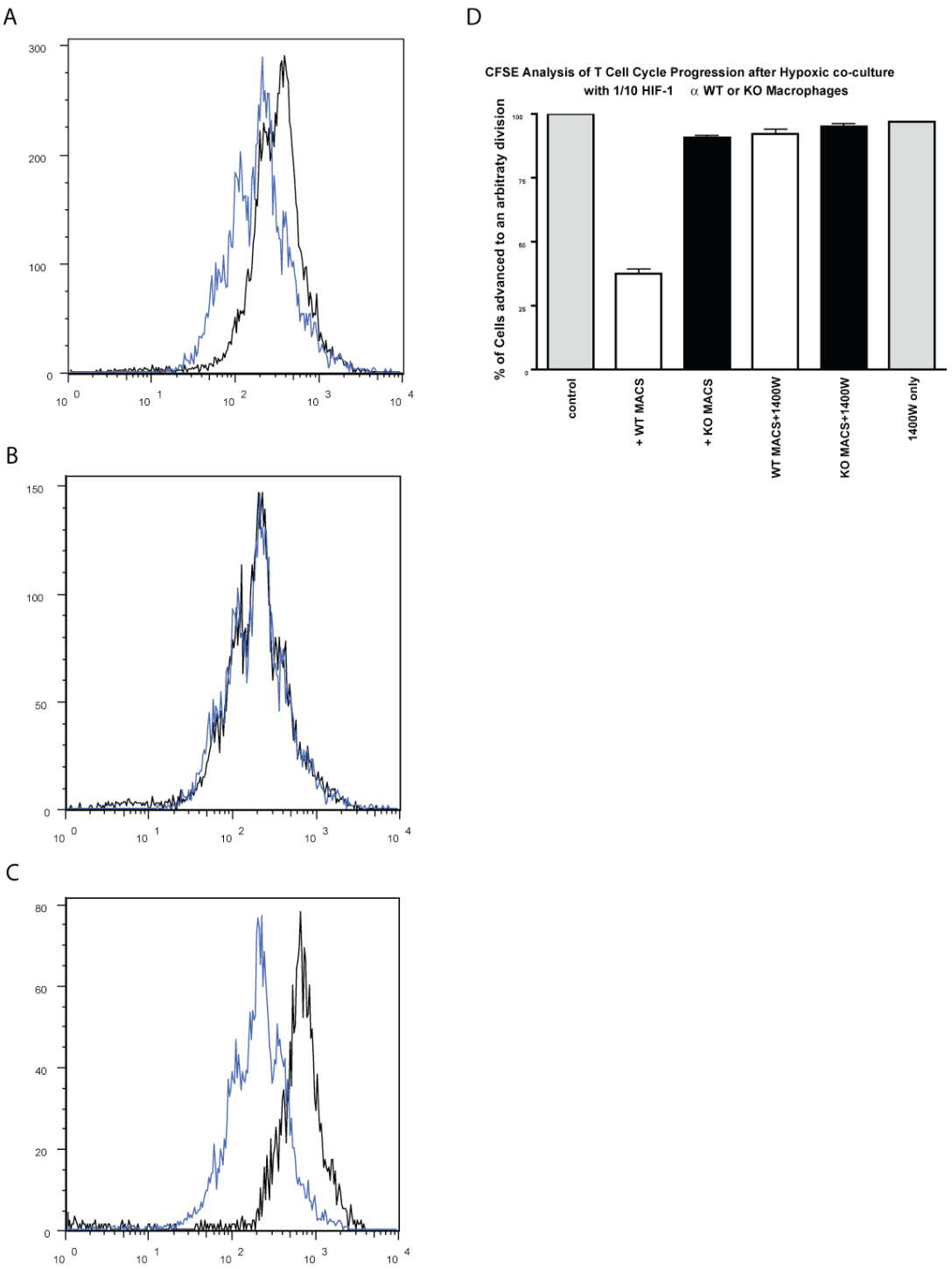


Figure 2.2: Addition of resident peritoneal macrophages to CD3/28 activated splenocytes 1:10 blocks T cell proliferation as measured by CFSE in a hypoxia/HIF-1alpha/iNOS dependent fashion. (a) Splenocytes + WT macs in black vs. splenocytes - macs in blue. (b) Splenocytes + KO macs in black vs. plenocytes - macs in blue. (c) Splenocytes + thioglycollate elicted WT macs in black vs. splenocytes + inos-/- thioglycollate macs in blue. (d) bar graph representation of suppression of CD8+ cell cycle progression.

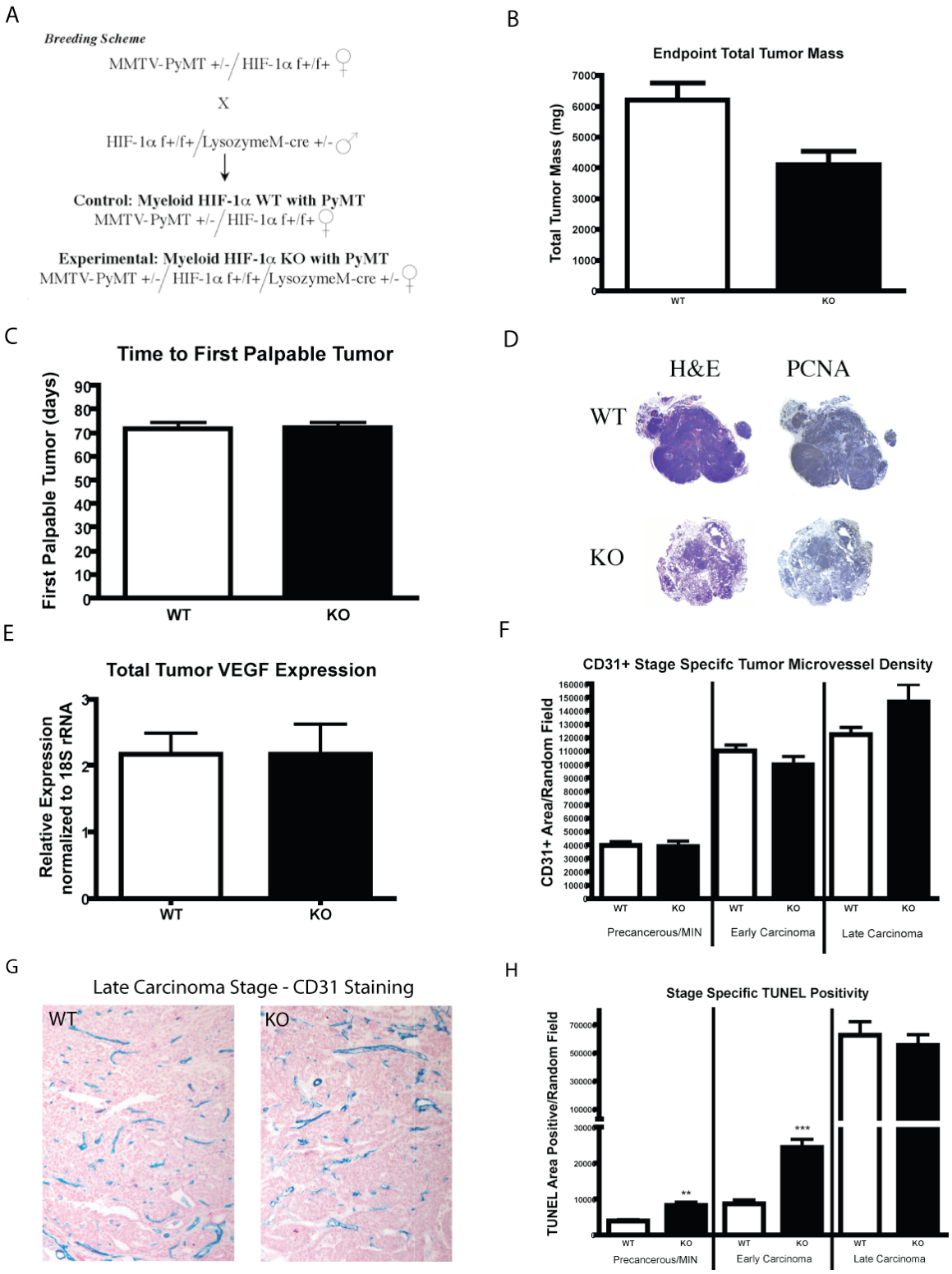


Figure 2.3: Myeloid specific HIF-1alpha knockout bred to PyMT model of breast cancer.

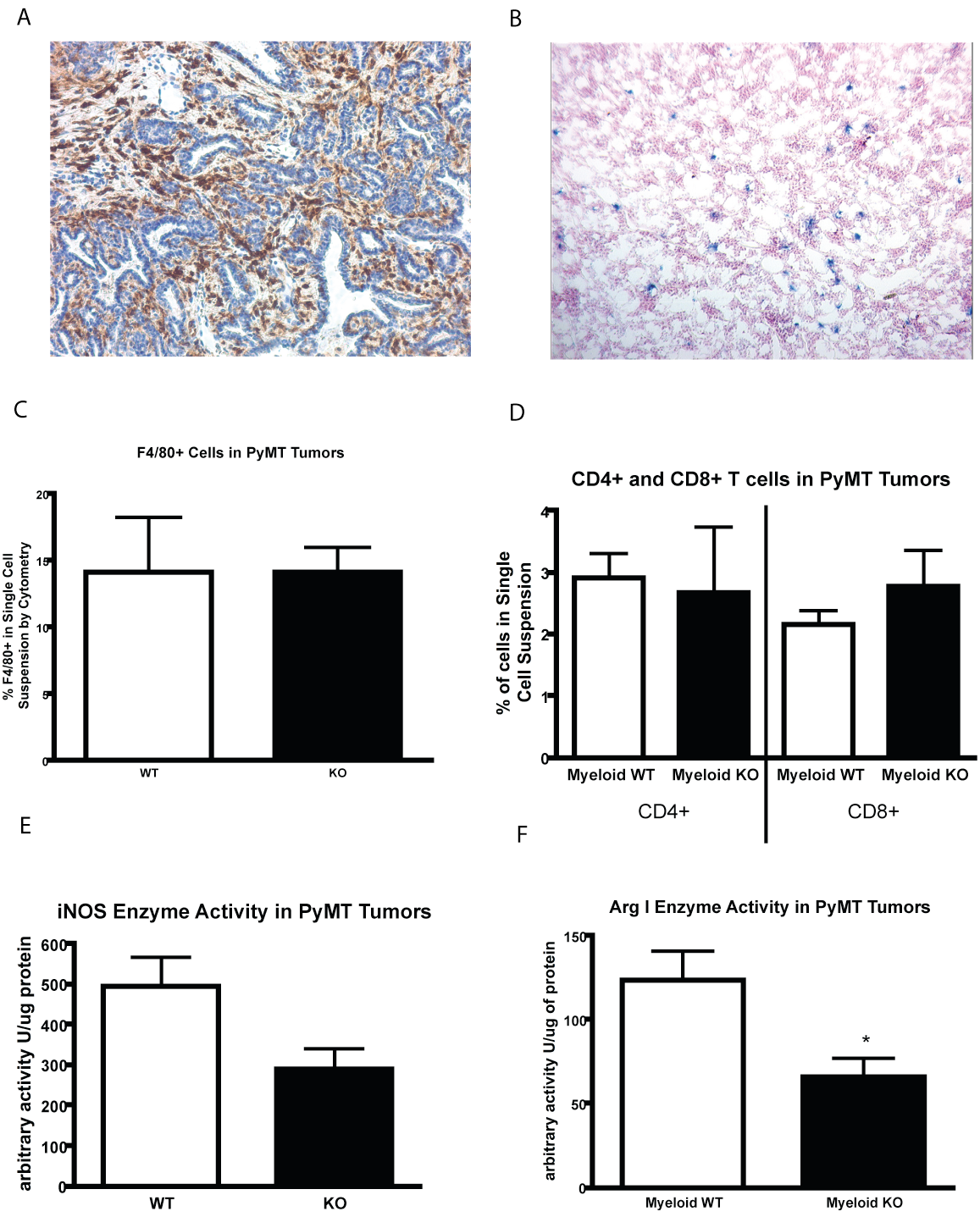


Figure 2.4: Quantitation of macrophage and T cell infiltration and HIF-1 target enzymatic activity in PyMT WT and myeloid HIF-1alpha KO. (a) F4/80 macrophage staining (brown) in hematoxylin counterstained PyMT tumor. (b) CD8+ T cell infiltrate (blue). (c,d) F4/80+ and CD4/8 infiltration by flow cytometry. (e-f) iNOS and arginase I enzyme activity from PyMT tumor

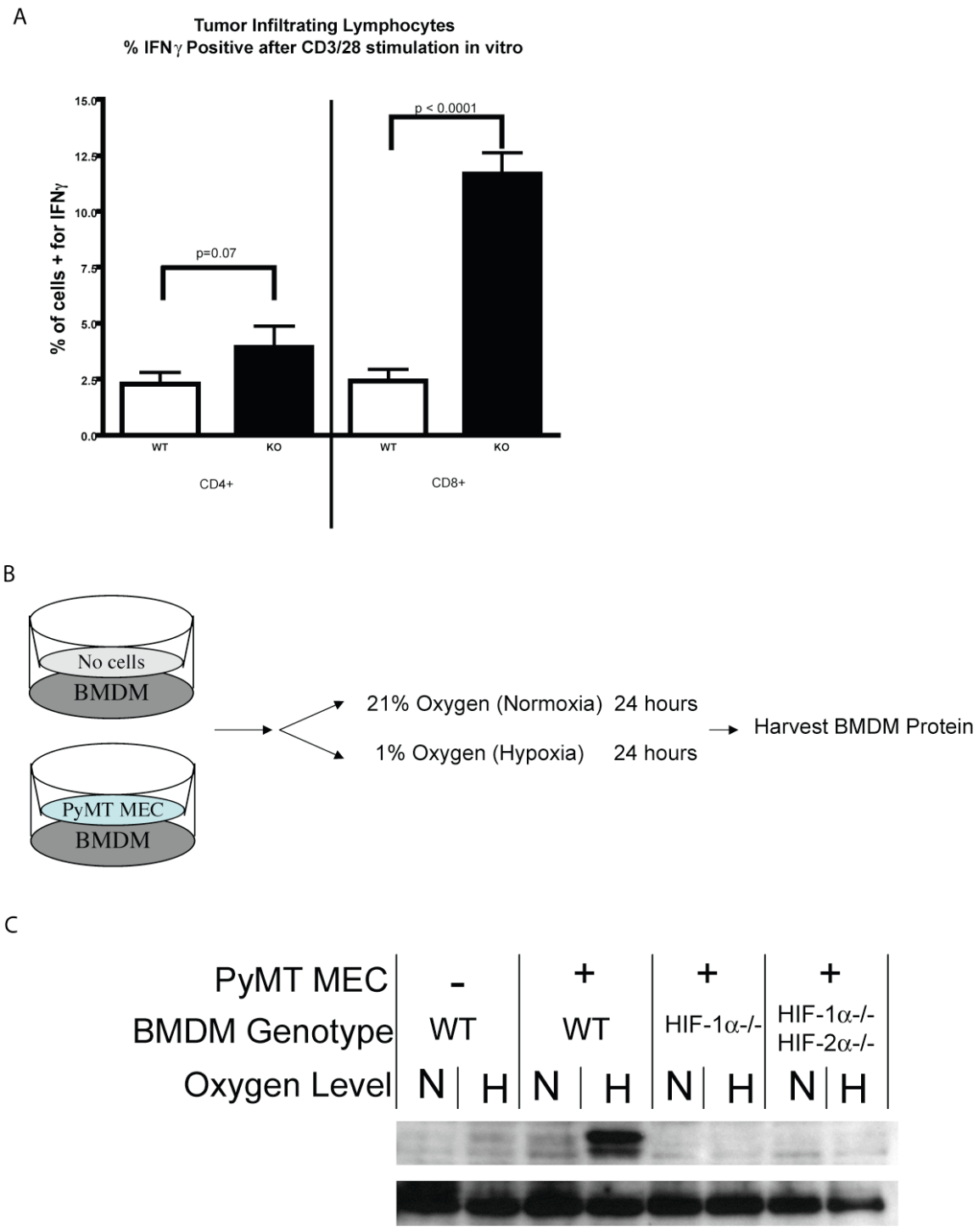


Figure 2.5: PyMT T cell responsiveness and arginase I induction in macrophages by diffusible mediator from PyMT cells. (a) T cells isolated from PyMT tumors lacking myeloid HIF-1alpha are more responsive to CD3/28 stimulation. (b) Method of co-culture of macrophages and PyMT MEC cell line. (c) Western blot for argI shows strong induction depends on MEC PyMT cells, macrophage HIF-1alpha, and hypoxia.

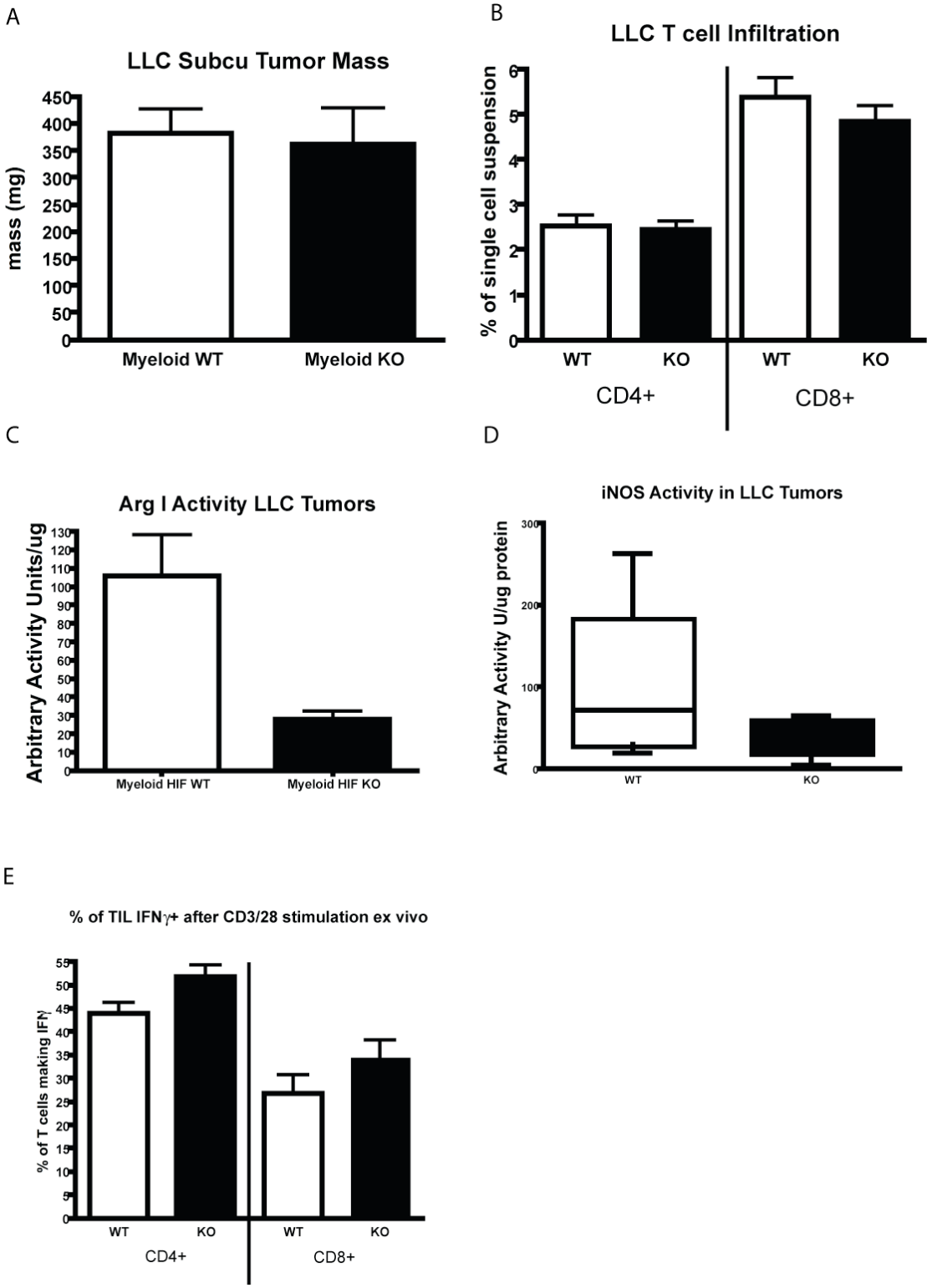


Figure 2.6: Injection of Lewis Lung Carcinoma cells into myeloid HIF-1alpha WT and KO mice. (a,b) Similar tumor mass and T cell infiltration. (c,d) HIF-1alpha dependent argI and iNOS activity. (e) Increased % of IFNg producting T cells from KO.

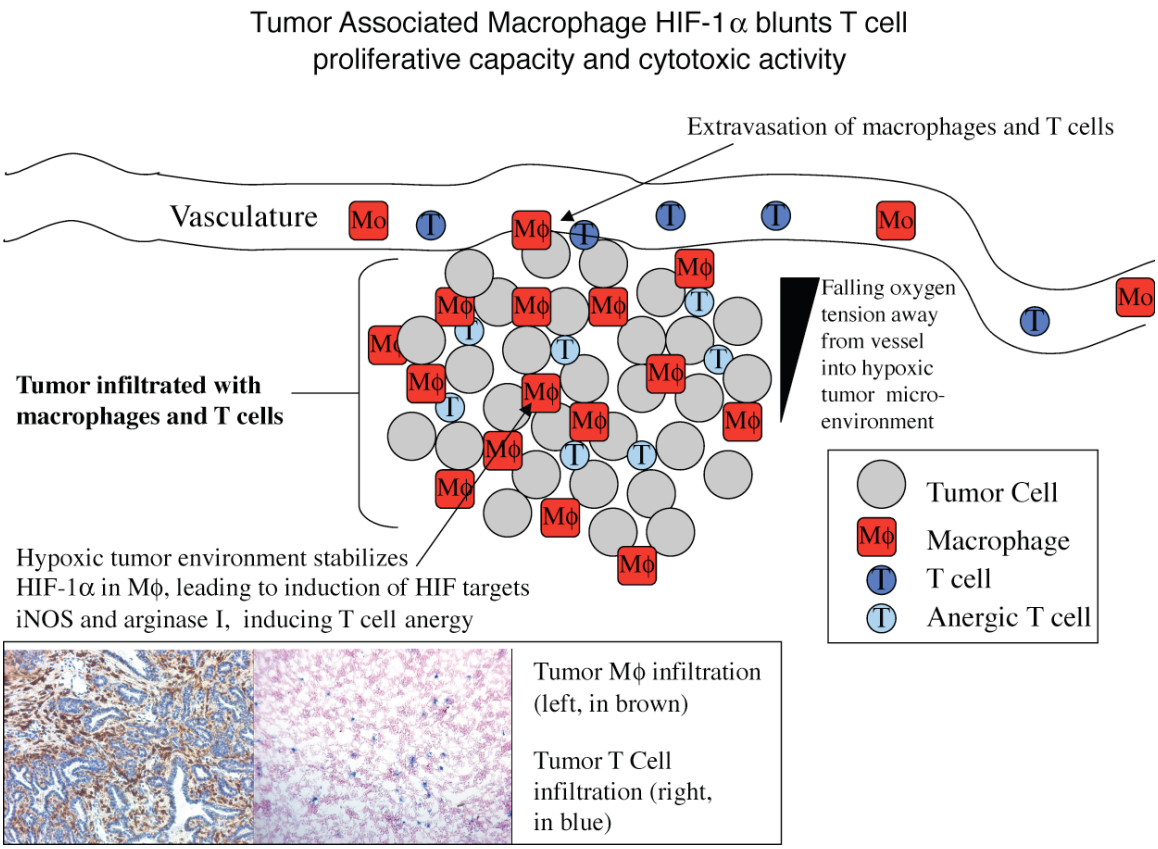


Figure 2.7: Model of macrophage HIF-1alpha dependent suppression of T cell activation.

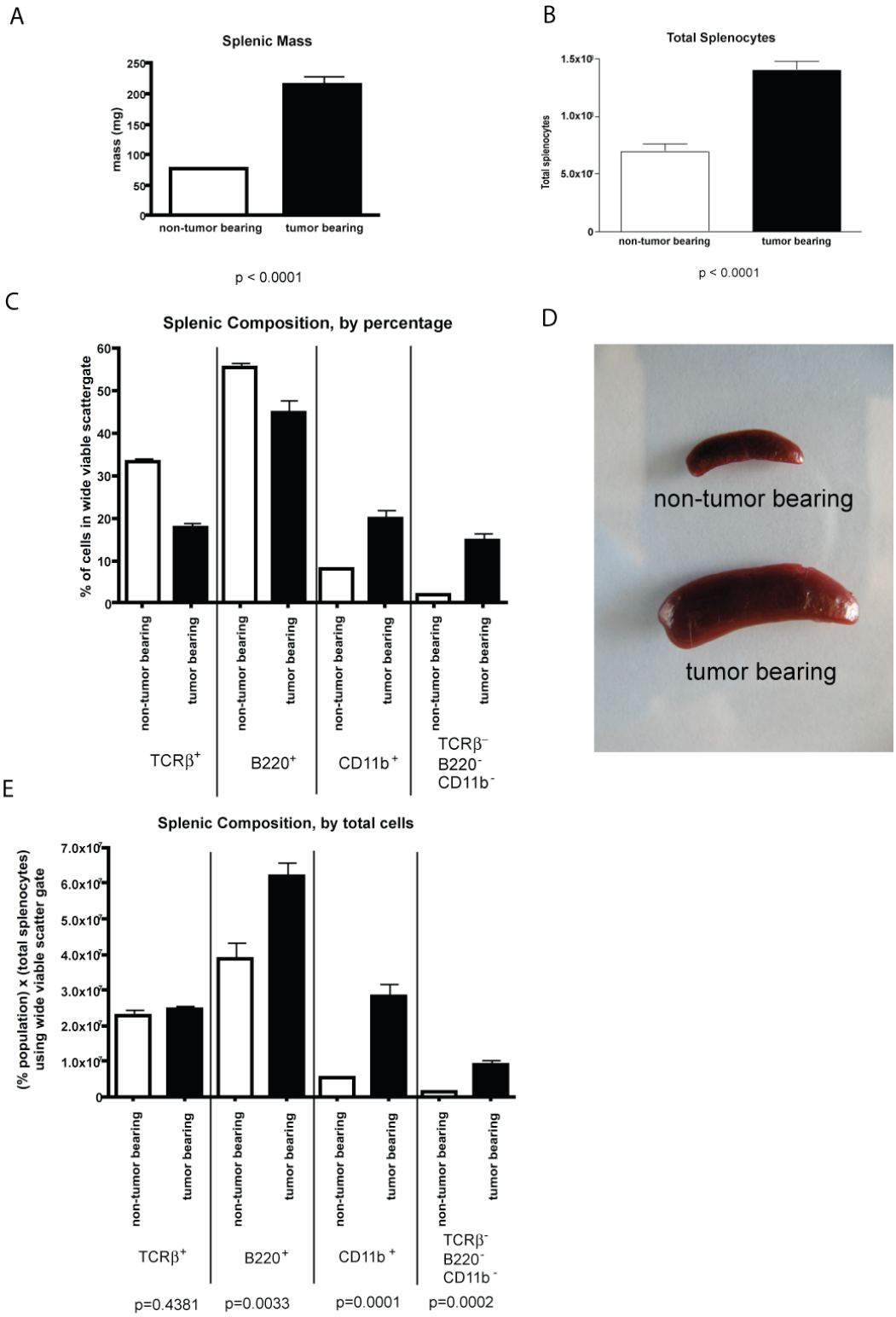


Figure 2.8: Perturbation of splenic composition in PyMT tumor bearing mice vs. littermate non-tumor bearing controls.

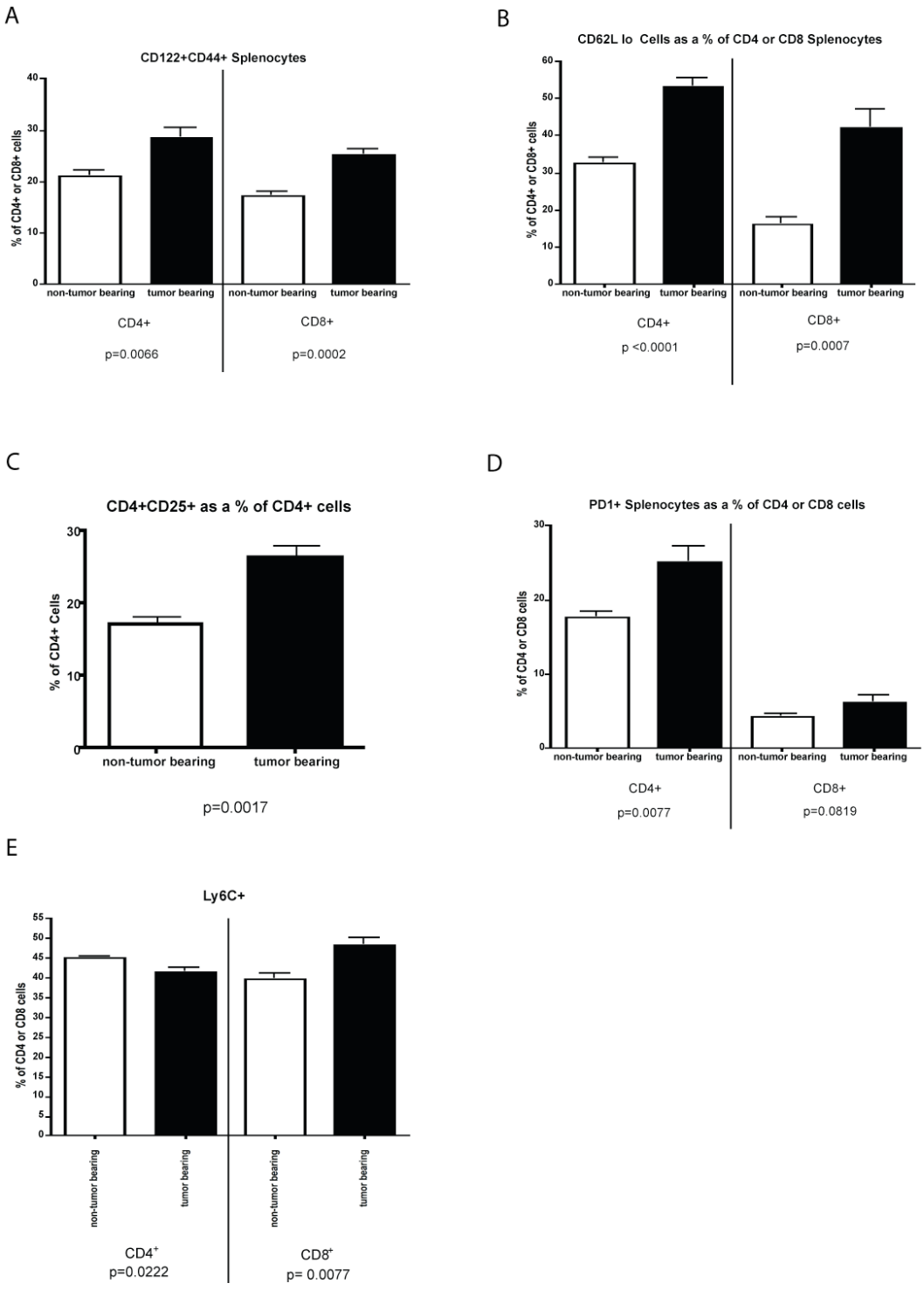


Figure 2.9: Activation and other immunophenotypic markers from splenic T cells of PyMT tumor bearing mice vs. littermate controls.

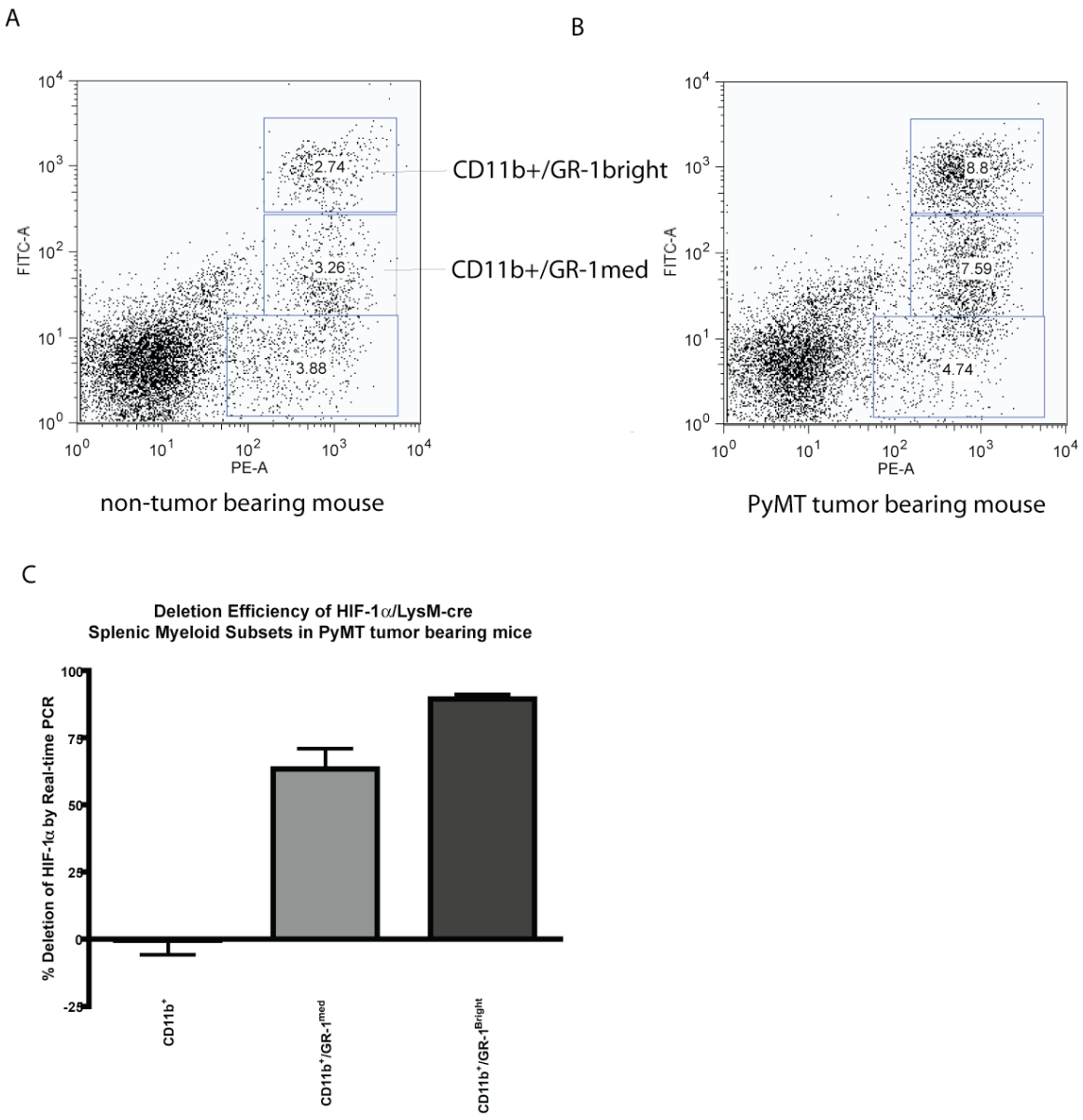


Figure 2.10: Deletion of HIF-1alpha in CD11b+/GR1med and CD11b+/GR1bright, but not in CD11b+/GR1- cells using the LysM-cre recombinase.

References

Acker T, Diez-Juan A, Aragones J, Tjwa M, Brusselmans K, Moons L, Fukumura D, Moreno-Murciano MP, Herbert JM, Burger A, Riedel J, Elvert G, Flamme I, Maxwell PH, Collen D, Dewerchin M, Jain RK, Plate KH, Carmeliet P: Genetic evidence for a tumor suppressor role of HIF-2alpha. Cancer Cell *8*: 131-141, 2005

Albina JE, Abate JA, Henry WL, Jr.: Nitric oxide production is required for murine resident peritoneal macrophages to suppress mitogen-stimulated T cell proliferation. Role of IFN-gamma in the induction of the nitric oxide-synthesizing pathway. J Immunol *147*: 144-148, 1991

Atochina O, Daly-Engel T, Piskorska D, McGuire E, Harn DA: A schistosome-expressed immunomodulatory glycoconjugate expands peritoneal Gr1(+) macrophages that suppress naive CD4(+) T cell proliferation via an IFN-gamma and nitric oxide-dependent mechanism. J Immunol *167*: 4293-4302, 2001

Auvinen M, Paasinen A, Andersson LC, Holtta E: Ornithine decarboxylase activity is critical for cell transformation. Nature *360*: 355-358, 1992

Beck I, Ramirez S, Weinmann R, Caro J: Enhancer element at the 3'-flanking region controls transcriptional response to hypoxia in the human erythropoietin gene. J Biol Chem *266*: 15563-15566, 1991

Biju MP, Neumann AK, Bensinger SJ, Johnson RS, Turka LA, Haase VH: Vhlh gene deletion induces Hif-1-mediated cell death in thymocytes. Mol Cell Biol *24*: 9038-9047, 2004

Blouw B, Song H, Tihan T, Bosze J, Ferrara N, Gerber HP, Johnson RS, Bergers G: The hypoxic response of tumors is dependent on their microenvironment. Cancer Cell *4*: 133-146, 2003

Bronte V, Serafini P, Mazzoni A, Segal DM, Zanovello P: L-arginine metabolism in myeloid cells controls T-lymphocyte functions. Trends Immunol *24*: 302-306, 2003

Bronte V, Zanovello P: Regulation of immune responses by L-arginine metabolism. Nat Rev Immunol *5*: 641-654, 2005

Bruick RK, McKnight SL: A conserved family of prolyl-4-hydroxylases that modify HIF.

Science *294*: 1337-1340, 2001

Brusselmans K, Compernolle V, Tjwa M, Wiesener MS, Maxwell PH, Collen D, Carmeliet P: Heterozygous deficiency of hypoxia-inducible factor-2alpha protects mice against pulmonary hypertension and right ventricular dysfunction during prolonged hypoxia. J Clin Invest *111*: 1519-1527, 2003

Bunger MK, Wilsbacher LD, Moran SM, Clendenin C, Radcliffe LA, Hogenesch JB, Simon MC, Takahashi JS, Bradfield CA: Mop3 is an essential component of the master circadian pacemaker in mammals. Cell *103*: 1009-1017, 2000

Carmeliet P, Dor Y, Herbert JM, Fukumura D, Brusselmans K, Dewerchin M, Neeman M, Bono F, Abramovitch R, Maxwell P, Koch CJ, Ratcliffe P, Moons L, Jain RK, Collen D, Keshert E: Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. Nature *394*: 485-490, 1998

Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoeck A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A: Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature *380*: 435-439, 1996

Clausen BE, Burkhardt C, Reith W, Renkawitz R, Forster I: Conditional gene targeting in macrophages and granulocytes using LysMcre mice. Transgenic Res *8*: 265-277, 1999

Compernolle V, Brusselmans K, Acker T, Hoet P, Tjwa M, Beck H, Plaisance S, Dor Y, Keshet E, Lupu F, Nemery B, Dewerchin M, Van Veldhoven P, Plate K, Moons L, Collen D, Carmeliet P: Loss of HIF-2alpha and inhibition of VEGF impair fetal lung maturation, whereas treatment with VEGF prevents fatal respiratory distress in premature mice. Nat Med *8*: 702-710, 2002

Compernolle V, Brusselmans K, Franco D, Moorman A, Dewerchin M, Collen D, Carmeliet P: Cardia bifida, defective heart development and abnormal neural crest migration in embryos lacking hypoxia-inducible factor-1alpha. Cardiovasc Res *60*: 569-579, 2003

Corraliza IM, Campo ML, Soler G, Modolell M: Determination of arginase activity in macrophages: a micromethod. J Immunol Methods *174*: 231-235, 1994

Cowden KD, Simon MC: The bHLH/PAS factor MOP3 does not participate in hypoxia

responses. Biochem Biophys Res Commun *290*: 1228-1236, 2002

Cramer T, Johnson RS: A novel role for the hypoxia inducible transcription factor HIF-1alpha: critical regulation of inflammatory cell function. Cell Cycle *2*: 192-193, 2003

Cramer T, Yamanishi Y, Clausen BE, Forster I, Pawlinski R, Mackman N, Haase VH, Jaenisch R, Corr M, Nizet V, Firestein GS, Gerber HP, Ferrara N, Johnson RS: HIF-1alpha is essential for myeloid cell-mediated inflammation. Cell *112*: 645-657, 2003

Detwiller KY, Fernando NT, Segal NH, Ryeom SW, D'Amore PA, Yoon SS: Analysis of hypoxia-related gene expression in sarcomas and effect of hypoxia on RNA interference of vascular endothelial cell growth factor A. Cancer Res *65*: 5881-5889, 2005

Elson DA, Thurston G, Huang LE, Ginzinger DG, McDonald DM, Johnson RS, Arbeit JM: Induction of hypervascularity without leakage or inflammation in transgenic mice overexpressing hypoxia-inducible factor-1alpha. Genes Dev *15*: 2520-2532, 2001

Epstein AC, Gleadle JM, McNeill LA, Hewitson KS, O'Rourke J, Mole DR, Mukherji M, Metzen E, Wilson MI, Dhanda A, Tian YM, Masson N, Hamilton DL, Jaakkola P, Barstead R, Hodgkin J, Maxwell PH, Pugh CW, Schofield CJ, Ratcliffe PJ: C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell *107*: 43-54, 2001

Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, Hillan KJ, Moore MW: Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature *380*: 439-442, 1996

Gallina G, Dolcetti L, Serafini P, De Santo C, Marigo I, Colombo MP, Basso G, Brombacher F, Borrello I, Zanovello P, Bicciato S, Bronte V: Tumors induce a subset of inflammatory monocytes with immunosuppressive activity on CD8+ T cells. J Clin Invest *116*: 2777-2790, 2006

Gerber HP, Hillan KJ, Ryan AM, Kowalski J, Keller GA, Rangell L, Wright BD, Radtke F, Aguet M, Ferrara N: VEGF is required for growth and survival in neonatal mice. Development *126*: 1149-1159, 1999

Gnarra JR, Ward JM, Porter FD, Wagner JR, Devor DE, Grinberg A, Emmert-Buck MR, Westphal H, Klausner RD, Linehan WM: Defective placental vasculogenesis causes embryonic lethality in VHL-deficient mice. Proc Natl Acad Sci U S A *94*: 9102-9107,

1997

Gruber M, Hu CJ, Johnson RS, Brown EJ, Keith B, Simon MC: Acute postnatal ablation of Hif-2{alpha} results in anemia. Proc Natl Acad Sci U S A *104*: 2301-2306, 2007

Guy CT, Cardiff RD, Muller WJ: Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease. Mol Cell Biol *12*: 954-961, 1992

Haase VH, Glickman JN, Socolovsky M, Jaenisch R: Vascular tumors in livers with targeted inactivation of the von Hippel-Lindau tumor suppressor. Proc Natl Acad Sci U S A *98*: 1583-1588, 2001

Helton R, Cui J, Scheel JR, Ellison JA, Ames C, Gibson C, Blouw B, Ouyang L, Dragatsis I, Zeitlin S, Johnson RS, Lipton SA, Barlow C: Brain-specific knock-out of hypoxia-inducible factor-1alpha reduces rather than increases hypoxic-ischemic damage. J Neurosci *25*: 4099-4107, 2005

Hewitson KS, McNeill LA, Riordan MV, Tian YM, Bullock AN, Welford RW, Elkins JM, Oldham NJ, Bhattacharya S, Gleadle JM, Ratcliffe PJ, Pugh CW, Schofield CJ: Hypoxia-inducible factor (HIF) asparagine hydroxylase is identical to factor inhibiting HIF (FIH) and is related to the cupin structural family. J Biol Chem *277*: 26351-26355, 2002

Hogenesch JB, Gu YZ, Jain S, Bradfield CA: The basic-helix-loop-helix-PAS orphan MOP3 forms transcriptionally active complexes with circadian and hypoxia factors. Proc Natl Acad Sci U S A *95*: 5474-5479, 1998

Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS, Kaelin WG, Jr.: HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. Science *292*: 464-468, 2001

Iyer NV, Kotch LE, Agani F, Leung SW, Laughner E, Wenger RH, Gassmann M, Gearhart JD, Lawler AM, Yu AY, Semenza GL: Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1 alpha. Genes Dev *12*: 149-162, 1998

Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, Kriegsheim A, Hebestreit HF, Mukherji M, Schofield CJ, Maxwell PH, Pugh CW, Ratcliffe PJ: Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-

regulated prolyl hydroxylation. Science *292*: 468-472, 2001

Jensen RL, Ragel BT, Whang K, Gillespie D: Inhibition of hypoxia inducible factor-1alpha (HIF-1alpha) decreases vascular endothelial growth factor (VEGF) secretion and tumor growth in malignant gliomas. J Neurooncol *78*: 233-247, 2006

Kaelin WG, Jr.: Molecular basis of the VHL hereditary cancer syndrome. Nat Rev Cancer *2*: 673-682, 2002

Kaelin WG, Jr.: The von Hippel-Lindau tumor suppressor protein and clear cell renal carcinoma. Clin Cancer Res *13*: 680s-684s, 2007

Kakuda DK, Sweet MJ, Mac Leod CL, Hume DA, Markovich D: CAT2-mediated L-arginine transport and nitric oxide production in activated macrophages. Biochem J *340 (Pt 2)*: 549-553, 1999

Karhausen J, Furuta GT, Tomaszewski JE, Johnson RS, Colgan SP, Haase VH: Epithelial hypoxia-inducible factor-1 is protective in murine experimental colitis. J Clin Invest *114*: 1098-1106, 2004

Keith B, Adelman DM, Simon MC: Targeted mutation of the murine arylhydrocarbon receptor nuclear translocator 2 (Arnt2) gene reveals partial redundancy with Arnt. Proc Natl Acad Sci U S A *98*: 6692-6697, 2001

Kim WY, Safran M, Buckley MR, Ebert BL, Glickman J, Bosenberg M, Regan M, Kaelin WG, Jr.: Failure to prolyl hydroxylate hypoxia-inducible factor alpha phenocopies VHL inactivation in vivo. Embo J *25*: 4650-4662, 2006

Kozak KR, Abbott B, Hankinson O: ARNT-deficient mice and placental differentiation. Dev Biol *191*: 297-305, 1997

Kropf P, Baud D, Marshall SE, Munder M, Mosley A, Fuentes JM, Bangham CR, Taylor GP, Herath S, Choi BS, Soler G, Teoh T, Modolell M, Muller I: Arginase activity mediates reversible T cell hyporesponsiveness in human pregnancy. Eur J Immunol *37*: 935-945, 2007

Larcher F, Murillas R, Bolontrade M, Conti CJ, Jorcano JL: VEGF/VPF overexpression in skin of transgenic mice induces angiogenesis, vascular hyperpermeability and

accelerated tumor development. Oncogene *17*: 303-311, 1998

Leen AM, Rooney CM, Foster AE: Improving T cell therapy for cancer. Annu Rev Immunol *25*: 243-265, 2007

Li L, Lin X, Staver M, Shoemaker A, Semizarov D, Fesik SW, Shen Y: Evaluating hypoxia-inducible factor-1alpha as a cancer therapeutic target via inducible RNA interference in vivo. Cancer Res *65*: 7249-7258, 2005

Liao D, Corle C, Seagroves TN, Johnson RS: Hypoxia-inducible factor-1alpha is a key regulator of metastasis in a transgenic model of cancer initiation and progression. Cancer Res *67*: 563-572, 2007

Lin EY, Jones JG, Li P, Zhu L, Whitney KD, Muller WJ, Pollard JW: Progression to malignancy in the polyoma middle T oncoprotein mouse breast cancer model provides a reliable model for human diseases. Am J Pathol *163*: 2113-2126, 2003

Lin EY, Pollard JW: Role of infiltrated leucocytes in tumour growth and spread. Br J Cancer *90*: 2053-2058, 2004

Lob S, Konigsrainer A: Is IDO a key enzyme bridging the gap between tumor escape and tolerance induction? Langenbecks Arch Surg, 2007

Louis CA, Reichner JS, Henry WL, Jr., Mastrofrancesco B, Gotoh T, Mori M, Albina JE: Distinct arginase isoforms expressed in primary and transformed macrophages: regulation by oxygen tension. Am J Physiol *274*: R775-782, 1998

Mack FA, Patel JH, Biju MP, Haase VH, Simon MC: Decreased growth of Vhl-/- fibrosarcomas is associated with elevated levels of cyclin kinase inhibitors p21 and p27. Mol Cell Biol *25*: 4565-4578, 2005

Mahon PC, Hirota K, Semenza GL: FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. Genes Dev *15*: 2675-2686, 2001

Maltepe E, Keith B, Arsham AM, Brorson JR, Simon MC: The role of ARNT2 in tumor angiogenesis and the neural response to hypoxia. Biochem Biophys Res Commun *273*: 231-238, 2000

Maltepe E, Schmidt JV, Baunoch D, Bradfield CA, Simon MC: Abnormal angiogenesis and responses to glucose and oxygen deprivation in mice lacking the protein ARNT. Nature *386*: 403-407, 1997

Manni A, Grove R, Kunselman S, Aldaz M: Involvement of the polyamine pathway in breast cancer progression. Cancer Lett *92*: 49-57, 1995

Manni A, Wechter R, Gilmour S, Verderame MF, Mauger D, Demers LM: Ornithine decarboxylase over-expression stimulates mitogen-activated protein kinase and anchorage-independent growth of human breast epithelial cells. Int J Cancer *70*: 175-182, 1997

Mason SD, Howlett RA, Kim MJ, Olfert IM, Hogan MC, McNulty W, Hickey RP, Wagner PD, Kahn CR, Giordano FJ, Johnson RS: Loss of skeletal muscle HIF-1alpha results in altered exercise endurance. PLoS Biol *2*: e288, 2004

Matlack R, Yeh K, Rosini L, Gonzalez D, Taylor J, Silberman D, Pennello A, Riggs J: Peritoneal macrophages suppress T-cell activation by amino acid catabolism. Immunology *117*: 386-395, 2006

Maxwell PH, Pugh CW, Ratcliffe PJ: Inducible operation of the erythropoietin 3' enhancer in multiple cell lines: evidence for a widespread oxygen-sensing mechanism. Proc Natl Acad Sci U S A *90*: 2423-2427, 1993

Mazzoni A, Bronte V, Visintin A, Spitzer JH, Apolloni E, Serafini P, Zanovello P, Segal DM: Myeloid suppressor lines inhibit T cell responses by an NO-dependent mechanism. J Immunol *168*: 689-695, 2002

Melillo G, Musso T, Sica A, Taylor LS, Cox GW, Varesio L: A hypoxia-responsive element mediates a novel pathway of activation of the inducible nitric oxide synthase promoter. J Exp Med *182*: 1683-1693, 1995

Modolell M, Corraliza IM, Link F, Soler G, Eichmann K: Reciprocal regulation of the nitric oxide synthase/arginase balance in mouse bone marrow-derived macrophages by TH1 and TH2 cytokines. Eur J Immunol *25*: 1101-1104, 1995

Munder M, Schneider H, Luckner C, Giese T, Langhans CD, Fuentes JM, Kropf P, Mueller I, Kolb A, Modolell M, Ho AD: Suppression of T-cell functions by human granulocyte arginase. Blood *108*: 1627-1634, 2006

Munn DH, Sharma MD, Baban B, Harding HP, Zhang Y, Ron D, Mellor AL: GCN2 kinase in T cells mediates proliferative arrest and anergy induction in response to indoleamine 2,3-dioxygenase. Immunity *22*: 633-642, 2005

Neumann AK, Yang J, Biju MP, Joseph SK, Johnson RS, Haase VH, Freedman BD, Turka LA: Hypoxia inducible factor 1 alpha regulates T cell receptor signal transduction. Proc Natl Acad Sci U S A *102*: 17071-17076, 2005

Nicholson B, Manner CK, Kleeman J, MacLeod CL: Sustained nitric oxide production in macrophages requires the arginine transporter CAT2. J Biol Chem *276*: 15881-15885, 2001

Ohh M, Yauch RL, Lonergan KM, Whaley JM, Stemmer-Rachamimov AO, Louis DN, Gavin BJ, Kley N, Kaelin WG, Jr., Iliopoulos O: The von Hippel-Lindau tumor suppressor protein is required for proper assembly of an extracellular fibronectin matrix. Mol Cell *1*: 959-968, 1998

Ohtani Y, Hiyoshi M, Ohkubo T, Tsuji K, Hagihara M, Nakasaki H, Makuuchi H, Nagata N, Mine T, Takada S, Yamamura M, Tsuda M: A liver-derived immunosuppressive factor is an arginase: identification and mechanism of immunosuppression. Biomed Res *28*: 17-23, 2007

Oosthuyse B, Moons L, Storkebaum E, Beck H, Nuyens D, Brusselmans K, Van Dorpe J, Hellings P, Gorselink M, Heymans S, Theilmeier G, Dewerchin M, Laudenbach V, Vermylen P, Raat H, Acker T, Vleminckx V, Van Den Bosch L, Cashman N, Fujisawa H, Drost MR, Sciot R, Bruyninckx F, Hicklin DJ, Ince C, Gressens P, Lupu F, Plate KH, Robberecht W, Herbert JM, Collen D, Carmeliet P: Deletion of the hypoxia-response element in the vascular endothelial growth factor promoter causes motor neuron degeneration. Nat Genet *28*: 131-138, 2001

Orban PC, Chui D, Marth JD: Tissue- and site-specific DNA recombination in transgenic mice. Proc Natl Acad Sci U S A *89*: 6861-6865, 1992

Papandreou I, Cairns RA, Fontana L, Lim AL, Denko NC: HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. Cell Metab *3*: 187-197, 2006

Park SK, Dadak AM, Haase VH, Fontana L, Giaccia AJ, Johnson RS: Hypoxia-induced gene expression occurs solely through the action of hypoxia-inducible factor 1alpha

(HIF-1alpha): role of cytoplasmic trapping of HIF-2alpha. Mol Cell Biol *23*: 4959-4971, 2003

Park SK, Haase VH, Johnson RS: von Hippel Lindau tumor suppressor regulates hepatic glucose metabolism by controlling expression of glucose transporter 2 and glucose 6-phosphatase. Int J Oncol *30*: 341-348, 2007

Peng J, Zhang L, Drysdale L, Fong GH: The transcription factor EPAS-1/hypoxia-inducible factor 2alpha plays an important role in vascular remodeling. Proc Natl Acad Sci U S A *97*: 8386-8391, 2000

Peng YJ, Yuan G, Ramakrishnan D, Sharma SD, Bosch-Marce M, Kumar GK, Semenza GL, Prabhakar NR: Heterozygous HIF-1alpha deficiency impairs carotid body-mediated systemic responses and reactive oxygen species generation in mice exposed to intermittent hypoxia. J Physiol *577*: 705-716, 2006

Peyssonnaux C, Datta V, Cramer T, Doedens A, Theodorakis EA, Gallo RL, Hurtado-Ziola N, Nizet V, Johnson RS: HIF-1alpha expression regulates the bactericidal capacity of phagocytes. J Clin Invest *115*: 1806-1815, 2005

Pugh CW, Tan CC, Jones RW, Ratcliffe PJ: Functional analysis of an oxygen-regulated transcriptional enhancer lying 3' to the mouse erythropoietin gene. Proc Natl Acad Sci U S A *88*: 10553-10557, 1991

Rankin EB, Tomaszewski JE, Haase VH: Renal cyst development in mice with conditional inactivation of the von Hippel-Lindau tumor suppressor. Cancer Res *66*: 2576-2583, 2006

Richardson RS, Noyszewski EA, Kendrick KF, Leigh JS, Wagner PD: Myoglobin O2 desaturation during exercise. Evidence of limited O2 transport. J Clin Invest *96*: 1916-1926, 1995

Rodriguez PC, Hernandez CP, Quiceno D, Dubinett SM, Zabaleta J, Ochoa JB, Gilbert J, Ochoa AC: Arginase I in myeloid suppressor cells is induced by COX-2 in lung carcinoma. J Exp Med *202*: 931-939, 2005

Rodriguez PC, Quiceno DG, Zabaleta J, Ortiz B, Zea AH, Piazuelo MB, Delgado A, Correa P, Brayer J, Sotomayor EM, Antonia S, Ochoa JB, Ochoa AC: Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell

receptor expression and antigen-specific T-cell responses. Cancer Res *64*: 5839-5849, 2004

Rodriguez PC, Zea AH, Culotta KS, Zabaleta J, Ochoa JB, Ochoa AC: Regulation of T cell receptor CD3zeta chain expression by L-arginine. J Biol Chem *277*: 21123-21129, 2002

Rodriguez PC, Zea AH, DeSalvo J, Culotta KS, Zabaleta J, Quiceno DG, Ochoa JB, Ochoa AC: L-arginine consumption by macrophages modulates the expression of CD3 zeta chain in T lymphocytes. J Immunol *171*: 1232-1239, 2003

Rothenberg ME, Doepker MP, Lewkowich IP, Chiaramonte MG, Stringer KF, Finkelman FD, MacLeod CL, Ellies LG, Zimmermann N: Cationic amino acid transporter 2 regulates inflammatory homeostasis in the lung. Proc Natl Acad Sci U S A *103*: 14895-14900, 2006

Ryan HE, Lo J, Johnson RS: HIF-1 alpha is required for solid tumor formation and embryonic vascularization. Embo J *17*: 3005-3015, 1998

Ryan HE, Poloni M, McNulty W, Elson D, Gassmann M, Arbeit JM, Johnson RS: Hypoxia-inducible factor-1alpha is a positive factor in solid tumor growth. Cancer Res *60*: 4010-4015, 2000

Sauer B: Inducible gene targeting in mice using the Cre/lox system. Methods *14*: 381-392, 1998

Schipani E, Ryan HE, Didrickson S, Kobayashi T, Knight M, Johnson RS: Hypoxia in cartilage: HIF-1alpha is essential for chondrocyte growth arrest and survival. Genes Dev *15*: 2865-2876, 2001

Scortegagna M, Ding K, Oktay Y, Gaur A, Thurmond F, Yan LJ, Marck BT, Matsumoto AM, Shelton JM, Richardson JA, Bennett MJ, Garcia JA: Multiple organ pathology, metabolic abnormalities and impaired homeostasis of reactive oxygen species in Epas1-/- mice. Nat Genet *35*: 331-340, 2003a

Scortegagna M, Ding K, Zhang Q, Oktay Y, Bennett MJ, Bennett M, Shelton JM, Richardson JA, Moe O, Garcia JA: HIF-2alpha regulates murine hematopoietic development in an erythropoietin-dependent manner. Blood *105*: 3133-3140, 2005

Scortegagna M, Morris MA, Oktay Y, Bennett M, Garcia JA: The HIF family member EPAS1/HIF-2alpha is required for normal hematopoiesis in mice. Blood *102*: 1634-1640, 2003b

Seagroves TN, Hadsell D, McManaman J, Palmer C, Liao D, McNulty W, Welm B, Wagner KU, Neville M, Johnson RS: HIF1alpha is a critical regulator of secretory differentiation and activation, but not vascular expansion, in the mouse mammary gland. Development *130*: 1713-1724, 2003

Semenza GL: Hypoxia-inducible factor 1: oxygen homeostasis and disease pathophysiology. Trends Mol Med *7*: 345-350, 2001

Semenza GL: Regulation of physiological responses to continuous and intermittent hypoxia by hypoxia-inducible factor 1. Exp Physiol *91*: 803-806, 2006

Semenza GL, Roth PH, Fang HM, Wang GL: Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. J Biol Chem *269*: 23757-23763, 1994

Semenza GL, Wang GL: A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol Cell Biol *12*: 5447-5454, 1992

Takeda K, Ho VC, Takeda H, Duan LJ, Nagy A, Fong GH: Placental but not heart defects are associated with elevated hypoxia-inducible factor alpha levels in mice lacking prolyl hydroxylase domain protein 2. Mol Cell Biol *26*: 8336-8346, 2006

Talks KL, Turley H, Gatter KC, Maxwell PH, Pugh CW, Ratcliffe PJ, Harris AL: The expression and distribution of the hypoxia-inducible factors HIF-1alpha and HIF-2alpha in normal human tissues, cancers, and tumor-associated macrophages. Am J Pathol *157*: 411-421, 2000

Talmadge JE: Pathways mediating the expansion and immunosuppressive activity of myeloid-derived suppressor cells and their relevance to cancer therapy. Clin Cancer Res *13*: 5243-5248, 2007

Tang N, Mack F, Haase VH, Simon MC, Johnson RS: pVHL function is essential for endothelial extracellular matrix deposition. Mol Cell Biol *26*: 2519-2530, 2006

Tian H, Hammer RE, Matsumoto AM, Russell DW, McKnight SL: The hypoxia-responsive transcription factor EPAS1 is essential for catecholamine homeostasis and protection against heart failure during embryonic development. Genes Dev *12*: 3320-3324, 1998

Tian H, McKnight SL, Russell DW: Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. Genes Dev *11*: 72-82, 1997

Vaupel P, Hockel M, Mayer A: Detection and characterization of tumor hypoxia using pO2 histography. Antioxid Redox Signal *9*: 1221-1235, 2007

Vaupel P, Mayer A: Hypoxia in cancer: significance and impact on clinical outcome. Cancer Metastasis Rev *26*: 225-239, 2007

Vig M, Srivastava S, Kandpal U, Sade H, Lewis V, Sarin A, George A, Bal V, Durdik JM, Rath S: Inducible nitric oxide synthase in T cells regulates T cell death and immune memory. J Clin Invest *113*: 1734-1742, 2004

Wang GL, Jiang BH, Rue EA, Semenza GL: Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proc Natl Acad Sci U S A *92*: 5510-5514, 1995

Wang GL, Semenza GL: Characterization of hypoxia-inducible factor 1 and regulation of DNA binding activity by hypoxia. J Biol Chem *268*: 21513-21518, 1993

Wiener CM, Booth G, Semenza GL: In vivo expression of mRNAs encoding hypoxia-inducible factor 1. Biochem Biophys Res Commun *225*: 485-488, 1996

Yu AY, Shimoda LA, Iyer NV, Huso DL, Sun X, McWilliams R, Beaty T, Sham JS, Wiener CM, Sylvester JT, Semenza GL: Impaired physiological responses to chronic hypoxia in mice partially deficient for hypoxia-inducible factor 1alpha. J Clin Invest *103*: 691-696, 1999

Zhang Q, Zhang ZF, Rao JY, Sato JD, Brown J, Messadi DV, Le AD: Treatment with siRNA and antisense oligonucleotides targeted to HIF-1alpha induced apoptosis in human tongue squamous cell carcinomas. Int J Cancer *111*: 849-857, 2004