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The Role of Hypoxia-Inducible Factor in the Immune Response to Infection

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by

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The Dissertation of Tamara Schneider Bhandari is approved, and it is acceptable in

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DEDICATION

I dedicate this thesis to my parents, who never doubted my abilities; to my son Dion, whose dogged determination to explore and understand his world provided a daily reminder of what a scientist ought to be; to my daughter Kaya, whose arrival gave me the motivation to make the final push across the finish line; and to my husband Shom who provided me with these two wonderful children and without whose support, love, and assistance none of this would have been possible.

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LIST OF ABBREVIATIONS

DC: Dendritic cell

HIF: Hypoxia-inducible factor

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ABSTRACT OF THE DISSERTATION

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by

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The twentieth century saw great improvements in our ability to control infectious diseases through the use of antibiotics and vaccinations. In recent decades, however, some of these improvements have eroded as pathogens evolve antibiotic resistance. Further progress in controlling infectious disease will likely require not just new antibiotics, but also strategies to strengthen and target the host immune response more effectively. To this end, a more nuanced understanding of host immune response regulation is necessary. One key regulatory factor is hypoxia-inducible factor (HIF), which plays a pivotal role in the antimicrobial function of myeloid cells. Here we explore

the role of HIF in other aspects of the immune response to infection, and evaluate its potential as a target for new anti-infective therapies.

Using a genetic model in which *Hif-1α* was ablated specifically in dendritic cells, we studied the role of HIF in initiating the T-cell response. We found that HIF was important for the expression of antigen-presenting and costimulatory surface molecules, the ability to stimulate T cell proliferation, and the overall response to vaccination. Furthermore, pharmacological augmentation of HIF improved both the humoral and cell-mediated response to vaccination, indicating that HIF could be a target for vaccine adjuvants.

Previous work by the Nizet lab and others has shown that HIF is crucial for the control of skin infections. Like HIF, the active form of vitamin D increases in the skin upon exposure to molecular signals of infection, leading to increased antimicrobial activity of skin epithelial and immune cells. We used a human skin cell line to elucidate the relationship between HIF and vitamin D in the regulation of skin immunity. We found that vitamin D increases HIF expression and function, and HIF modulates vitamin D-induced expression of the antimicrobial peptide LL-37. These data provide the first suggestion that drugs which augment HIF may help treat infections in vitamin D-deficient patients.

It is clear that HIF plays an important and complex role in the immune response to infection. As our understanding of HIF grows, we will be better able to manipulate the host immune response to prevent or control infectious diseases.

CHAPTER 1:

HYPOXIA-INDUCIBLE FACTOR AND THE HOST RESPONSE TO INFECTION

The golden age of antibiotics may be nearing its end, as more and more pathogens acquire resistance to an ever-widening range of antibiotics. New ways to prevent and treat infectious diseases are urgently needed. One possible solution is to focus not on killing microbes with antibiotics but on strengthening host defenses, the other side of host-pathogen interactions. Just as vaccines harness the power of the adaptive immune system to prevent infectious disease, treatments that activate the innate immune system could potentially help to cure acute infections. Hypoxia-inducible factor (HIF)—a key regulator of myeloid cell immune function—is a promising target for such immune boosting treatments.

Myeloid cells such as neutrophils and macrophages form the first line of cellular defense against infection. Neutrophils respond within minutes to tissue injury or infection, and macrophages follow within the first few hours. Myeloid cells phagocytose microorganisms and damaged tissue, and release a diverse array of antimicrobial mole-

cules and proinflammatory mediators which attract and activate other immune cells. As such, they serve to localize and eradicate pathogens and prevent the systemic spread of infection. People with insufficient numbers of myeloid cells (for example, following cancer chemotherapy) or whose myeloid cells have impaired function (for example, in the inherited immunodeficiency known as chronic granulomatous disease) are vastly more susceptible to recurrent or severe infections.

Myeloid cell function is tightly regulated to avoid excessive tissue damage and immune activation, and one of the key regulators is HIF. However, a growing body of research reveals that the HIF plays multiple roles in immune regulation, with differing effects in different cell types. Attempts to modulate HIF levels for infectious disease therapy must take these complexities into consideration.

HIF BIOLOGY AND REGULATION

HIF is a basic helix-loop-helix transcription factor[1] first identified for its role in erythropoietin regulation,[2] but later discovered to also regulated genes involved in glycolysis, angiogenesis, cell differentiation, apoptosis, and other cellular pathways.[3] HIF is a heterodimer composed of a HIF-α subunit and HIF-1β subunit. HIF-α is actually a

family of three genes: HIF-1α, HIF-2α, and HIF-3α. HIF-3α is distantly related to HIF-1α

and HIF-2α and little is known about its function, although it may inhibit the activity of

HIF-1α and HIF-2α.[4] The HIF-1α and HIF-2α subunits are closely related, sharing 48%

overall amino acid identity.[5] The two subunits are very similar in their DNA binding and

dimerization domains but differ in their transactivation domains, implying that they may

regulate unique sets of target genes.[5] Whereas HIF-1α is ubiquitously expressed, HIF-2α

is most abundantly expressed in vascular endothelial cells during embryonic development

and in endothelial, lung, heart,[6] and bone-marrow cells[7] in the adult. HIF-2α levels are

closely correlated with vascular endothelial growth factor (*VEGF*) mRNA expression[6]

and are frequently elevated in solid tumors,[7] suggesting that its most important functions

may lie in vascularization.[6] Since only a small fraction of published research focuses

specifically on HIF-2α or HIF-3α, this review will not address their roles.

In the presence of oxygen and the absence of inflammatory stimuli, the level of

HIF-α is kept low by two mechanisms. In one, HIF-α is hydroxylated by prolyl

hydroxylases.[8] The hydroxylated HIF-α is recognized by the ubiquitin ligase von Hippel-

Lindau factor (vHL) which ubiquitinates HIF-α, targeting it for destruction via the

proteosome.[9] In the second mechanism, factor inhibiting HIF (FIH) hydroxylates HIF-α,

blocking its ability to associate with p300-CREB binding protein (CREB-BP), which in turn inhibits the ability of the HIF complex to bind DNA and promote transcription.[10] When oxygen tension is low, neither hydroxylation event occurs, HIF-α and HIF-1β dimerize, combine with CREB-BP and bind to hypoxia-response elements (HRE) in the promoter regions of over a hundred target genes.[3]

The NF-κB pathway appears to be crucial for the induction of HIF in response to hypoxia.[11] The human *Hif-1α* promoter contains a canonical NF-κB binding site at a site -197/-188 base pairs upstream of the transcriptional start site, the mutation of which leads to a loss of hypoxic HIF-1α upregulation.[11] There is evidence that NF-κB family members bind to the HIF-1α promoter,[12] and an inhibitor of NF-κB derepresses HIF-1 by sequestering FIH.[13] Basal NF-κB activity is required for HIF-1α protein accumulation under hypoxia in cultured cells and in the liver and brain of hypoxic animals.[11] A deficiency of the NF-κB activator IKK-β results in defective induction of HIF-1α target genes including VEGF. IKK-β is also essential for HIF-1α accumulation in macrophages during the response to bacterial infection. Hence, IKK-β is an important physiological contributor to the hypoxic response, linking it to innate immunity and inflammation.[11]

Though HIF was first identified and named for its role in hypoxia, later work

showed that a variety of molecular signals of infection and inflammation can also increase HIF activity under normoxic conditions. Growth hormones such as insulin-like growth factor,[14] cytokines such as IL-1β[15] and viral proteins[16] all activate HIF. This regulation can occur at the transcriptional, translational, or post-translational levels. For example, lipopolysaccharide (LPS) induces *Hif-1α* mRNA expression in a TLR4-dependent manner that involves members of the NF-κB, MAPK, and ERK pathways.[17-19] TLR7/8 ligation also leads to *Hif-1α* transcript accumulation[20] and to protein stabilization in macrophages.[20, 21]

Cytokines, on the other hand, often increase HIF activity by post-translational mechanisms. TGF-β1 enhances HIF-1α protein stability by inhibiting the expression of prolyl hydroxylase PHD2, which hydroxylates HIF and targets it for proteolytic destruction.[22] TNF-α[23, 24] and IL-1β[23] [15] induce HIF-1α protein stabilization in an NF-κB-dependent mechanism without affecting its mRNA level.

HIF AS A REGULATOR OF IMMUNE FUNCTION

Why should a ubiquitous transcription factor be induced by both hypoxia and molecular signals of infection? Tissue foci of inflammation represent hypoxic

microenvironments, with oxygen tensions measured under 1%.[25] Hypoxia reflects increased metabolic demands due to a high density of inflammatory cells and microorganisms, and limited perfusion because of thrombosis, damage to the vasculature, or compression of blood vessels due to interstitial hypertension. Immune cells therefore need to be able to carry out their functions under conditions of reduced oxygen tension, a situation made even more challenging since many leading bacterial pathogens proliferate readily even in anaerobic microenvironments. Since infection and hypoxia are so often encountered together, it perhaps stands to reason that HIF would be induced not only by hypoxia also in response to a broad range of infections: viral, bacterial, protozoan, and fungal.[26, 27]

HIF in Innate Immunity

HIF has been proposed as a master regulator of innate immunity.[3, 28] It increases the recruitment of neutrophils[29] and macrophages. DC exposed to hypoxia upregulate genes coding for proteins chemotactic for neutrophils, such as CXCL2, CXCL3, CXCL5, CXCL, and CXCL8[30]. HIF induces $\beta2$ integrin expression in neutrophils[31], and Cdc42 and Rac1 expression in macrophages,[32] enhancing migration of both cell types to the site

of infection. Hypoxia also increases CXCR4[33] and inhibits CCR5[34] expression in macrophages in a HIF-dependent manner, which increases retention of macrophages at the site of infection.

Not only are more immune cells recruited and retained, but those cells live longer. HIF extends the functional neutrophil lifespan by inhibiting apoptotic pathways in an NF-κB-dependent manner.[35, 36] People with mutations in vHL—and therefore constitutively elevated HIF levels—have neutrophils with longer lifespans.[37] Hypoxia also promotes survival of monocytes and macrophages.[38] The immune cells are also more active. Hypoxia leads to TLR-2,[39] TLR-4,[40] and TLR-6[39] upregulation in a HIF-dependent manner, making them better able to detect the presence of pathogens. Hypoxic macrophages from mice also exhibit increased phagocytosis[41] and those from humans who have mutations in vHL show increased phagocytosis as well.[37]

In an *in vivo* model of innate infection, mice lacking *Hif-1α* in myeloid cells were less able to fight off a skin infection with the pathogen group A *Streptococcus* (GAS).[42] Conversely, those in which HIF was elevated by drug treatment were better able to control skin infection by methicillin-resistant *Staphylococcus aureus* (MRSA).[43, 44] Overall, augmenting HIF in macrophages increases bactericidal activity[43] by increasing a

wide range of antimicrobial factors. Hypoxia leads myeloid cells to release more nitric oxide (NO), granule proteases, neutrophil[45] and mast cell[46] extracellular traps, antimicrobial peptides, and proinflammatory cytokines.[42, 47] One notable exception is superoxide generation via the oxidative burst, which appears to transpire with equal efficiency in wild-type and *Hif-1α* null macrophages.[42] It is perhaps unsurprising that superoxide generation is not elevated by hypoxia, given that it requires the presence of oxygen, which is by definition in short supply.

HIF in Antigen-Presenting Cells

Research to examine potential roles of HIF in APCs has yielded contradictory results (reviewed by Sica[48]), and much of the previous work has focused more closely upon the effects of hypoxia *per se*, rather than *Hif-1α* transcriptional regulation, on DC activities. While some investigators have produced data that would indicate that hypoxia inhibits DC differentiation, maturation markers and antigen capture[49-51], others have come to exactly the opposite conclusion, namely that hypoxia promoted DC maturation both alone[52] and in combination with lipopolysaccharide (LPS) stimulus[50].

HIF in T Cells

Perhaps surprisingly, HIF appears to have opposite effects on cells of the adaptive immune system as it does on cells of the innate immune systems. Hypoxia and HIF-1α elevation reduces T cell survival[53, 54] and proliferation.[54, 55] The paradoxical behavior of HIF in activated T cells may derive from alternative splicing and expression of an isoform, known as I.1, of HIF-1α that inhibits T cell activation in a delayed feedback manner[56]. When isoform I.1 was deleted in T cells in a cecal ligation and puncture model of sepsis, the overall ability to fight infection was improved, with reduced bacterial load, increased resistance to sepsis, enhanced M1 macrophage polarization, and the release of more proinflammatory cytokines and less of the anti-inflammatory IL-10.[57] Following T cell receptor activation, the release of TNF and interferon-γ (IFNγ) by T cells with targeted deletion of *Hif-1α* was higher than release by wild-type T cells[58]. This result held for both CD4$^+$ and CD8$^+$ T cells.[58]

Data from studies involving the deletion of *Hif-1α* specifically in T cells indicate that tight regulation of HIF function in these cells is crucial to prevent cell death, and imply that T cells may have a minor role in hypoxic tissues owing to hypoxia-mediated and HIF-dependent cell death.[59] One way in which hypoxia and HIF may play an

important role is in tipping the balance between regulatory T cells (T_{reg}) and T_H17 cells

towards the T_{reg} lineage. T_{regs} and T_H17 cells derive from naïve $CD4^+$ T cells, with T_{regs}

characterized by expression of the transcription factor Foxp3[60] and T_H17s characterized

by the expression of RORγT.[61] Hypoxia leads to induction of Foxp3 in a HIF-dependent

manner[62] and increased numbers of T_{reg} cells *in vivo*[62] and more potent T_{regs} *in vitro*.[63]

Knockout of *Hif-1α* in $CD4^+$ T cells leads to an increase in the numbers of T_H1 and T_H17

cells.[64] Others have found that differentiating naïve $CD4^+$ T cells under hypoxia followed

by reoxygenation increases the number of T_H17 cells,[65] and *Hif-1α* knockout in $CD4^+$ T

cells results in increased T_{regs} and fewer T_H17 cells,[66, 67] possibly by transcriptional

activation of RORγT and degradation of Foxp3.[66] However, these latter studies looked at

the effect of HIF in the presence of IL-6, which biases towards a T_H17 response, or using

the autoimmune disease model of experimental autoimmune encephalomyelitis, which

creates the same bias.[66, 67] In the absence of conditions that bias towards the development

of T_H17, T_{regs} are produced.[62]

HIF in Skin Immunity

The skin provides a highly effective physical, cellular, and chemical barrier against

microbial penetration.[68] In response to bacterial pathogens, keratinocytes produce peptides of the cathelicidin and β-defensin families that can directly kill microbial pathogens.[69] HIF is expressed at high levels in the skin, which is a hypoxic organ even in noninflammatory settings.[70] Following targeted deletion of *Hif-1α* in keratinocytes, mice show a defect in controlling necrotic skin infection by group A streptococci.[70] Specific RNA interference studies confirmed that HIF-1α-mediated regulation of keratinocyte cathelicidin production is crucial for cutaneous defense against infection with this invasive pathogen.[70] HIF in keratinocytes increases release of the macrophage chemotactic factor MIP-2, keratinocyte chemoattractant, and TNFα in an NF-κB dependent manner.[71]

Complex Effects of HIF in the Immune Response to Infection

The HIF pathway represents an elegant control mechanism for the specialized activities of circulating immune cells, which must transit from the oxygen-rich bloodstream to hypoxic sites of infection. This system allows leukocyte bactericidal and proinflammatory capacities to be maintained in an 'off' state while the cells circulate in the blood, and gradually activate in response to the decreasing oxygen gradient that is

encountered as the cells migrate to the site of infection. Thus primed by low oxygen

tension, the leukocyte is prepared to respond even more strongly when it encounters the

pathogens directly with activation of NF-κB and upregulation of *Hif-1α* mRNA. The

maximal 'on state' of bactericidal capacities is enhanced by the release of

proinflammatory cytokines and upregulation of TLR expression. This system links HIF to

the myeloid cell response, ensuring that proinflammatory mediators, degradative

enzymes and antimicrobial peptides are expressed preferentially at sites of infection, but

not in healthy tissues where they would cause unwanted damage to host cells.

This elegant control system for the innate immune response does not seem to be

mirrored in the adaptive immune response. The evidence suggests that HIF negatively

regulates the activity of T cells, with effects on APCs that still require experimental

clarification. These differences may be understood in the context of the role that each cell

type plays in fighting an infection. Innate immune cells start arriving at sites of injury and

infection in a matter of minutes, and they must immediately be active in the hypoxic

environment to kill bacteria before they can establish a foothold. Meanwhile, T cells are

positioned in the relatively normoxic lymphoid organs awaiting signals from the APCs.

The process of T cell maturation and activation transpires in these lymphoid organs and

the cells may not encounter hypoxia until relatively late in the immune response. The effect of hypoxia to reduce T cell activity and promote the development of T_{regs} may aid in preventing an uncontrolled immune response that provokes autoimmunity or pathological tissue damage.

Kominsky *et al.* have argued[72] that the differential HIF response mechanisms in myeloid cells versus T cells has to do with the fundamental metabolism exhibited by each cell type. Myeloid lineage cells tend to glycolysis whereas lymphoid lineage cells tend to oxidative phosphorylation.[72] HIF, which promotes glycolysis in the absence of sufficient oxygen for oxidative phosphorylation, would therefore be most important for supporting glycolysis in myeloid cells, which are best adapted for taking advantage of increased glycolysis. Conversely, supporting glycolysis in lymphoid cells may be a less effective way of increasing their metabolic activity. DC can belong to either lineage, but the often contradictory research studies discussed above have all been performed using experimental models of myeloid DC.

HIF: A TARGET FOR IMMUNE REGULATION

A wealth of emerging information shows that HIF and the hypoxic response are

deeply involved with the regulatory pathways of innate immune defense. The key implication of these findings is that the nature and magnitude of host bactericidal and inflammatory activities are highly dependent on factors in the local tissue microenvironment such as oxygen tension and cannot be simply extrapolated from *in vitro* model systems under ambient conditions. Through HIF control of immune cell energetics and gene expression pathways, antimicrobial activities can be focused and amplified where they are needed most, namely foci of tissue infection, which are harsh and threatening microenvironments where oxygen and nutrients are limiting and cytotoxic molecules abound. A detailed understanding of the relationships between HIF, pathways of innate immune signal transduction such as TLR–NF-κB signaling and the deployment of various immune effector molecules will provide a clearer and more physiological understanding of infectious and inflammatory disease pathogenesis. It is also necessary to learn more about the role of HIF in triggering the adaptive immune response, and the relationship between HIF and other regulators of immune function such as vitamin D in the skin. Because of the short half-life and well-understood mechanism for post-translational regulation of HIF levels, HIF is an attractive pharmacological and vaccine target to fine tune immune cell functions for the prevention and treatment of

human disease.

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CHAPTER 2:

HIF-1α INFLUENCES MYELOID CELL ANTIGEN PRESENTATION AND RESPONSE TO SUBCUTANEOUS OVA VACCINATION

ABSTRACT

Hypoxia-inducible factor (HIF)-1 is a transcription factor known to play an important role in regulating the innate immune response to infection. Under baseline conditions, cellular HIF-1 levels in leukocytes are scarce, but levels rise rapidly in response to hypoxia or molecular signals of infection or inflammation such as microbial surface molecules and host-derived cytokines. Innate immune cells such as macrophages, neutrophils and mast cells exhibit increased microbicidal activity when HIF-1 levels are increased, and mice lacking HIF-1 are more susceptible to invasive bacterial infection. In this study, we used genetic and pharmacologic means to determine whether HIF-1 also plays an important role in the adaptive immune response to infection. *Hif-1α/Tie-2* Cre$^+$ mice harboring a >90% knockdown of HIF-1 in myeloid cells were studied. We found antigen-presenting cells from these mice expressed lower levels of MHC-II and the costimulatory molecules CD80 and CD86, and were less able to induce T cell

proliferation. These differences were present at baseline and persisted after activation.

Increasing HIF-1 levels in WT cells by using the prolyl hydroxylase inhibitor drug AKB-4924 had the opposite effect, increasing MHC and co-stimulatory molecule expression and T cell proliferation. In experimental vaccination, *Hif-1α/Tie-2* Cre$^+$ mice exhibited a weaker T cell response and lower antibody levels in response to vaccination than wild-type mice, while WT mice treated with a drug to elevate HIF-1 responded more strongly to vaccination. Thus HIF-1 participates in bridging the innate and adaptive immune responses, and may merit further exploration as an adjuvant target.

INTRODUCTION

Antibiotics and childhood vaccinations drastically lowered the burden of infectious diseases in the 20th century, but in recent decades progress in combating infectious diseases has slowed. A better understanding of the factors which control the immune response to infection would help us design treatments or vaccine adjuvants that would improve our ability to control infectious diseases.

Hypoxia-inducible factor (HIF) has been called a "master regulator" of innate immune function[28] because it plays a crucial role in enhancing the bactericidal activity of

myeloid cells such as macrophages and neutrophils[73]. Through HIF-1 induction under conditions of hypoxia, myeloid cells release more nitric oxide (NO), granule proteases and antimicrobial peptides, survive longer because of reduced apoptosis, and kill Gram-positive and -negative bacterial pathogens more efficiently than at normoxia[42, 47]. HIF activation is apparent during the differentiation of circulating monocytes into tissue macrophages[74], and may promote phagocytic uptake of bacteria under hypoxia[41]

Along with their function in the innate response to infection, myeloid cells such as macrophages and dendritic cells (DC) also play a crucial role in triggering the adaptive response to infection through their function as antigen-presenting cells (APCs). Our understanding of the role of HIF-1 in this critical aspect of host defense is much less developed. APCs provide three signals to T cells: the antigen, presented in the context of MHC-I or MHC-II; costimulatory signals through ligation of surface molecules; and cytokines and other soluble mediators. The combination of signals alerts the T cells to the foreign antigen, activates them, and modulates the strength and polarization of the adaptive immune response. The most important APC is the dendritic cell, a myeloid cell type closely related to macrophages.

Dendritic Cell Biology

DCs are a functionally and phenotypically diverse group of cells. They can be derived from the myeloid or lymphoid lineages.[75] For the purposes of this work, it is useful to focus on myeloid DCs and divide them into pre-dendritic cells (pre-DCs), conventional dendritic cells (cDCs), and inflammatory dendritic cells (iDCs). cDCs can be further divided into migratory and lymphoid-tissue resident dendritic cells.

Pre-dendritic cells (pre-DCs) are cells without the classic dendritic form and antigen-presenting function, but with a capacity to develop into DCs with little or no division. An inflammatory or microbial stimulus might be required. For example, monocytes can be considered pre-DCs because they can give rise to inflammatory DC upon exposure to inflammatory stimuli. [76]

Conventional dendritic cells already have DC form and function. Migratory DCs fit the profile of the textbook dendritic cells, and can be immature or mature. Immature migratory DCs (iDCs) are specialized for antigen capture and processing and have limited ability to stimulate T-cells. Under steady-state conditions, iDCs mostly reside at sites of contact between the host and the environment, such as the skin and the respiratory or gastrointestinal mucosa. These sentinel cells continuously scan the surroundings for

the presence of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). Upon antigen uptake and activation by proinflammatory cytokines and DAMPs or PAMPs, iDCs undergo phenotypic and functional changes called maturation.

Maturation prepares the DC to fulfill the second half of their sentinel duty: to take the antigen they have captured while immature to the lymph nodes and present it to T cells. At the molecular level, maturation manifests as increased expression of MHC antigens and co-stimulatory molecules (such as CD83, CD80, CD86, and CD40), decreased expression of phagocytic/endocytic receptors, and a switch in the chemokine receptor repertoire to down-regulate receptors for inflammatory chemokines (e.g. CCR1, CCR2, CCR5, CCR6, and CXCR1) and upregulate receptors for chemokines required for homing to secondary lymphoid organs, namely CCR7 and CXCR4.

Lymphoid-tissue-resident DCs are the other kind of cDCs. They collect and present foreign and self-antigens in their home organ. These cells play crucial roles in maintaining tolerance to self-antigens, harmless environmental antigens, and commensal microorganisms.

Inflammatory dendritic cells are not normally present in the steady state, but

appear as a consequence of inflammation or microbial stimuli. An example is the tumor-necrosis factor- and inducible nitric-oxide synthase-producing DCs (Tip DCs).

Dendritic Cells and HIF

Research into the role of HIF in DCs is complicated by the fact that DCs are a rare cell type and it is difficult to obtain adequate numbers of primary cells for experimentation. Consequently, much of the *in vitro* work on DCs and HIF has been performed on human peripheral blood monocytes or mouse bone marrow cells differentiated into DCs by treatment with GM-CSF and IL-4 for periods of 7 to 11 days. Both methods produce DCs most similar to inflammatory DCs,[77] and not the migratory cDCs that are likely to play an important sentinel role *in vivo.*

Previous attempts to determine the role of HIF in DC maturation have yielded contradictory results. Various investigators have produced data indicating that hypoxia promotes DC maturation both alone[78, 79] and in combination with LPS stimulus[52, 80], as measured by decreased phagocytosis,[81, 82] increased migration,[50, 51] and increased expression of MHC and costimulatory molecules.[50, 80, 82, 83] Others have come to exactly the opposite conclusion, namely that hypoxia inhibits DC maturation[81], migration[49, 81, 84]

(possibly by reduced expression of MMP-9, which helps DC migrate[85, 86]), and expression of costimulatory molecules.[49, 87, 88]

When it comes to the effect of hypoxia and HIF on the ability of APC to prime T cells, the literature is no less mixed. Some groups have shown that hypoxia and HIF increase the ability of APCs to stimulate a T cell response[52, 82, 89, 90] and lead to the expression of more proinflammatory cytokines[49, 52, 83, 87, 88, 91, 92] that bias towards a T_H1 response[89] while others have found the opposite.[81 93] Still others have reported a mixed phenotype among the DC in their *in* vitro model system.[49]

Hypoxia and HIF induction cannot be approached as functional equivalents in studies of the immune responses, because HIF is also induced at the transcriptional level by a variety of signals other than hypoxia, including markers of infection and inflammation such as cytokines[15, 23] and viral proteins.[94, 95] Furthermore, when HIF is activated by hypoxia, its contribution to transcriptional regulation comprises a different set of target genes than when it is activated by a TLR ligand such as LPS[96].

From the above literature survey, it is safe to say the jury is still out on the role of HIF in priming the adaptive immune response. Some of the variation in reported results may be due to differences in stimuli. Critically, the context within which HIF is activated

(hypoxia versus inflammation) affects the results of HIF activation. When HIF is activated by hypoxia, it enhances transcription from a different set of target genes than when it is activated by a TLR ligand such as LPS.[96] Furthermore, when hypoxia is used as a stimulus in the antigen presentation readouts, it affects not only the APC but the T cells themselves, further influencing the results of the experiments.

Here we contribute a new approach to our understanding of HIF in DC biology, by employing the genetic system of myeloid *Hif-1α*-null deficient mice. Coupled with pharmacological studies with a potent HIF-1 specific pharmacological agonist, we examine antigen presentation function *in vitro* and *in vivo*, lending support to a significant role of HIF regulation in these phenotypes.

MATERIALS AND METHODS

Cell Culture

Bone marrow (BM) was obtained from the hind legs of mice age 8-16 weeks and grown in RPMI with 10% endotoxin-free FBS, 50 U/ml penicillin, 50 µg/ml streptomycin, 50 µg/ml gentamicin, 50 µM β_2-mercaptoethanol, 10% conditioned media from GM-CSF-expressing B16 cells, and 20 ng/ml IL-4 (Peprotech). Media was changed

on day 3 and 5 and the nonadherent cells were used between days 8-11. RAW264.7 macrophages were grown in RPMI with 10% FBS, 50 U/ml penicillin, and 50 μg/ml streptomycin.

Surface Marker Analysis

BM-derived DC from *Hif-1α/Tie-2* Cre[+] or Cre[-] mice differentiated for 8-11 d were plated in triplicate at 10^6 cells/well in 12-well plates and treated with LPS (10 or 100 ng/ml, as indicated) TNF (500 U/ml), or media alone, overnight. RAW264.7 macrophages were also plated in triplicate at 10^6 cells/well in 12-well plates and treated with AKB-4924 (10 or 100 μM, as indicated), the vehicle for AKB-4924 (cyclodextrin, at equivalent concentration), both LPS (100 ng/ml) and 4924, or vehicle alone overnight. The following day, the nonadherent DC or RAW macrophages were harvested, washed, and stained with the fluorescent anti-mouse antibodies CD11c-FITC, MHC-II-PE, CD86-PECy7, and CD80-APC. Fluorescence was measured by flow cytometry (FacsCalibur, BD Biosciences) and analyzed using FlowJo (TreeStar). Mean fluorescence was calculated on cells gated on CD11c^{+} cells.

Cytokine Protein Measurement

BM-derived DC from *Hif-1α/Tie-2* Cre$^+$ or Cre$^-$ mice differentiated for 8-11 d were plated in triplicate at 10^6 cells/well in 12-well plates and treated with LPS (10 or 100 ng/ml, as indicated) or media alone, overnight. For some experiments, BM-derived DC from C57BL/6 mice (Charles River) between days 8-11 of differentiation were treated with AKB-4924 (10 or 100 μM, as indicated), the vehicle for AKB-4924 (cyclodextrin, at equivalent concentration), both LPS (10 ng/ml) and AKB-4924, or vehicle alone overnight. The following day, the supernatant was harvested and protein levels measured by ELISA (IL-6, BD Biosciences #555240; IL-10, BD Biosciences #555252; IL-12 p70, BD Biosciences #555256; TNFα R&D Biosciences #DY410).

Cytokine RT-PCR

BM-derived DC from *Hif-1α/Tie-2* Cre$^+$ or Cre$^-$ mice that had been differentiated for 8 to11 days were plated in triplicate at 10^6 cells/well in 12-well plates and treated with LPS (10 or 100 ng/ml, as indicated) or media alone for 4 h. For some experiments, bone-marrow-derived dendritic cells from C57BL/6 mice (Charles River) between day 8 and day 11 of differentiation were treated with AKB-4924 (10 or 100 μM, as indicated), the

vehicle for AKB-4924 (cyclodextrin, at equivalent concentration), both LPS (10 ng/ml)

and 4924, or vehicle alone for four hours. RNA was obtained using the RNEasy Mini kit

(Qiagen, Cat #74104), reverse transcribed to DNA using the iScript cDNA synthesis kit

(BioRad, Cat#170-8890), and RT-PCR run using iQ SYBR Green Supermix (BioRad,

Cat#170-8882). RT-PCR reaction conditions: 50°C for 10 min, 95°C for 5 min, 40 cycles

of 95°C for 10 sec followed by 56°C for 30 sec, 95°C for 10 sec, and a melt curve from

55°C to 90°C in increments of 0.5°C for 5 sec each. Data was normalized using β-actin.

Primer sequences and annealing temperatures used for RT-PCR analysis of β-actin

(housekeeping control), IL-6, IL-12 and TNF-α are available on request.

T-Cell Proliferation Assay

BM-derived DC from *Hif-1α/Tie-2* Cre$^+$ or Cre$^-$ mice differentiated for 8-11 days

were harvested, washed, plated at 2 x 10^4 cells/well in a 96-well plate, and incubated

overnight with the OT-I peptide SIINFEKL at 20 μg/ml or 0 μg/ml, or with the OT-II

peptide OVA$_{323-337}$ at 20 μg/ml or 0 μg/ml. The next day, CD8 T cells were obtained

from the spleen and lymph nodes of naïve OT-I mice, and CD4$^+$ T cells obtained from

the spleen and lymph nodes of naïve OT-II mice using MACS purification kits (Miltenyi

Biotec, CD4^{+} Cat #130-095-248; CD8α+ Cat#130-095-236). The T cells were added at 2

x 10^5 cells/well (a 1:10 DC:T cell ratio) in RPMI with 10% FBS, 2 mM L-glutamine, 50

U/ml penicillin, 50 μg/ml streptomycin, 50 μM β-mercaptoethanol, 10 mM HEPES and

100 μM MEM-NEAA. After 3 d incubation, 0.25 μCi/well of ^{3}H-thymidine was added

and the plate was incubated for an additional 8-16 h, at which point the cells were lysed

by freezing at -80°C and read on an automated harvester (Becton Dickinson).

Vaccination Experiments

Hif-1α/Tie-2 Cre[+] mice and age- and sex-matched Cre[-] controls were immunized

subcutaneously (s.c.) on the back with 50 μg antigen (SIINFEKL or OVA) in 50 μl PBS

emulsified with 50 μl IFA, for a total volume of 100 μl. In other experiments, wild-type

C57BL/6 mice were treated with 5 mg/kg AKB-4924 or vehicle control by intraperitoneal

(i.p.) injection 24 h before, 1 h before, and 16 h after immunization as above. The

SIINFEKL-immunized mice were sacrificed on day 8, spleens were harvested, plated at 2

x 10^6 cells/well in a 96-well ELISPOT plate pre-coated with anti-IFNγ antibody, then re-

stimulated with SIINFEKL at 2.5 μg/ml or with irrelevant OT-II peptide as a negative

control. The number of IFNγ-producing cells in the whole splenocyte population was

measured by ELISPOT (Mabtech). The OVA-immunized mice were bled on day 14 after immunization. ELISA plates were coated with 2 μg/ml OVA in PBS, and the OVA-specific IgG serum antibody titer was measured using anti-mouse IgG-HRP linked antibodies.

Statistical Analysis

Statistical significance was calculated by Student's *t* test or two-way ANOVA, as appropriate for the data, using GraphPad Prism.

RESULTS

DCs provide three signals to T cells: the antigen, presented in the context of MHC-I or MHC-II; costimulatory signals, achieved through ligation of surface molecules; and lastly, the release of cytokines and other soluble mediators. The combination of signals alerts the T cells to the foreign antigen, activates them, and modulates the strength and polarization of the adaptive immune response. To find out what role HIF plays in the ability of antigen-presenting cells (APCs) to stimulate the adaptive immune response, we used *Hif-1α/Tie-2* Cre mice. These mice harbor a targeted

deletion of *Hif-1α* in endothelial cells and hematopoietic precursors; *Hif-1α/Tie2*-Cre line

exhibits 98% deletion efficiency in bone-marrow cells.[97]
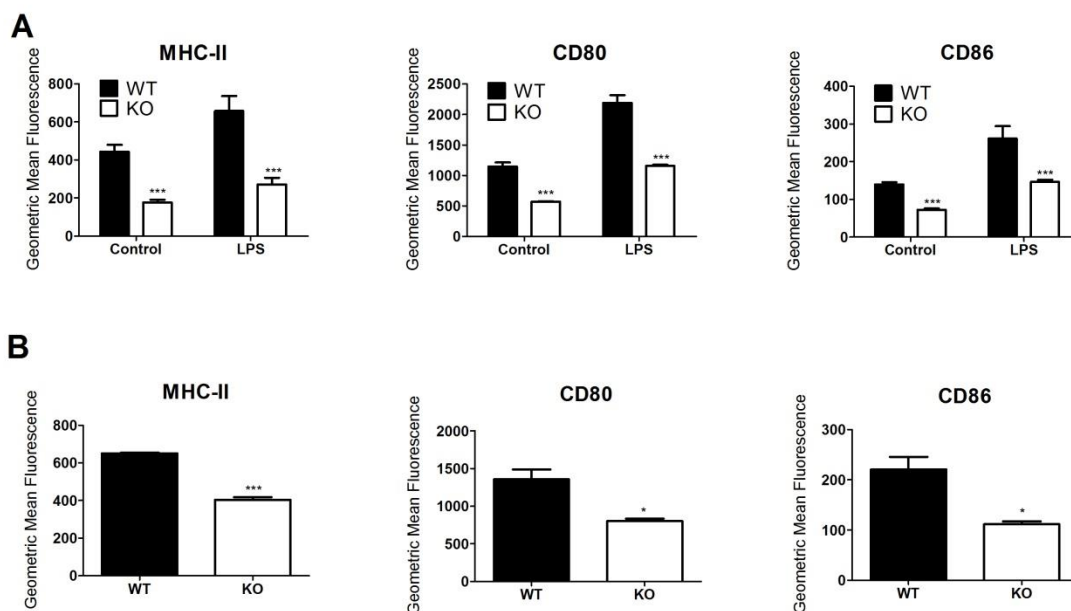
Figure 1. Dendritic cells (DC) that lack HIF-1α express lower levels of MHC-II and co-stimulatory molecules. Immature BM-derived DC stimulated with 100 ng/ml LPS (A) or matured with 500 U/ml TNF-α (B) overnight and were evaluated for MHC-II, CD80 and CD86 expression using specific antibodies and FACS analysis. Statistical significance was calculated using (A) two-way ANOVA and (B) unpaired, two-tailed *t*-test; *P < .05, ***P < .001.

We first looked at the effect of *Hif-1α* ablation on the DC lineage itself. BM-

derived DC from *Hif-1α* KO mice and Cre- controls showed decreased expression of key

surface molecules. Immature DC lacking HIF expressed less MHC-II and the

costimulatory molecules CD80 and CD86 at baseline, and when they were activated by

overnight exposure to LPS the deficit persisted (**Fig. 1A**). Likewise, DC that had been

matured by exposure to TNFα also expressed lower levels of MHC-II, CD80, and CD86

(**Fig. 1B**). These findings suggest that *Hif-1α* is important for the ability of activated DC

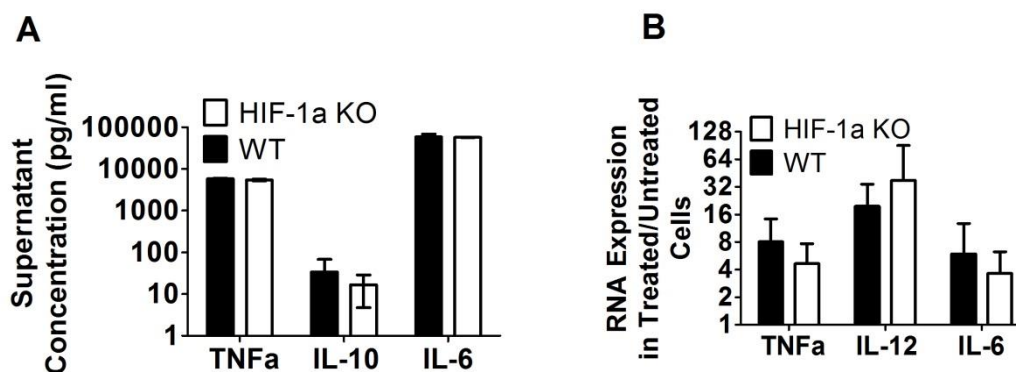to deliver the first and second activation signals to T cells.

Figure 2. HIF-1α is not essential for release of key cytokines from DC. (A) BM-derived dendritic cells (DC) from WT and HIF-1α myeloid null mice were stimulated with LPS (10 ng/ml) overnight; cytokine release was quantified by ELISA. Data is representative experiment with triplicate samples, representative of 5 experiments with similar results. (B) DC were stimulated with LPS (10 ng/ml) for 4 h and cytokine transcripts monitored by real-time RT-PCR. Data shown is the pooled results of 5 experiments. Statistical significance was calculated using two-tailed *t* tests.

In contrast, we did not identify a requirement for *Hif-1α* in the LPS-stimulated

release of cytokines by DC. Key cytokines known to play a role in the adaptive immune

response were examined. IL-6 is a B cell differentiation factor, and it also may play a role

in polarizing the T helper cell response, while IL-12 is a proinflammatory cytokine that

promotes T_H1 polarization and IL-10 is an anti-inflammatory cytokine that promotes T_H2

polarization[98, 99]. TNFα enhances the proliferation of T cells. No differences were found

for any of these cytokines at the level of protein (**Fig. 2A**) or mRNA transcript (**Fig. 2B**).

This result distinguishes DC from other myeloid cells such as macrophages and

neutrophils, in which we reported that deletion of HIF-1α modulated the release of
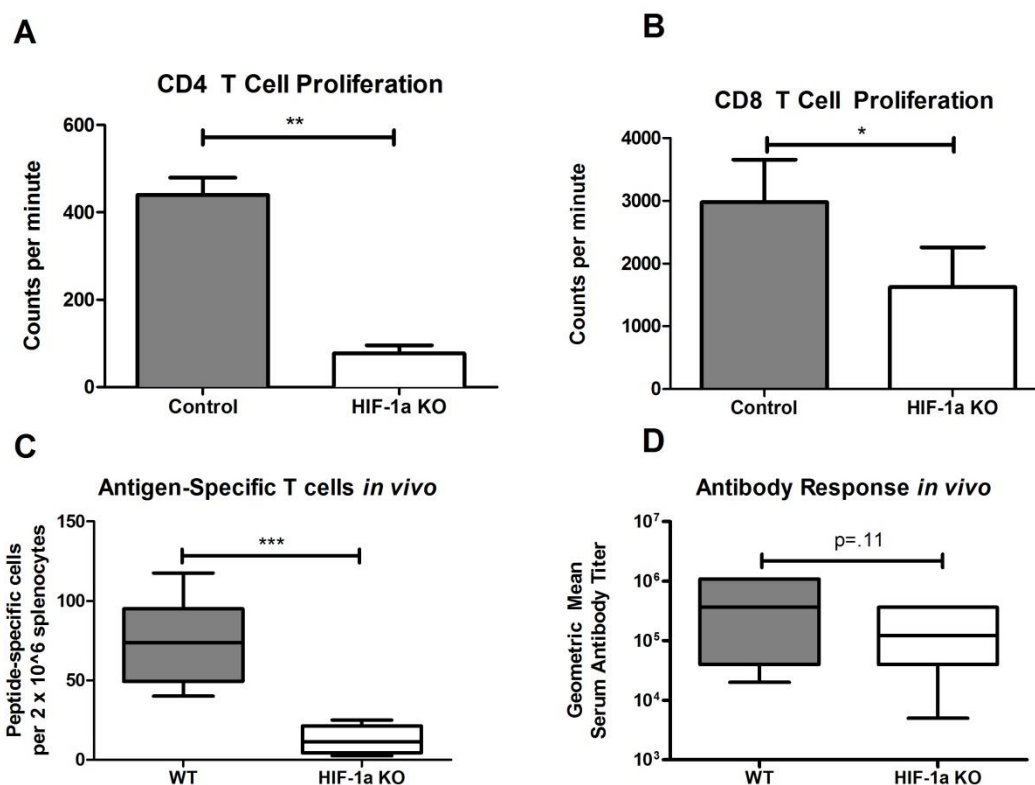
certain pro-inflammatory cytokines.[42]

Figure 3. HIF-1α is important for DC-triggered T cell proliferation and HIF-1α myeloid-KO mice have weakened responses to vaccination. BM-derived DC were coated with the MHC II-restricted OVA subpeptide $OVA_{323-329}$ and incubated with $CD4^+$ T cells from OT-II mice (A) or coated with the MHC-I restricted OVA subpeptide SIINFEKL and incubated with $CD8^+$ T cells from OT-I mice (B) for 3 days. T cell proliferation was measured by the incorporation of ^3H thymidine. Data represent the normalized results of 3 (A) or 4 (B) experiments. (C) Six myeloid HIF-1α/Tie-2 Cre^+ and 6 Cre^- littermate controls were vaccinated with 50 µg/mouse SIINFEKL peptide. Eight days after vaccination, mice were sacrificed and specific T cells in their spleens measured by IFNγ ELISPOT. (D) Myeloid HIF-1α/Tie-2 Cre^+ and Cre^- mice were vaccinated with 50 µg/mouse OVA protein. Blood was collected 14 days after vaccination and OVA titer was measured using a specific ELISA. Data shown is the combined results of two separate experiments, for a total of 11 WT and 9 KO mice. Statistical significance was calculated by two-tailed t test. $*P < .05$, $**P < .01$, $***P < .001$.

As a primary function of DC is to activate T cells, we next asked whether DC

lacking HIF-1α were able to stimulate T cell proliferation. BM-derived DC were coated

with the MHC-I restricted peptide SIINFEKL or the MHC-II restricted peptide OVA_{323-}

337, and then incubated with CD8^{+} T cells or CD4^{+} T cells from OT-I or OT-II mice, respectively. Both CD4^{+} and CD8^{+} T cells incubated with KO DC exhibited significantly less proliferation than those incubated with Cre^{-} control DC (**Fig. 3A, B**), indicating that HIF-1α plays an important role in the ability of DC to trigger T cell proliferation.

Since the loss of HIF-1α impairs the ability of APCs to present antigen, provide costimulatory signals, and stimulate T cell proliferation *in vitro*, we hypothesized that it should also impair the *in vivo* response to vaccination. Indeed, when HIF-1α KO mice were vaccinated with the ovalbumin subpeptide SIINFEKL, they produced fewer antigen-specific T cells than Cre[-] control mice (**Fig. 3C**). However, when serum antibody titers were examined, the effect was weaker. Although a trend of diminished response could be discerned, the effect of *Hif-1α* ablation on serum IgG titer in response to vaccination with OVA did not reach statistical significance (**Fig. 3D**).

Our evidence in the genetic model systems to this point suggested that loss of HIF-1α impairs the function of DC in promoting adaptive immune response. For the purpose of preventing or treating infectious disease, one could contemplate whether pharmacological agents that increase cellular HIF-1α levels could improve DC function in the immune response. Proof-of-principle studies were performed with RAW264.7
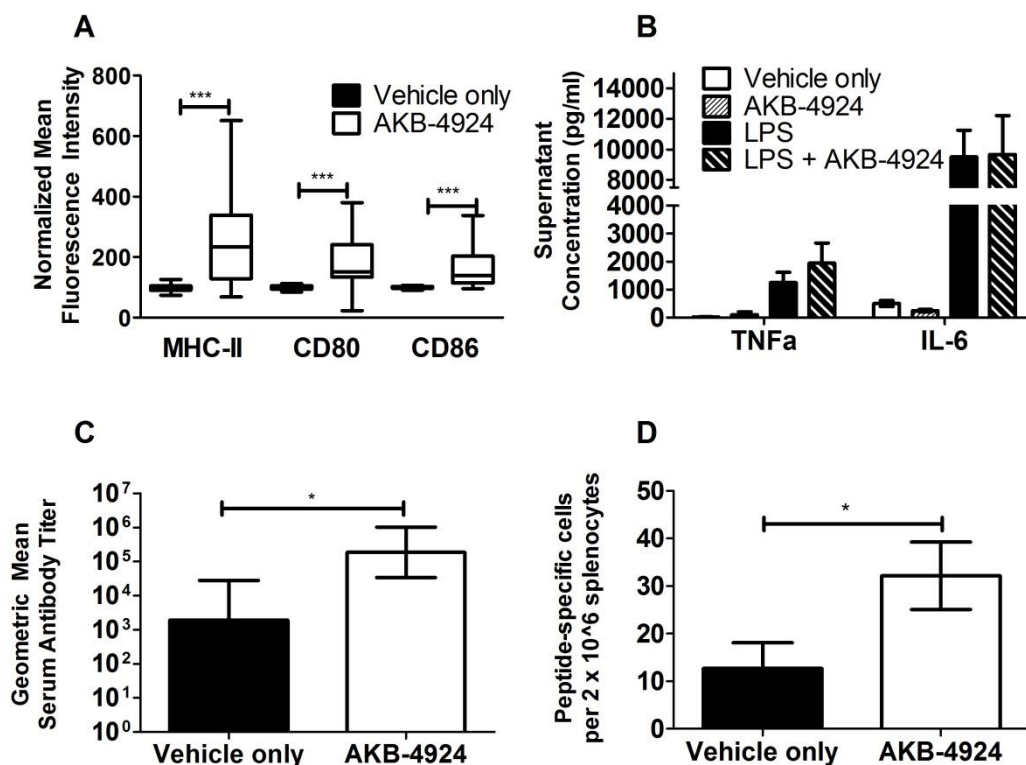
Figure 4. Pharmacological augmentation of HIF-1α increases DC activation and response to vaccination. (A) RAW murine macrophages were treated with 100 μM AKB-4924 or vehicle (cyclodextrin) control overnight and surface marker expression measured by flow cytometry. (B) WT BM-derived DC were treated with 100 μM AKB-4924, 10 ng/ml LPS, both AKB-4924 and LPS, or vehicle control, and cytokine release measured by ELISA. (C and D) WT C57BL/6 mice (5 per group) were pretreated with AKB-4924 (5 mg/kg intraperitoneal) or vehicle control before immunization with 50 μg of (C) SIINFEKL peptide or (D) OVA protein. (C) Splenic T cell response was measured by IFNγ ELISPOT 8 days after immunization; (D) Anti-OVA serum IgG was measured by ELISA 14 days after immunization. Statistical significance was determined by two-tailed t-test; *$P <.05$, ***$P < .001$.

macrophages, showing that when HIF levels are increased with HIF-1α boosting drug AKB-4924[44], the macrophages express higher levels of MHC-II, CD80, and CD86 (**Fig. 4A**), a result in agreement with the genetic studies in Figure 1. AKB-4924 inhibits the

prolyl hydroxylases[44] which negatively regulate HIF-1α[8], and preferentially elevates HIF-1α over HIF-1β[44]. Also in agreement with our data from the knockout mice, we found that AKB-4924 had no effect on cytokine release from WT bone-marrow derived DC (**Fig. 4B**), but did strengthen both the humoral (**Fig. 4C**) and cell-mediated (**Fig. 4D**) response to OVA immunization.

DISCUSSION

The data presented above indicates that HIF-1α plays a modest but discernable role in the ability of DC to trigger the adaptive immune response to infection. Loss of HIF-1α reduces the surface expression of major histocompatibility and costimulatory molecules on DC, leading to an impaired ability to stimulate both $CD4^+$ and $CD8^+$ T cell proliferation, without significant effects upon the release of cytokines. These changes correlate to a weaker cell-mediated and humoral response to vaccination. Elevating HIF with the drug AKB-4924 produces the opposite effects, enhancing DC antigen presentation and T cell stimulatory functions.

Our results provide new information relevant to a somewhat confounding literature regarding the influence of reduced oxygen tension on DC activity. Using

murine[52] or human[80] DC, two groups separately found that while hypoxia alone did not affect antigen presentation or T cell activation, it did synergize with LPS to create a stronger effect than LPS alone. Along those lines, it was reported that hypoxic macrophages were better at activating T cells while releasing more of the T_H1-polarizing cytokine IL-12[89]; however, other groups arrived at opposite conclusions, suggesting hypoxic DC had impaired antigen presentation and T cell activation[100] and promoted a T_H2 phenotype[81]. Still others have reported a mixed phenotype[49]. Differences experimental approaches used with regard to source and purity of DC precursors, differentiation/maturation protocols, degree and duration of the hypoxic stimulus, have been suggested as potential explanations for these discrepancies[48]. Our research was the first to couple genetically modified mice and pharmacological tools to distinguish the effect of the transcription factor HIF-1α specifically from more global, hypoxia-dependent effects.

The compound AKB-4924 has been found to stabilize HIF-1α activity in macrophages and neutrophils and to promote their direct bactericidal activity against drug-resistant pathogens[44]. This project was motivated in part by an interest in determining whether a similar approach to pharmacologically augment HIF-1α could be

contemplated as an adjuvant strategy for vaccines. Drugs related to AKB-4924 that modulate prolyl hydroxylases have advanced in clinical trials of extended therapy to boost erythropoietin levels in anemia, showing a favorable safety profile to date[101]; thus their short term use in vaccines would likewise be feasible. Our preliminary results show that pharmacologically increasing HIF-1 levels has a modest effect to improve the response to experimental vaccination. Whether this effect could be harnessed and optimized to prove useful in adjuvant formulations merits further exploration.

Chapter 2, in full, is a reprint of material that appears in the *Journal of Molecular*

Medicine (Berlin, Germany), 2013, 92:1199-1205, Bhandari, Tamara; Olson, Joshua; Johnson, Randall; Nizet, Victor. The dissertation author was the primary investigator and author of this paper.

CHAPTER 3:

VITAMIN D AND HIF IN THE CONTROL OF CATHELICIDIN EXPRESSION

ABSTRACT

The skin is the first line of defense against infection with many pathogens, including major ones such as *Staphylococcus* and *Streptocococcus*. Both the transcription factor hypoxia-inducible factor (HIF) and the secosteroid hormone dihydroxyvitamin D_3 (vitamin D) have been shown to be key regulators of skin immunity. Here we investigate the overlap between HIF and vitamin D_3 in the regulation of cathelicidin, a key effector molecule in skin immunity.

INTRODUCTION

The skin, continually exposed to the external environment, functions as a critical first line of defense against infection. The skin epithelium serves as a barrier to invasion by external pathogens by also a barrier to innate immune cells that must respond rapidly to signs of pathogen entry. A growing body of literature has demonstrated that adequate

levels of vitamin D are important for maintaining an optimal immune response in the host. A separate but equally strong body of literature has demonstrated that the transcription factor HIF is a key regulator of innate immune cell function. In particular, both vitamin D and HIF strongly regulate the production of cathelicidin, an antimicrobial peptide central to the skin immune response. These findings raise the question of how vitamin D and HIF interact in the regulation of cathelicidin. A better understanding of cathelicidin regulation would help in the task of developing targeted strategies for therapeutic enhancement of skin immunity.

The Skin and its Immune System

The skin represents the first host barrier against many environmental pathogens. Skin provides four kinds of defense: physical, chemical, microbial, and cellular. The outermost layer of the epidermis is the stratum corneum—a cornified layer of dead keratinocytes—which forms a watertight physical barrier. Underneath the stratum corneum lies the stratum granulosum, where keratinocytes are bound together with tight junctions. Together, the stratum corneum and stratum granulosum form a strong physical barrier to microbial entry. Chemically, the skin is dry with a low pH rendering it

inhospitable to many microorganisms. The normal skin microflora also provide a layer of

protection against infection, as would-be pathogens must compete for space and nutrients

within a dense lawn of commensal microbes.

The epidermis is composed mostly of keratinocytes, interspersed with a few

myeloid immune cells. Keratinocytes are often overlooked for their role in immune

defense, but they are crucial for the control of bacterial infection.[69, 102] Keratinocytes

express many pattern recognition receptors, including Toll-like receptors (TLRs)[103] that

recognize conserved microbial components, Nod-like receptors (NLRs) Nod1 and

Nod2,[104] which sense bacterial peptidoglycan, and NLR pyrin domain–containing

proteins[105] that respond to viral, fungal and self components. Upon detection of microbial

products, keratinocytes are capable of generating a vast array of antimicrobial effector

molecules, including antimicrobial peptides such as cathelicidins and defensins.[69, 106, 107]

Keratinocytes can also produce chemokines and cytokines in response to pathogenic

stimuli.[102] Chemokines released from the skin recruit immune cells such as monocytes,

dendritic cells and T cells, and cytokines activate those cells and help shape the immune

response.

Within the network of keratinocytes are found memory $\alpha\beta$ T cells[108, 109] which are

important for a rapid response to recurrent infections, and epidermal dendritic cells known as Langerhans cells. Beneath the epidermis lies the dermis, which is also composed mostly of keratinocytes, but with a greater diversity of other cell types, including mast cells, macrophages, various DC subsets, innate lymphoid cells, γδ T cells and αβ T cells. All these cells communicate with one another to provide a coordinated immune response.

Antimicrobial peptides (AMPs) are a key component of the skin innate immune system. Also called host defense peptides, AMPs are an ancient form of host defense found among all classes of life. The human antimicrobial peptides include 6 human α-defensins (HNP1-4 and HD-5,6), 4 human β-defensins (hBD-1 to 4) and cathelicidin (LL-37).[110]

AMPs are very structurally diverse, sharing little but their function as microbicides. Many are unstructured in free solution, and fold into their final configuration upon partitioning into biological membranes. The modes of action by which antimicrobial peptides kill bacteria is varied and includes disrupting membranes, interfering with metabolism, and targeting cytoplasmic components.[111]

Mice and humans both express just one cathelicidin, known as LL-37 in humans

and CRAMP in mice. They are α-helical peptides.[112] Using a global cathelicidin

knockout mouse (Camp [-/-]), Nizet *et al.* showed that absence of this single AMP gene

led to a large increase in susceptibility to invasive bacterial infection.[113] Knockout

mice subcutaneously infected with group A *Streptococcus* (GAS) had much bigger

lesions and a higher bacterial burden than wild-type mice.[113] Subsequent research showed

that cathelicidin and other AMPs are effective against a wide variety of pathogens:

bacteria such as *S. aureus*[114] and *M. tuberculosis,*[115] protozoa such as *Leishmania,*[116] and

viruses such as herpes simplex virus,[117] among others.

The role of AMPs in host defense extends beyond their antibiotic effects. They

also play a role in wound healing, an adaptive bifunctionality since wounding and skin

infection so often go together. AMPs are strongly upregulated by the epithelium shortly

after wounding and LL-37 induces epithelial proliferation.[118]

AMPs also function as immunomodulators. Cathelicidins attract other cell types

to the site of infection or wounding, including human and mouse neutrophils, monocytes,

mast cells, T cells, and DCs.[119-124] Cathelicidin LL-37 enhances the expression of co-

stimulatory molecules and T_H1 cytokines by monocyte-derived dendritic cells,[125]

promotes the release of α-defensins from neutrophils,[126] enhances the vascular

permeability of mast cells,[124] and stimulates keratinocytes[127] and dendritic cells.[128]

HIF in Skin Immunity

HIF is strongly expressed in skin, which is a hypoxic organ even at baseline,[129] and plays an important role in skin immunity. Mice that lack HIF-1α in their keratinocytes (K14-Cre$^+$) are more susceptible to bacterial skin infection[69] and treatment of mice with HIF stabilizers such as mimosine or AKB-4924 boosts defense against *S. aureus* skin infection *in vivo*.[44] This effect is due at least in part to the immunomodulatory role of HIF in keratinocytes, as Increasing HIF levels with the drug AKB-4924 stimulates keratinocyte production of pro-inflammatory cytokines *in vitro*,[29] enhances neutrophil recruitment *in vivo*,[29] and boosts the bactericidal activity of mast cells.[46]

Most importantly for skin immunity, HIF plays a key role in the control of cathelicidin expression. HIF regulates expression of human and murine cathelicidins through HREs in their 5' promoter domains, and to further promote expression of granule proteases that process cathelicidins from their prepropeptide to mature active forms.[42] Specific RNAi studies confirm that HIF-1α regulation of keratinocyte

cathelicidin production is critical in cutaneous defense against invasive bacterial infection.[70]

Vitamin D in Immunity

Vitamin D is a group of fat-soluble secosteroid hormones. Despite its name, vitamin D is not properly called a vitamin because it can be synthesized *in vivo* upon exposure to sunlight as well as obtained through the diet. Vitamin D can be ingested as cholecalciferol (vitamin D_3) or ergocalciferol (vitamin D_2); the latter is synthesized by plants, fungi, and fish. Vitamin D_3 is the form synthesized by mammals. Dietary D_2 and D_3 seem to have similar effects, although vitamin D_3 is more potent.[130] There is no vitamin D_1; the isolate originally named vitamin D_1 was later shown not to be a unique compound.

Vitamin D_3 is produced in the skin from 7-dehydrocholesterol after exposure to UVB light. This form is not active. To activate vitamin D, it must be hydroxylated twice. The first hydroxylation typically takes place in the liver, forming calcidiol, also known as 25-hydroxyvitamin D and abbreviated $25(OH)D_3$. This is the form of vitamin D that is measured in the serum to determine whether a patient suffers from vitamin D deficiency.

Calcidiol is further hydroxylated, typically by the kidney, to calcitriol, abbreviated

$1,25(OH)_2D_3$. The enzyme that hydroxylates calcidiol is known as 1α hydroxylase.[131]

Although the liver and kidney are the primary sites of the first and second hydroxylation,

respectively, it is important to note that keratinocytes and myeloid cells can also

hydroxylate vitamin D.[132, 133] A lipophilic molecule, vitamin D slips easily through the

cellular and nuclear membranes to bind its receptor in the nucleus. There is a single

vitamin D receptor (VDR), which, together with the required cofactor retinoid X

receptor, forms a heterodimer and binds to vitamin D response elements (VDREs).

Vitamin D deficiency has long been known to cause rickets and osteoporosis, but

there is growing evidence that it also plays a role in susceptibility to infectious

diseases.[134-136] Immune cells such as T cells, B cells, NK cells, and monocytes express

the VDR.[137] In monocytes, vitamin D induces differentiation[138, 139] and leads to increased

phagocytic[140] and oxidative burst ability.[141] Macrophages also synthesize active vitamin

D themselves[142] in response to immune signals such as IFNγ and LPS,[143] or upon

detection of viral infection.[144]

Relevant to skin immunity, vitamin D enhances barrier function by upregulating

genes such as occludin (required for tight junctions), connexin 43 (required for gap

junctions), and E-cadherin (required for adherens junctions).[145] In keratinocytes, vitamin D is part of a positive feedback loop. Wounding or TLR activation leads to activation of 1α-hydroxylase[146] and production of active vitamin D, which in turn upregulate expression of pattern recognition receptors such as TLR-2 and CD14,[147] making the keratinocytes even more sensitive to danger signals. Perhaps most importantly, the human cathelicidin gene contains three VDREs in its promoter,[148] which explains why vitamin D induces the expression of cathelicidin by a variety of immune cells including neutrophils,[149] monocytes and macrophages,[146] and keratinocytes.[150]

However, vitamin D seems to have the opposite effect on T cells as it does on monocytes and macrophages. High levels of vitamin D activate antigen-specific T cell division, but also inhibit activation and IFNγ and IL-2 production in $CD4^+$ T cells.[151] Vitamin D also limits excessive production of the proinflammatory cytokine IL-12.[152] It seems that proper vitamin D signaling both activates the innate immune response and helps prevent it from getting out of control.

Crosstalk between HIF and Vitamin D

Comparing the roles of HIF and vitamin D in immunity highlights the fact that

they participate in regulating many of the same processes. Both molecules are involved in both immunity and wound healing, and both positively regulate a suite of myeloid cell functions, while suppressing T cell activity. Both HIF and vitamin D strongly regulate cathelicidin production in cells relevant to skin immunity. The two factors even act through some of the same molecular pathways, notably the NF-κB and MAPK cascades.[138, 139, 153] Thus circumstantial evidence that vitamin D and HIF may interact with one another is strong.

Moreover, the human *Hif-1α* gene has a VDRE in its 5' promoter[154] and vitamin D has been shown to increase HIF-1α mRNA,[155] protein,[155] and function.[156] Most of this work was done in systems unrelated to infection such as bone mineralization,[156] blood vessel formation[157] and tumor biology,[155, 158, 159] but one group used the neutrophil-like cell line HL-60 to show that vitamin D treatment leads to increased *Hif-1α* mRNA expression.[160] It should be noted that there are independent reports that vitamin D has no effect on[157] or negatively regulates HIF.[159]

In light of the evidence presented above, we hypothesize that the effect of vitamin D on skin immunity could be mediated in part by HIF. To investigate this hypothesis, cathelicidin expression was chosen as the output measure. Cathelicidin is essential for

skin immunity, and its expression is strongly regulated by both HIF and vitamin D in humans. Furthermore, there is only one cathelicidin gene in humans, simplifying genetic studies. One limitation of studying cathelicidin in this regard is that it is regulated differently in mice and humans, decreasing the utility of *in vivo* experiments in a small animal model. Unlike in humans, vitamin D alone does not induce cathelicidin expression in the mouse;[148] instead, a combination of vitamin D and parathyroid hormone is required.[161]

MATERIALS AND METHODS

Cells and Reagents

The human keratinocyte HaCaT cell line was maintained in HaCaT maintenance media (RPMI + 10% FBS + 50 U/ml penicillin + 50 μg/ml streptomycin. Before beginning each experiment, the cells were plated at 10^6 cells/well in maintenance media. The following day, the cells were washed and the maintenance media was replaced with HaCaT experimental media (RPMI + 10% charcoal-stripped FBS + 50 U/ml penicillin + 50 μg/ml streptomycin) 8 to 16 hours before the start of the experiment. The experiment was conducted in experimental media. $1,25(OH)_2D_3$ (Sigma-Aldrich, Cat#D1530) was

suspended in 100% ethanol and used at 100 nM. AKB-4924 was provided by Akebia Therapeutics and suspended in 10% cyclodextrin, pH 3.7. LPS (Enzo Life Sciences, Cat#ALX=581-001-14005) was suspended in water, digoxin (Tokyo Chemical Company, Cat#D1828) was suspended in 100% DMSO, and YC-1 (Sigma-Aldrich, Cat#4102) was suspended in 100% DMSO.

Western Blot

HaCaT cells were treated overnight with vitamin D (100 nM), ethanol control, or LPS (100 ng/ml). Nuclear proteins were extracted using the NE-PER Nuclear and Cytoplasmic Protein Extraction Kit (Thermo Scientific, Cat#78833) and quantified using BCA assay (Pierce, Cat#23252). Samples were run on 10% bis-tris gel (Novex, Cat#NP0302). HIF was detected using anti-HIF antibody (Novus Biologics, Cat# NB100-449) at 1:500 dilution, followed by donkey α-rabbit IgG HRP secondary antibody at 1:10,000 (GE Healthcare, Cat#LNA934V). The membrane was stripped and washed and then P84 was detected using anti-P84 antibody (Genetex, Cat#GTX70220) at 1:10,000 followed by sheep α-mouse IgG HRP (GE Healthcare, Cat#NA931V) at 1:10,000.

RT-PCR

HaCaT cells were plated at 10^6 cells/well in maintenance media. RNA was obtained using the RNEasy Mini kit (Qiagen, Cat #74104), reverse transcribed to DNA using the iScript cDNA synthesis kit (BioRad, Cat#170-8890), and specific gene targets amplified using KAPA Sybr Fast qPCR kit (KAPA Biosystems, Cat#KK4602). The amplification conditions were 95° C for 3 min, followed by 40 cycles of 95°C for 3 sec and 60°C for 20 sec. Gene expression was normalized to the housekeeping gene 60S acidic ribosomal protein P1 (RPLP1), and relative transcript abundance expressed relative to vehicle-treated controls.

Table 1. Human RT-PCR primer sequences

Target		Sequence
RPLP1	F	5'-GGCGACCTGGAAGTCCAACT-3'
	R	5'-CCATCAGCACCACAGCCTTC-3'
HIF-1α	F	5'-TTCACCTGAGCCTAATAGTCC-3'
	R	5'-CAAGTCTAAATCTGTGTCCTG-3'
Glut-1	F	5'-ATTGGCTCCGGTATCGTCAAC-3'
	R	5'-GCTCAGATAGGACATCCAGGGTA-3'
LL-37	F	5'-ATCATTGCCCAGGTCCTCAG-3'
	R	5'-GTCCCCATACACCGCTTCAC-3'

HRE-Luciferase Reporter

An HRE-luciferase plasmid was kindly provided by Giovanni Melillo. This plasmid contained three copies of the HRE cloned into a thymidine kinase promoter in

front of the luciferase gene in a pGL2 backbone (Promega, Cat#E1641).[162] Using

restriction enzymes KpnI and HinDIII, the HRE-containing promoter was cut out and

cloned into a pGL4.17 backbone (Promega, Cat#E6721) The resulting plasmid was

transfected into HaCaT cells using Lipofectamine 2000 (Invitrogen, Cat#11668-027).

The transfected cells were selected using 200 μM G418 (Agilent, Cat#200049-21) for

two weeks and subsequently maintained in 100 μM G418. For experiments, HaCaT cells

were plated in a 96-well plate at 10^5 cells/well. At the indicated time points, cells were

lysed using Glo Lysis Buffer (Promega, Cat#E2661) and luciferase activity measured

using the Bright Glo Luciferase Assay System (Promega, Cat#E2610).

Statistical Tests

Statistical significance was determined by Student's *t* test performed in GraphPad

Prism.

RESULTS

Vitamin D Directly Induces HIF

To determine whether vitamin D directly induces HIF in a cell type important for

skin immunity, HaCaT cells were treated with $1,25(OH)_2D_3$. *Hif-1α* transcription

increased in a time-dependent fashion, with a peak 2 h after exposure **(Fig. 5A)**.
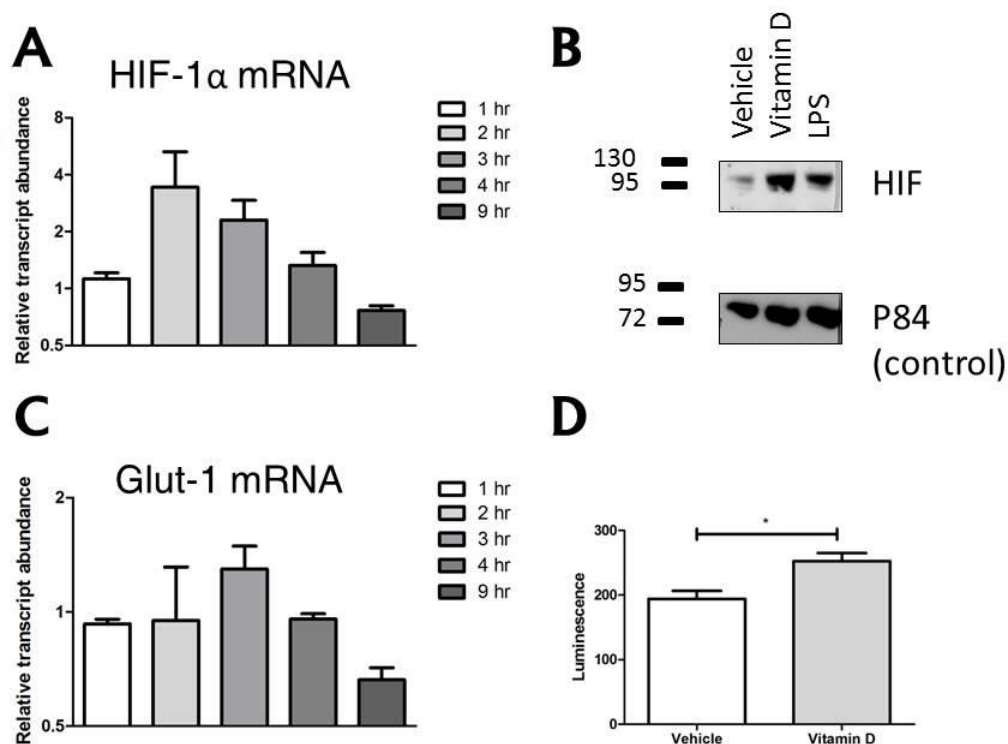

Figure 5. HIF is induced by vitamin D. A) HaCaT cells were treated with vitamin D (100 nM) for the indicated time periods or with vehicle control (ethanol). Relative transcript abundance is expressed relative to the vehicle control at each time point. B) HaCaT cells were treated with vitamin D (100 nM), LPS (10 ng/ml), or vehicle control (ethanol) for 24 hours. HIF protein was extracted from the nucleus and detected by Western blot. C) HaCaT cells were treated with vitamin D (100 nM) for the indicated time periods or with vehicle control (ethanol). Relative transcript abundance is expressed relative to the vehicle control at each time point. D) HaCaT cells stably transfected with an HRE-luciferase reporter plasmid were treated with vitamin D (100 nM) or vehicle control for 48 hours. Luciferase activity was measured by luminescence. Statistical significance was calculated by Student's *t* test. *: p<.05

Overnight treatment with $1,25(OH)_2D_3$ increased the nuclear abundance of HIF

protein more than LPS, a TLR-4 ligand known to increase HIF levels[18] **(Fig. 5B)**.

Furthermore, vitamin D treatment increased transcription of the glucose transporter gene

Glut-1 (**Fig. 5C**). The *Glut-1* promoter served as a control since it does not contain a VDRE[154] but it does contain HREs and is known to be strongly regulated by HIF.[163] *Glut-1* mRNA levels peaked about an hour later than *Hif-1α* levels, indicating that HIF was functionally active as a transcription factor. Vitamin D was also able to drive transcription from a thymidine kinase promoter containing three HREs which had been cloned in front of a luciferase gene (**Fig. 5D**), providing further evidence that vitamin D-induced HIF is functionally active as a transcription factor.

HIF Modulates Vitamin D-Induced Cathelicidin Expression

To determine the effect of HIF and vitamin D on cathelicidin expression, HaCaT cells were treated with vitamin D and the HIF stabilizer AKB-4924 or the HIF inhibitors digoxin or YC-1. HaCaT cells exposed to both vitamin D and elevated HIF expressed more LL-37 than those exposed to vitamin D alone (**Fig. 6A**). When the TLR-4 ligand LPS was added to simulate conditions of infection, the same pattern held (**Fig. 6C**). However, attempts at pharmacological inhibition of HIF by either digoxin or YC-1 did not reduce vitamin D-induced LL-37 mRNA expression, either alone or in the presence of LPS (**Figs. 6B and 6D**). These data suggest that vitamin D and HIF do not act

primarily through the same pathway.
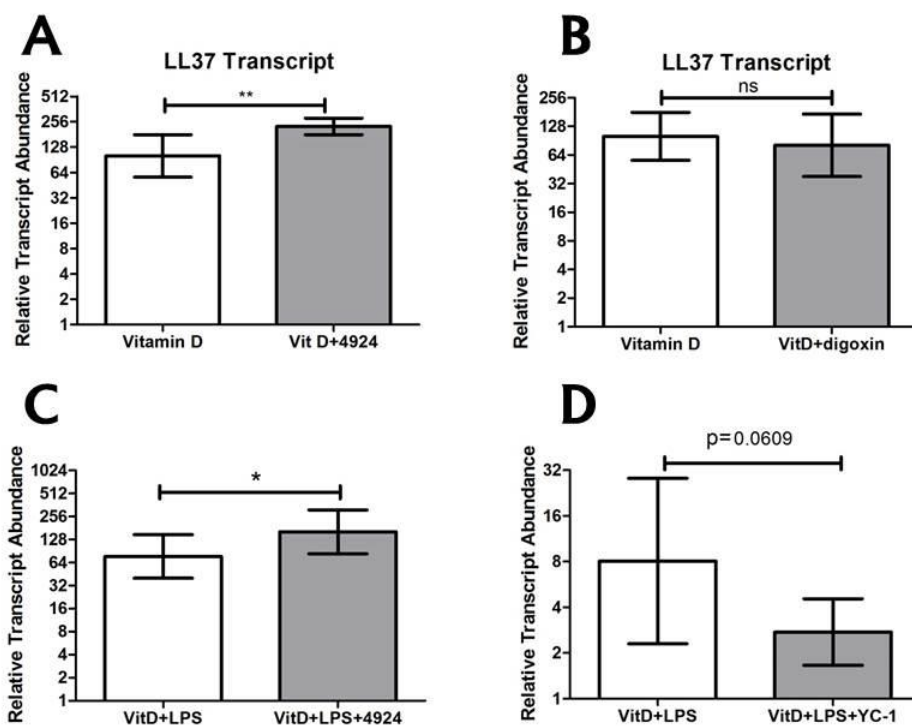
Figure 6. HIF modulates vitamin D-induced cathelicidin transcription. A) HaCaT cells were treated with vitamin D (100 nM) or vitamin D + AKB-4924 (10 uM), or vehicle controls in media containing charcoal-stripped FBS for 24 hours before the RNA was extracted. B) HaCaT cells were treated with vitamin D (100 nM) or vitamin D + digoxin (100 nM), or vehicle controls in media containing charcoal-stripped FBS for 24 hours before the RNA was extracted. C) HaCaT cells were treated with vitamin D (100 nM) + LPS (100 ng/ml) or vitamin D + LPS + AKB-4924 (10 uM), or vehicle controls for 24 hours before the RNA was extracted. D) HaCaT cells were treated with vitamin D (100 nM) + LPS (100 ng/ml) or vitamin D + LPS + YC-1 (10 uM), or vehicle controls for 2 hours before the RNA was extracted. Relative transcript abundance is expressed relative to the vehicle control. Statistical significance was calculated by Student's *t* test. *: p<.05 . **: p<.01

FURTHER RESEARCH

Much further work remains to be performed to confirm the preliminary results

described above. Although HIF was not shown to have an effect on vitamin D-induced

LL-37 transcription, it may still have an effect in regulating HIF at the post-transcriptional level. To explore this possibility, HaCaT cells could be treated with vitamin D and AKB-4924 or digoxin and expression of the LL-37 peptide detected via Western blot and intracellular flow cytometry.

The relationship between vitamin D and HIF may also differ depending on the presence or absence of infection. The preliminary experiments using LPS reported above could be complemented by experiments using live or heat-killed Gram-positive or Gram-negative bacteria, or cytokines such as IFNγ as markers of infection and the associated host response.

The pharmacologic studies can be accompanied by genetic studies. A plasmid containing the gene for a non-degradable form of HIF-1α[164] could be transfected into HaCaT cells and the experiments above repeated to study the effect of elevated HIF. Such experiments could be paired with siRNA to knockdown *Hif-1α*.

Vitamin D deficiency is widespread and is associated with increased risk of infectious disease. To determine whether HIF elevation could compensate for vitamin D deficiency, HaCaT cells in media containing low or normal concentrations of vitamin D could be treated with AKB-4924 and then challenged with skin pathogens such as GAS

or *S. aureus*. The ability of the keratinocytes to kill the bacteria could be measured by the bactericidal assay, and LL-37 expression could be measured by RT-PCR and Western blot.

Vitamin D and HIF are both major players in the control of the innate immune response to infection. This project promises to shed light on the regulation of cathelicidin, a crucial part of the skin immune system and provides insight into the relationship between these two key systems.

CHAPTER 4:

HARNESSING THE POWER OF HIF FOR THE PREVENTION AND TREATMENT OF INFECTIOUS DISEASE

HIF induction is a general part of the host response to infection. It is induced in response to both Gram-positive and Gram-negative bacteria[42] in a manner that depends on NF-κB activation,[11] as well as by viruses,[165, 166] protozoa,[27] and fungi.[27] Its importance makes it a target for pathogens which have developed strategies for avoiding HIF induction or subverting it to their own ends. As our understanding of HIF regulation and function grows, we come closer to being able to take a page from the pathogen playbook and learning to manipulate HIF to combat infectious diseases.

SUBVERSION OF HIF FUNCTION BY PATHOGENS

Some pathogens have developed strategies to protect themselves by preventing HIF induction. For example, oncolytic reovirus can prevent accumulation of HIF-1α in a proteasome-dependent manner, without affecting *Hif-1α* transcription.[167] Moloney

murine leukemia virus is able to prevent HIF-1α protein accumulation in infected mice

without affecting *Hif-1α* gene transcription by reducing the levels of the HIF-stabilizing

host protein Jab1.[168] *Chlamydia pneumoniae* degrades HIF by secreting the chlamydial

protease-like activity factor into the cytoplasm of infected cells.[169] *Pseudomonas*

aeruginosa expresses alkyl quinolones which target the HIF-1α protein for proteasomal

degradation.[170]

Infections by certain other viral pathogens may increase HIF levels or activity,

perhaps exerting an anti-apoptotic effect that promotes survival of the host cell they are

infecting. The carboxy terminus of HBx from hepatitis B virus was shown to enhance the

transactivation of HIF-1α by enhancing its association with CREB-BP.[171] The Kaposi's

sarcoma associated herpesvirus (KSHV) expresses a protein known as latency-associated

nuclear antigen (LANA) which targets vHL for degradation via ubiquitination, thereby

increasing HIF protein levels[172] and another part of LANA promotes HIF nuclear

accumulation.[173] Epstein-Barr virus (EBV) oncoprotein latent membrane protein 1

(LMP1) activates HIF-1α by upregulating Siah1 E3 ubiquitin ligase by enhancing its

stability, which allows it to increase the proteasomal degradation of prolyl hydroxylases 1

and 3 which normally mark HIF-1α for degradation.[174] As a result, LMP1 prevents

formation of the vHL/HIF complex, and HIF is not degraded.

Other viral and parasitic organisms are able to subvert HIF activity to their own benefit. HIF-1α stimulates the transcription of HIV-1 genes by associating with HIV-1 long terminal repeat,[175] and JCV genes by binding to the early promoter of the polyomavirus.[176] Other viruses seem to be using HIF as a marker of cellular stress to tell them when it is time to leave the cell. Murid herpesvirus 4[177] and EBV[178] switch from lysogenic to lytic when HIF levels are high. High levels of HIF leads to the expression of platelet activating factor, which some bacteria then use to increase translocation across the intestinal epithelium.[179] *Toxoplasma gondii* survives better when HIF is elevated,[180] and so *Toxoplasma* induces HIF stabilization via activin-like receptor kinase signaling.[181] *Leishmania,* too, survives better when HIF is elevated, and HIF inhibition reduces survival of the parasite.[182, 183]

HARNESSING THE POWER OF HIF FOR DISEASE PREVENTION AND CONTROL

As a "master regulator" of innate immune function,[184] boosting HIF activity through pharmacological strategies might provide a new approach to aid the treatment of certain infectious disease conditions.[28] In most infections, increasing HIF levels could be

expected to boost diverse myeloid cell antimicrobial activities and promote clearance of infection. Under certain conditions, particularly among viral pathogens, HIF stabilization may promote the survival of the pathogen, so care must be taken in determining when HIF augmentation can be a beneficial strategy.

The idea that treatments which increase HIF levels could be effective against infectious diseases is supported by the observation that macrophages with elevated levels of HIF are markedly more efficient than wild-type macrophages at killing Gram-positive and Gram-negative bacteria in vitro.[42] This implies that normal phagocytic cells could be more effective at killing bacteria than they are, mainly because their activation is tightly regulated to limit unnecessary inflammatory injury. Invasive bacterial infections are commonly linked to a failure of innate immunity to control the infecting pathogen, and it is possible that patients might benefit from augmentation of phagocytic cell bactericidal activity by HIF. Indeed, animal research has been published which shows that treatment with the HIF stabilizers mimosine[39] or AKB-4924[98] improves their ability to fight skin infections. No trials in humans have been initiated to date in which drugs that elevate HIF are used to treat acute bacterial infection. Nonetheless, such a strategy could be effective for difficult clinical scenarios such as opportunistic bacterial infections in patients with

weakened immune systems or with pathogens exhibiting multidrug resistance to conventional antibiotics. Theoretically, HIF boosting may also have an advantage in reducing the likelihood of drug resistance; it would be prohibitively difficult for bacteria to evolve resistance to the whole arsenal of antimicrobial factors that are increased when HIF activity increases.[3]

One promising starting point is local or topical administration for treatment or prophylaxis of bacterial skin and wound infections. In these cases, fortification of the antimicrobial barrier provided by keratinocytes and myeloid cells could be coupled with the reported enhancement of cutaneous wound healing associated with HIF augmentation.[185]

For those scenarios in which bacteriologic control is achievable by conventional antibiotics and in which pathology is being driven by an overactive immune response to bacterial components, HIF induction would have unclear utility and perhaps risk further exacerbation of the disease. In noninfectious LPS-induced sepsis, for example, which provokes an immunopathological cytokine storm, knocking out HIF in either myeloid cells[186] or T cells[187] reduces the severity of disease. Augmenting HIF under these conditions is likely to result in an increase in pro-inflammatory cytokine production and

nitric oxide release which would worsen symptoms.

For viral infections the landscape is a bit bumpier. On the one hand, HIF is a positive regulator of key immune response effectors against viral infections, just as against bacterial ones. On the other hand, with certain persistent viral infections the induction of HIF fails to result in eradication of the virus and may encourage lysogenic viruses to become lytic. Under those circumstances, activating HIF may have the undesirable effect of reactivating dormant infections. Furthermore, an unfortunate consequence of HIF activation in these circumstances is that increased VEGF and the accompanying proangiogenic program can contribute to oncogenic transformation. This seems to be the case in chronic infections with the hepatitis B and C viruses (HBV and HCV), which are epidemiologically associated with the development of hepatocellular carcinoma, a highly vascularized solid tumor. Experimental evidence of this includes the finding that HIF levels are increased in liver cells transfected with the oncogenic X protein of HBV (HBx) and in the livers of HBx-transgenic mice.[171] HBx interacts directly with HIF-1α to block its association with VHL and degradation by the ubiquitin-proteasome pathway.[16] Recently, HCV infection has also been found to stabilize HIF-1α protein, with the involvement of the NF-κB and MAPK signaling pathways, stimulating

VEGF production and neovascularization.[188]

Also, HIF treatment *in vivo* could benefit viruses by suppressing the activity of plasmacytoid DCs (pDCs). These DCs are another kind of DC not discussed earlier. They are specialized for the control of viral infections, and one group has shown that HIF-1α is a negative regulator of pDC development *in vitro* and *in vivo*.[189]

Although work is just beginning on the interrelationship between vitamin D and HIF as co-regulators of skin immunity, there is the possibility that treating skin infections with HIF agonists may be able to compensate for vitamin D deficiency, which is widespread.

The work in APCs suggests that HIF elevation could be effective not only in treating but also in preventing disease, through examination of adjuvant characteristics. To take advantage of the positive role of HIF in innate immune cells but avoid the negative effect of HIF on T cells, a HIF-stabilizing agent would have to be effective in the first hours of the immune response but be exhausted by 24 to 48 hours after immune stimulation when T cells begin activating. We have recently reported[190] proof-of-concept experiments using the HIF stabilizer AKB-4924 to strengthen the response to vaccination with a model antigen. Further research must be done to determine whether a HIF drug

could be developed as a vaccine adjuvant.

In summary, HIF plays a central and complex role in regulating the immune response to infection. As our understanding of HIF function becomes more nuanced, we move ever closer to being able to manipulate this crucial host factor to prevent and control human disease.

ACKNOWLEDGEMENTS

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