UNIVERSITY OF CALIFORNIA, SAN DIEGO

Hypoxia Inducible Factors: Redefining Metabolic Regulation of CD8 T cell Differentiation and Function

A Dissertation submitted in partial satisfaction of the
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in

Biology

by

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2016

The Dissertation of Anthony Phan is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2016

DEDICATION

I dedicate this dissertation to my loving parents Lang and George, who have sacrificed so much to guarantee me countless opportunities to further my dreams. I couldn't have done this without you both and your constant and unwavering belief in me.

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ABSTRACT OF THE DISSERTATION

Hypoxia Inducible Factors: Redefining Metabolic Regulation of CD8 T cell Differentiation and Function

by

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T cell responses are initiated by detection of cognate peptide presented by antigen presenting cells in secondary lymphoid tissues and result in migration of T cells to various tissues with disparate local conditions. Hypoxia Inducible Factors (HIFs) are uniquely poised to integrate physiological and disease-derived signals to modulate T cell responses making them an attractive target for further study. Understanding how T cells have taken advantage of the HIF pathway to interpret local conditions and integrate these with the array of mitogenic signals produced during infection is essential for the advancement of our understanding of T cell biology and critical for the development of novel therapeutic

approaches. This dissertation takes advantage of genetic models to modulate the oxygen-sensing HIF pathway to define how HIFs regulate $CD8^+$ T cell differentiation and function.

By employing genetic deletion of the von Hippel Lindau tumor suppressor protein (VHL), a crucial negative regulator of HIF signaling, we identified a novel role for HIF activity in modulating effector $CD8^+$ T cell function following chronic viral infection. We demonstrated that sustained HIF signaling not only enhances effector function in the face of persistent antigen, but drives resistance to immune exhaustion in antigen-specific $CD8^+$ T cells. Constitutive HIF signaling also drove enhanced glycolytic metabolism and pharmacological inhibition of glycolysis resulted in partial rescue of HIF-dependent alterations in expression of effector molecules, activation-associated receptors, and critical transcription factors suggesting that modulating cellular metabolism can directly impact T cell function and possibly fate decisions.

Extensive metabolic changes accompany T cell activation including a switch to glycolytic energy production and increased biosynthesis. Recent studies suggest that a subsequent return to reliance on fatty acid oxidation to fuel oxidative phosphorylation and increasing spare respiratory capacity are essential for the differentiation of memory $CD8^+$ T cells. Constitutive HIF activity in $CD8^+$ sustains glycolytic metabolism and suppresses oxidative phosphorylation providing a powerful model by which to define the role of metabolic pathway choice in $CD8^+$ T cell differentiation. Here we demonstrate that surprisingly, constitutive glycolytic metabolism does not inhibit the formation of long-lived memory $CD8^+$ T cells, but predominantly supports the formation of effector memory (T_{EM}) $CD8^+$ cells. Moreover, the departure from a reliance on oxidative phosphorylation also drives accelerated emergence of memory precursor cells following the peak of the response to acute viral infection. Importantly, these long-lived memory $CD8^+$ T cells upregulated IL-7 receptor, expressed critical transcription factors at similar levels to wildtype cells, and demonstrated a heightened response upon secondary challenge demonstrating that restricting

$CD8^+$ T cells to glycolytic metabolism does not inhibit the differentiation of a protective memory pool.

While constitutive HIF signaling has provided a powerful model to define targets of HIF activity in $CD8^+$ T cells, we also sought to understand how loss of HIF signaling and HIF-mediated glycolytic metabolism may impact $CD8^+$ T cell differentiation and function. In this dissertation we begin to explore how deletion of HIF-1α and HIF-2α impact $CD8^+$ T cell metabolism and function following acute viral infection and place these findings in the context of $CD8^+$ T cell immunity as well as propose areas of future study.

INTRODUCTION

The greatest innovation in public health has been the invention of vaccination, which relies on the bedrock concept of immunological memory to provide enduring protection against numerous diseases. Adaptive immune cells are critical for mediating vaccine-induced protection, however the vast majority of current vaccines fail to elicit potent protective memory T cell populations. $CD8^+$ T cells are a subset of T lymphocytes that are essential for the eradication of intracellular pathogens and play an important role in the clearance of tumors [17,41,43]. As such, the generation of $CD8^+$ T cell memory has been an area of intense study with significant progress made in defining regulators of memory differentiation with the ultimate goal of improving vaccine efficacy. This dissertation expands upon the field's understanding of transcriptional and metabolic regulation of $CD8^+$ T cell differentiation by defining the impact of the Hypoxia-Inducible Factor family of transcription factors (HIFs), which transcriptionally regulate immunity and cellular metabolism, in $CD8^+$ T cells during the response to viral infection.

$CD8^+$ T cell Differentiation

During infection, circulating antigen-specific naive $CD8^+$ T cells encounter cognate antigen presented in the context of MHC Class I molecules presented by antigen-presenting cells in secondary lymphoid tissues. Stimulation of the T cell receptor along with co-stimulation activate $CD8^+$ T cells promoting rapid proliferation, coincident differentiation,

acquisition of effector functions, and migration to sites of infection [17, 41, 43]. Once in infected tissues effector CD8$^+$ T cells produce cytokines (i.e. IFNγ and TNFα) and utilize cytolytic molecules (i.e. perforin and granzymes) to control the invading pathogen and kill infected cells. Following clearance of the pathogen the effector CD8$^+$ T cell population contracts precipitously, whereby the majority of antigen-specific CD8$^+$ T cells die via apoptosis, with a fraction (5-10%), surviving to become long-lived protective memory CD8$^+$ T cells [17, 41, 43].

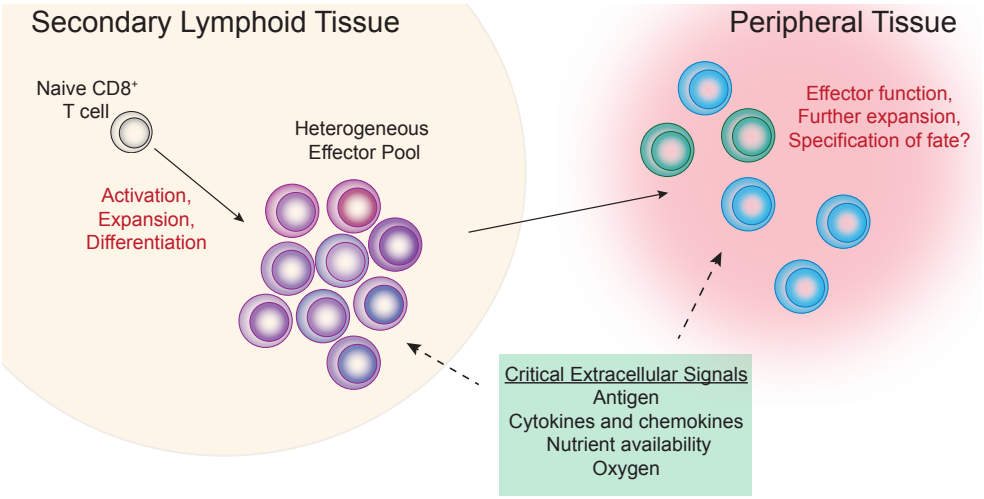

Figure 0.1: **Activation and migration of CD8$^+$ T cells.** Naive CD8$^+$ T cells encounter cognate antigen in secondary lymphoid tissues, active, expand, and acquire effector capacity then migrate to peripheral tissues. During this time CD8$^+$ T cells are exposed to numerous extracellular signals that can play a role in promoting migration, supporting effector function, and defining cell fate.

Tremendous progress has been made in identifying critical signals (i.e. antigen, IL-12, and TGF-β), transcription factors (i.e. T-bet, Eomes, Id2, Id3, and Foxo1), and cellular populations (i.e. distal and proximal daughter cells, terminal effector and memory precursor cells) that drive or lead to differentiation of memory CD8$^+$ T cell populations [17]. Useful cell-surface markers for studying the factors guiding CD8$^+$ T cell effector versus memory differentiation are IL-7-receptor-α (CD127) and Killer cell lectin-like receptor subfamily G member 1 (KLRG1). Following infection and activation all CD8$^+$ T cells downregulate

expression of CD127, however near the peak of the cellular response to infection a proportion of cells (dependent on inflammatory environment) upregulate KLRG1 while remaining $CD127^{lo}$, additionally another subset of cells re-express CD127 and express low levels of KLRG1. This shift in KLRG1 and CD127 expression correlates with memory and proliferative potential as $KLRG1^{hi}CD127^{lo}$ cells tend to be terminally differentiated effector cells, while $KLRG1^{lo}CD127^{hi}$ cells exhibit a higher potential to form long-lived memory cells. Importantly, these markers are correlative and not deterministic as recent studies have shown that these subsets consist of a heterogeneous population of cells with a continuum of memory forming potential [17, 43]. Mirroring the heterogeneity of the effector cells that eventually form memory $CD8^+$ populations, recent studies have highlighted that memory $CD8^+$ T cells are a heterogeneous population as well, consisting of circulating populations (central memory (T_{CM}) and effector memory (T_{EM})) and tissue resident memory populations (T_{RM}). With the continued characterization of these subsets, by genetic models (i.e. knockout-studies) and high throughput sequencing technologies (i.e. single cell RNA-seq and ATAC-seq), the ontogeny of memory cells and their subsets remains undefined and it appears evident that multiple distinct cell-intrinsic programs, influenced by cell-extrinsic signals, are likely at play in specifying the memory fate.

When considering the vast array of signals, transcription factors, and mechanisms regulating $CD8^+$ T cell differentiation it becomes clear that many unexplored factors potentially drive the bifurcation between effector and memory formation. Further defining the molecular mechanisms that drive $CD8^+$ T cell fate will provide unique strategies for improving current vaccines.

Protective immunity relies on coordinate responses from numerous cell types in order for host tissues to resist incursion by foreign agents. Infection of tissues by pathogens often drives vast changes in the tissue microenvironment as well as the upregulation of systemic signals necessary for marshalling immune cells to the fight. Recently it has become

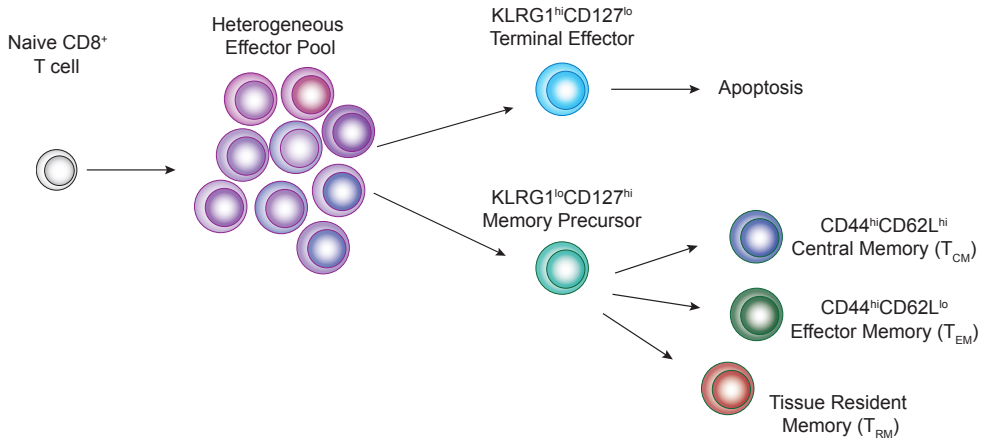

Figure 0.2: **A potential model of memory CD8$^+$ T cell differentiation.** Naive CD8$^+$ T cells activate, expand, and differentiate into a heterogenous pool of effector cells from which the ontogeny of memory generation is unclear. This figure illustrates one potential model where memory precursors further differentiate into all three described memory subsets.

appreciated that antigen, cytokines, chemokines, as well as the balance of nutrients and oxygen can play a role in modulating immune cell metabolism thereby affecting cellular fate and function [17, 75]. Further, T cells must traffic from secondary lymphoid tissues to infected sites, experiencing dramatic shifts in microenvironmental signals unique to tissue milieu, which may play a crucial role in influencing T cell metabolism, altering cellular function and cell fate decisions. How such microenvironmental signals are integrated at the transcriptional level is a topic of considerable importance in understanding T cell immunity.

T cell metabolism - a modulator of T cell responses

It has been noted for decades that the massive proliferation that occurs following lymphocyte activation is accompanied by marked shifts in cellular metabolism [99]. Quiescent naive T cells primarily rely on oxidative phosophorylation for their energetic demands, but upon activation by cognate peptide and costimulation from antigen presenting cells, T cells rapidly shift towards a reliance on glycolytic metabolism [17, 75]. Glycolytic supply of energy proves to be critical for differentiation and effector function of activated T cells. Following

clearance of the pathogen, most effector T cells die, however a small proportion survive and differentiate into long-lived memory populations that exhibit a coordinated return to a reliance on oxidative phosphorylation [17, 75]. Recent work has demonstrated that these metabolic transitions accompany key cell-fate decisions impacting differentiation and effector function of T cell subsets [16, 22, 45, 56, 61, 69, 89, 91, 94, 95, 102]. Most intriguingly, data from some of these studies argue that differentiation of memory as well as function of effector cells are dependent on utilization of specific metabolic pathways [16,31,69,91,94,95]. For example, the transition from predominantly glycolytic metabolism to oxidative phosphorylation, fatty acid metabolism, and generation of spare respiratory capacity by antigen-specific $CD8^+$ T cells may be essential for the formation of long-lived functional memory $CD8^+$ T cells [94]. However, understanding molecular regulators of T cell metabolism has emerged as a topic of intense study, and in this dissertation we will discuss the master regulators of oxygen homeostasis, the HIFs, and their role in regulating T cell metabolism and function.

HIF - a regulator of cellular metabolism

The importance of cellular metabolism in altering T cell differentiation and function has propelled research into the role of well-established metabolic regulators such as c-Myc, PI3K/AKT, mTOR, Foxo1, and the HIFs in controlling T cell function. HIFs are a family of transcription factors consisting of three alpha subunits, HIF-1α, HIF-2α, and HIF-3α, with differing tissue-specific expression patterns that when stabilized can heterodimerize with HIF-1β also known as the aryl hydrocarbon receptor nuclear translocator (ARNT), and in the case of HIF-1α and HIF-2α, bind hypoxia response element (HRE) sequences and activate a transcriptional program dedicated to mediating the adaptation of cells to reduced oxygen availability [8, 71, 85]. HIF-3α's lack of a c-terminal activation domain suggests that it is unable to activate transcription despite being capable of heterodimerizing

with HIF-1β and binding HRE sequences. It is currently unclear what role HIF-3α plays in modulating cellular responses to hypoxia [54, 57]. HIFs promote the adaptation of cells to hypoxia primarily by lowering oxygen consumption through the increased expression of two critical glycolytic enzymes, lactate dehydrogenase A (LDHa), which increases the capacity to regenerate NAD+ following reduction of pyruvate produced by glycolysis, and pyruvate dehydrogenase kinase 1 (PDK1), which actively prevents pyruvate from shunting into the TCA cycle [29, 46]. Altering these two metabolic checkpoints along with increased expression of other glycolytic enzymes dramatically shifts cellular metabolism away from oxygen-consuming pathways and drives a reliance on glycolysis for the generation of ATP.

Emerging literature has demonstrated a critical role for HIFs in modulating T cell metabolism via both oxygen-dependent and oxygen-independent pathways and highlighted their regulation of other novel gene targets influencing effector T cell function and differentiation [21, 23, 26, 28, 56, 89]. This introduction will first examine many of the regulators of HIF in T cells, and then what is known about HIF's impact on T cell metabolism, differentiation, and function.

Regulators of HIF activity in T cells

Interest in HIF-regulated transcription in T cells stems from HIF's function as a modulator of metabolism in response to changes in oxygen tension; however, studies in T cells and other immune cell types have revealed that while a primary function of HIFs is the detection and response to low oxygen tensions, a number of other mechanisms regulate HIF activity in an oxygen-independent fashion, allowing T cells to co-opt the HIF pathway for regulation of cellular metabolism and other known transcriptional targets in response to immune stimuli independent of oxygen availability. Here we will cover many of the known regulators of HIF activity in T cells as well as some that remain unexplored in T cell biology,

but likely play a role in T cell function and fate given their described function in other cell types.

Oxygen

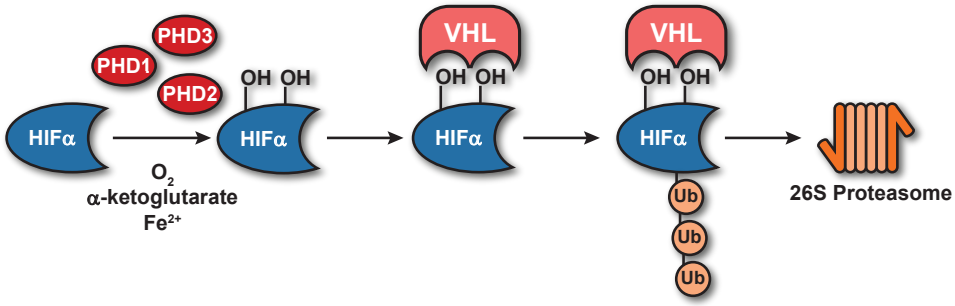

Figure 0.3: **Oxygen-dependent regulation of HIF stability.** The canonical oxygen-dependent degradation pathway of HIFα requires prolyl-hydroxylation by PHDs and subsequent polyubiquitination by VHL resulting in proteosomal degradation.

As in other cell types, the HIF pathway serves as the central sensor of oxygen tension in T cells and is regulated in an oxygen-dependent fashion [59, 71]. When T cells are in oxygen-sufficient environments HIFα subunits are rapidly degraded by the proteasome following hydroxylation by prolyl hydroxylase domain proteins (PHDs), primarily PHD2, and ubiquitination by the von Hippel-Lindau tumor suppressor protein complex (pVHL), an E3 ubiquitin ligase that specifically targets hydroxylated HIFα subunits (Figure 0.3) [39, 40]. Conversely, when cells enter hypoxic environments, (∼1% O_2), PHDs, which require oxygen (O_2) as a cofactor for their function, along with iron(II) and α-ketoglutarate, are unable to hydroxylate HIF on the appropriate proline residues resulting in a loss of pVHL-dependent ubiquitination and subsequent stabilization of HIFα subunits [39,40]. HIFα can then dimerize with HIF-1β upon translocation into the nucleus and activate HIF target genes. While canonical oxygen-dependent regulation of the HIF pathway remains integral to regulating HIFα stability, interestingly, activation by cognate antigen of T cells appears to "unlock" the oxygen-dependent responsiveness of T cells amplifying stabilization of HIFα subunits as

naive cells cultured in 1% O_2 exhibit mild stabilization of HIFα subunits in comparison to those activated and cultured in 1% O_2 (unpublished observation A. Phan and A. Goldrath and [97]).

Prolyl hydroxylase domain proteins and Factor Inhibiting HIF-1

Hydroxylation of HIFα subunits is the canonical mechanism for regulating HIF activity [87]. Therefore regulation of PHD expression and activity is critical for regulating HIF function. Three PHD isoforms are present in mice and examination of PHD function shows that PHD2 is the default regulator of HIFα stability in most cell types [1]. *Egln1* (PHD2) is constitutively expressed in T cells and hydroxylates HIFα subunits for subsequent degradation at the steady state. Interestingly, a conserved HRE has been identified and shown in mouse embryonic fibroblasts to drive increased PHD2 expression following extended exposure of cells to hypoxia, suggesting a self-regulating circuit designed to prepare cells for re-entry into oxygen rich environments following hypoxia [55, 60]. *Egln3*, which encodes PHD3, is dynamically expressed in T cells following activation suggesting an importance for hydroxylase activity during T cell responses [34]. PHD3 also appears to be directly regulated by a HRE in a HIF-dependent fashion in *in vitro* experiments in human cancer cell lines [55, 77]. Further exploration of PHD expression and activity in the context of T cell activation will be informative for defining regulators of HIF activity in the immune response.

In addition to PHDs, another hydroxylase, the Factor Inhibiting HIF-1 (FIH), hydroxylates an asparagine residue in the c-terminal activation domain of both HIF-1α and HIF-2α subunits in normoxia [47, 53]. Asparaginyl-hydroxylation blocks the ability of HIFs to bind transcriptional coactivators CREB-binding protein and p300 [47, 53]. This prevents HIF-mediated transcription, providing an additional layer of post-translational regulation of HIFs that escape degradation by the proteasome.

PHDs and FIH rely on O_2, iron(II), and α-ketoglutarate as cofactors. As such, hypoxia

or use of competitive inhibitors of α-ketoglutarate or iron chelators have been shown to inhibit prolyl- and asparaginyl- hydroxylase activity and stabilize HIFα subunits [9,27,40]. In addition, accumulation of TCA cycle intermediates succinate and fumarate, due to mutations in TCA cycle enzymes, have been shown in renal cell carcinoma cells to competitively inhibit hydroxylase activity by preventing PHD access to α-ketoglutarate thereby promoting HIFα stabilization [38, 86]. This suggests that alterations in T cell metabolism may serve as an additional mechanism regulating HIF stability and activity through modulation of PHD activity.

T cell receptor

Macrophages have been shown to stabilize HIFα subunits in response to bacterial antigens in an oxygen-independent, TLR-dependent fashion that requires NF-kB activation [6,78]. Much like macrophages, T cells have been shown to stabilize HIFs regardless of oxygen tension in response to activation of antigen receptors [12, 23, 26, 50, 51, 64, 89]. T cell receptor (TCR) signaling and costimulation through CD28 results in robust HIFα protein stabilization regardless of oxygen tension which can be further potentiated by hypoxia [26, Chapter 1 of this dissertation] [64]. Microarray analysis comparing naive and activated $CD8^+$ T cells show increased expression of mRNA for both HIF-1α and HIF-2α following activation in antigen-specific $CD8^+$ T cells responding to viral and bacterial infections, suggesting that TCR signaling regulates both HIF-1α and HIF-2α expression *in vivo* [34]. Induction of HIF-1α is thought to be mediated by PI3K/mTOR activity downstream of TCR and CD28 signaling which promotes transcription of two splice isoforms of HIF-1α mRNA in human and mouse T cells along with driving increased protein translation [50, 64]. Oxygen-independent stabilization of HIF-2α also occurs at low levels following TCR and CD28 stimulation of $CD8^+$ T cells [26, Chapter 1]. However, it is unknown if this occurs through PI3K/mTOR activity similarly to HIF-1α stabilization or if unique molecular pathways drive this stabilization

independently. TCR and CD28 signaling have also been shown to activate NF-kB signaling in T cells and given the importance of NF-kB activity in promoting antigen receptor-dependent activation of HIFs in macrophages it stands to reason that NF-kB activity may play a critical role in regulating HIF activity following TCR and CD28 engagement [19, 74]. Additionally, initial studies of TCR-dependent stabilization of HIFα subunits utilized rapamycin, a broad spectrum mTOR inhibitor, to assess mTOR-dependency [64]. However, recent advances in our understanding of the PI3K/mTOR pathway in T cells has revealed additional complexity in the regulation and activity of mTOR (i.e. mTORC1 versus mTORC2, cross-talk with other metabolic pathways) [81]. Further examination of TCR-dependent regulation of HIFα stability in the context of critical T cell activation pathways is necessary to clarify when and where HIF-mediated transcription will influence T cell immunity.

Cytokines

As interest in the impact of HIFα activity in T cells has increased, the effects of cytokine signaling on HIFα stabilization/activity has begun to be explored. Previous work in human cancer cell lines has demonstrated that TGF-β may drive oxygen-independent regulation of HIF demonstrated by normoxic stabilization of HIFs through Smad-dependent inhibition of PHD2 expression [58]. Intriguingly, in $CD4^+$ T cells, pro-inflammatory IL-6 and anti-inflammatory TGF-β have been implicated in *in vitro* normoxic stabilization of HIF-1α in a STAT3-dependent manner [23]. However, an additional study demonstrated that HIF-1α stabilization is STAT3 independent suggesting that other cytokines, possibly IL-23, could also play a role in influencing HIF-1α activity in $CD4^+$ T cell differentiation [89]. In macrophages, T_H1 and T_H2 cytokines could stabilize HIF-1α promoting M1-polarization or HIF-2α driving M2-polarization respectively [92]. Similarly, *in vitro* activation of $CD8^+$ T cells followed by culture with IL-2 potentiated normoxic stabilization of HIF-1α and little to no stabilization of HIF-2α while culturing with IL-4 promoted normoxic stabilization of

both HIF-1α and HIF-2α in expanding CD8s [26, Chapter 1]. Culturing activated $CD8^+$ T cells with IL-2 or IL-4 also altered HIF-1α- or HIF-2α-dependence of several differentially expressed effector molecules, transcription factors, and activation-associated receptors. This suggests that cytokine-dependent stabilization of HIFs could be an additional context-specific mechanism for modulating T cell function and fate similar to the bifurcation of M1 and M2 macrophages [26, 92, Chapter 1]. Deciphering how individual cytokines affect the different layers of HIFα regulation will provide important insight into the potential impact hypoxia and the inflammatory microenvironment have on T cell differentiation and function.

Non-coding RNAs

With genome-wide sequencing, it has recently become appreciated that non-coding RNAs (ncRNAs) make up a large proportion of differentially expressed genes following T cell activation. Examination of microRNAs (miRNAs) and long noncoding RNAs (lncRNAs) in T cells has demonstrated important roles for both in the regulation of T cell biology downstream of numerous signals [42, 70, 73]. Notably, a number of miRNAs and lncRNAs have been implicated in regulating HIFα accumulation. miR155 and miR210 are hypoxia-regulated which, in mouse intestinal epithelial cells and T cells respectively, provide negative feedback to HIF activity following extended hypoxic signaling [11, 97]. A recent study demonstrated that miR210, but not miR155, is upregulated upon T cell activation in a CD28- and HIF-1α-dependent manner in $CD4^+$ and $CD8^+$ T cells [97]. Surprisingly, computational analysis predicted HIF-1α as a target of miR210 and further study of T_H17 $CD4^+$ T cells revealed a negative feedback loop mediated by miR210 for T cells cultured in prolonged hypoxia. Transfer of miR210-deficient $CD4^+$ T cells in a T cell transfer model of colitis resulted in increased severity of disease that correlated with increased stabilization of HIF-1α in $CD4^+$ T cells isolated from the lamina propria of mice. These data suggest that miR210-dependent degradation of HIF-1α mRNA dampens wildtype T_H17 responses

in chronically hypoxic tissues and may serve as a critical regulator of T cell immunity [97]. Importantly, the impact of miR210 on HIF-1α stability was most striking in conditions of limiting oxygen such as the gut, suggesting that non-canonical regulators of the HIF pathway may provide context-specific tuning of the HIF pathway for regulation of unique cellular functions, such as $CD4^+$ T_H subset differentiation.

In contrast to miRNAs, p21, a lncRNA, has been shown to play a novel role in blocking pVHL-HIFα interactions in transformed and non-transformed human cell lines [101]. *In vitro* culture of HeLa cells in hypoxia inhibited PHD function and stabilized HIF-1α protein as expected, however, knockdown of p21 lncRNA resulted in an inability to maintain HIF-1α protein levels after 24 hours of hypoxia [101]. Intriguingly, in this study HIF-1α-dependent transcription of *egln1* increased PHD2 levels sufficiently to potentiate hypoxic degradation of HIF-1α. Maintenance of HIF-1α stability following increased PHD2 levels required HIF-1α-dependent expression of p21 lncRNA and disruption of pVHL-HIF-1α interactions [101]. Control of HIFα stability by ncRNAs is a recent addition to the HIF pathway's regulatory roadmap and significant work will need to be done to understand why these loops have evolved and how they impact canonical HIF regulation (i.e. PHD- and VHL-dependent regulation). Also important to note is that the majority of studies on ncRNA regulation of HIFα subunit stability has focused on stabilization of HIF-1α and it remains unclear how other subunits are impacted. Elucidating subunit specific regulation of HIFs will be critical in understanding their individual roles in T cell biology. Due to the emerging importance of ncRNAs in T cell biology, investigation into ncRNAs could also provide additional responsive flexibility to the HIF pathway in T cells for the regulation of effector functions.

Reactive oxygen species (ROS)

T cell activation has been shown to induce mitochondrial activity driving complex III-dependent production of ROS that is essential for activation of nuclear factor of activated T cells (NFAT) and subsequent IL-2 induction [88]. ROS additionally has been implicated in the stabilization of HIFs. However intense study has resulted in two prevailing interpretations of available data regarding the role of mitochondria-derived ROS in regulation of HIF (reviewed [93]). One model argues that mitochondrial ROS is necessary for regulation of HIFα stability. Initial reports examining the role of mitochondria in HIFα stabilization found that loss of mitochondria (Rho cells) or inhibition of electron transport chain (ETC) complexes responsible for ROS production prevented stabilization of HIF-1α in hypoxia (1.5% O_2) due to a lack of superoxide (SO) production from the ETC [14, 15]. Additional studies carefully dissecting the contribution of individual ROS species in human cell lines through exogenous addition of ROS or overexpression of ROS-producing or -depleting enzymes revealed that HIFα stabilization was dependent on mitochondrial derived H_2O_2 rather than SO in hypoxia [10, 32]. Direct mechanistic evidence of how ROS inhibited PHD or VHL function, however, has yet to be shown. These studies are contrasted by experiments which argue that mitochondrial metabolism is essential for HIFα stabilization, but in a ROS-independent fashion. Inhibition of complex III of the ETC of human kidney epithelial cells in the presence of 2,2,4-trimethyl-1,3-pentanediol (TMPD), which allows for continued ETC activity and oxygen consumption without production of SO, resulted in no difference in hypoxic HIFα stabilization arguing that mitochondria-dependent HIFα stabilization in hypoxia is ROS-independent and driven primarily by O_2 usage by the ETC which reduces cytosolic O_2 availability, thereby reducing PHD activity and culminating in HIFα stabilization [20]. While these two models appear mutually exclusive, a possible explanation for the current data is that a combination of both decreased O_2 availability due to mitochondrial metabolism as well as alterations in cellular redox state and iron(II)

availability due to ROS production may synergize to promote HIFα stabilization in cells [93]. As mitochondrial metabolism and ROS production are essential for the activation of T cells, this supports a potential role for mitochondrial ROS in regulating stability of HIFα subunits in T cells, similar to observations in other cell types [88, 93]. Experiments demonstrating a direct link between mitochondrial ROS production and HIFα stabilization in T cells could provide mechanistic insight to metabolism-dependent transcriptional programming.

The sheer diversity of signals that have been identified and continue to be implicated in HIF stability and activity reflect the complex role of HIF in the integration of microenvironmental stimuli for the regulation of T cell metabolism, differentiation, and ultimately, function (Figure 0.4).

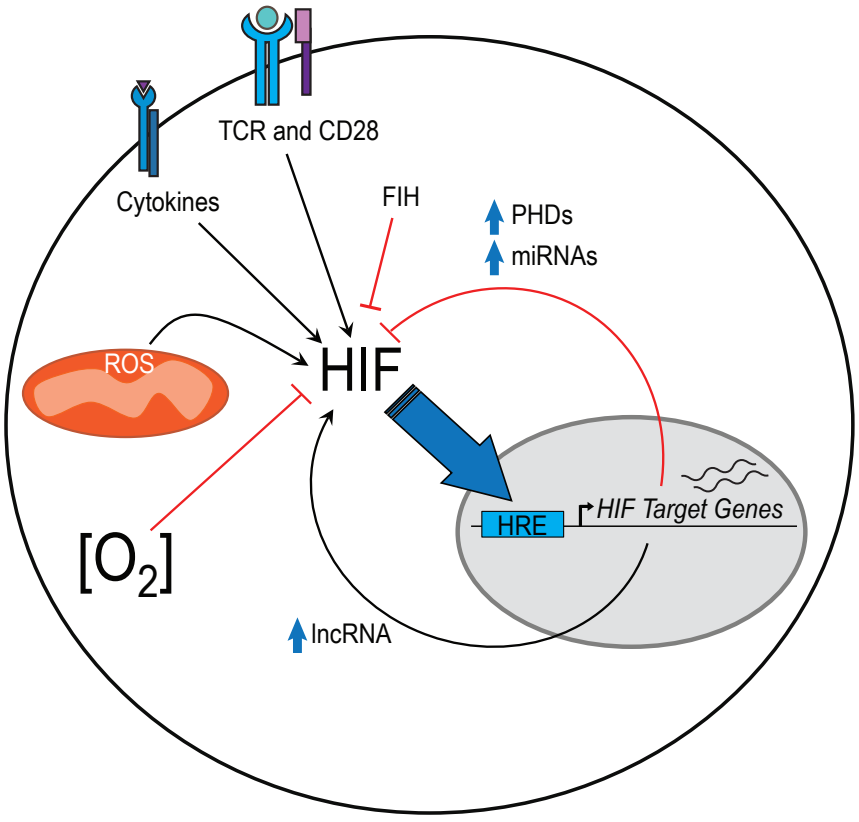

Figure 0.4: **Regulators of HIF activity in T cells.** In addition to oxygen, many signals regulate HIF activity in T cells, activating HIF-dependent transcription and potentiating a variety of self-regulatory pathways.

Regulation of T cell metabolism, differentiation, and function.

T cells undergo rapid metabolic reprogramming within the first 24 hours of activation, and recent work has focused on elucidating key regulators of T cell metabolism in an effort to delineate the direct impact of such changes on T cell immunity [17, 75]. Interestingly, it has been found that HIF-1α activity is unnecessary while c-Myc is essential for metabolic reprogramming of T cells within the first 24 hours of activation [98]. HIF-1α deletion did not impact proliferation, glutaminolysis, glycolysis, or fatty acid oxidation by T cells following activation with anti-CD3 and anti-CD28 *in vitro* [98]. Induction of LDHa and hexokinase-2 gene expression, two well characterized HIF-1α targets, were moderately downregulated by HIF-1α deletion and mild impairment of glycolytic rate was observed 72 hours after activation. Thus, while unnecessary for the initial transition to glycolytic metabolism, HIF-1α may be involved in sustaining glycolytic throughput during the T cell response [98]. It is important to note these data are from T cells activated in atmospheric oxygen concentrations, leaving open the possibility that in secondary lymphoid tissues, under physiologic oxygen tensions (0.5-4.5% O_2), HIFs may play a more significant or different role in regulating early T cell metabolic programming while integrating additional microenvironmental signals to direct trafficking, function, and differentiation [12].

Indeed in support of HIF-1α maintaining glycolytic metabolism in T cells, a recent study proposes that reduced exposure to IL-2 following T cell activation results in increased expression of Bcl-6 which directly represses glycolytic genes regulated by HIF-1α and c-Myc providing a mechanism for dampening glycolytic metabolism and effector function, thereby altering T cell fate decisions in a microenvironment-dependent fashion [66]. These data support a model where HIFs are capable of maintaining glycolytic metabolism in T cells shortly after activation driven by oxygen-independent mechanisms. Identifying additional factors that regulate HIF stability or activity in order to modulate glycolytic maintenance

may lend important insights about the molecular "switches" that drive context-dependent metabolic shifts critical for cell fate decisions.

While metabolic reprogramming occurs in all T cells upon activation, recent work has shown that $CD4^+$ and $CD8^+$ T cells show distinct metabolic changes [13]. Following 72 hours of *in vitro* stimulation with anti-CD3 and anti-CD28 glycolytic and oxidative phosphorylation rates were measured via extracellular flux by $CD4^+$ and $CD8^+$ T cells [13]. Both $CD4^+$ and $CD8^+$ T cells increased total metabolic rates following activation, indicated by increased extracellular acidification rate (ECAR) and oxygen consumption rate (OCR), proxies for glycolysis and oxidative phosphorylation respectively, in comparison to naive cells. However, examination of ECAR/OCR ratios as a measure of reliance on glycolysis or oxidative phosphorylation revealed that while both $CD8^+$ and $CD4^+$ T cells skew towards glycolysis following activation, $CD8^+$ T cells relied more heavily on glycolytic metabolism relative to $CD4^+$ T cells [13]. Interestingly, $CD4^+$ T cell proliferation exhibited greater sensitivity to inhibition of both glycolysis or oxidative phosphorylation despite arguably greater metabolic flexibility relative to $CD8^+$ T cells [13]. These data support a role for both glycolysis and oxidative phosphorylation in promoting T cell proliferation and are supported by studies in both $CD4^+$ and $CD8^+$ T cells where inhibition of glycolytic metabolism reduces but does not abolish proliferation, whereas inhibition of oxidative phosphorylation, surprisingly, produces a more dramatic defect in proliferation by comparison [16, 45, 88]. These data argue that in T cells metabolic reprogramming to a reliance on glycolytic metabolism primarily occurs to promote effector function and likely additional unidentified T cell processes rather than to support proliferation [16]. Importantly, Cao *et al* also emphasize that context-specific cues will play an essential role in modulating T cell metabolism *in vivo*. Experiments comparing the effect of fuel availability (glucose versus glutamine), cytokine signaling (IL-2, IL-15, IL-7), titrated levels of TCR stimulation, and addition of co-receptor signaling (4-1BB or OX40) during activation of T cells found that all microenvironmental

signals tuned metabolic pathway skewing, mitochondrial mass, and ROS production in $CD4^+$ and $CD8^+$ T cells [13]. Further studies will need to be done to assess whether these differences in metabolic profiles directly specify lineage and whether HIF regulates these differences. It will be important to determine the differences in energetic demands between $CD4^+$ and $CD8^+$ T cells and how they correlate with unique usage of the HIF pathway in each subset for regulation of metabolism, cellular functions, and fate decisions.

Regulation of $CD4^+$ T cell metabolism

$CD4^+$ T cells have been shown to differentiate into a wide range of helper subsets following activation. These subsets exhibit distinct effector functions and differentiate following upregulation of key transcription factors. For example in the case of T_H1 $CD4^+$ T cells, the transcription factor T-bet is induced upon activation or for T_H17 $CD4^+$ T cells the transcription factor RORγt. The wide range of phenotype and function exhibited by these $CD4^+$ helper subsets suggests the possibility for varying metabolic demands, and in line with this hypothesis, culturing activated $CD4^+$ T cells in T_H17 conditions drove significantly higher glycolytic rates in comparison to $CD4^+$ T cells cultured in other T_H conditions [89]. Notably, HIF-1α protein levels were also high in $CD4^+$ T cells cultured in T_H17 conditions [23, 89]. Analysis of gene expression of HIF-1α-deficient $CD4^+$ T cells cultured in T_H17 conditions found reduced expression of known HIF-1α targets in the glycolytic pathway in comparison to wildtype cells. Decreased gene expression of genes promoting glycolysis corresponded with greatly reduced glycolytic metabolism, in comparison to HIF-1α-sufficient $CD4^+$ T cells, demonstrating a reliance on HIF-1α in regulating $CD4^+$ T cell metabolism during T_H17 differentiation [89]. In support of a role for HIF-1α in regulating $CD4^+$ T cell metabolism, a recent study of $Foxp3^-$ regulatory $CD4^+$ T cells (Tr1), that produce IL-10 to control inflammation, demonstrated that HIF-1α also participates in maintaining a shift towards glycolytic metabolism in $CD4^+$ T cells activated and cultured

in vitro in the presence of IL-27 [56].

Impact on CD4$^+$ T cell differentiation and function

The impact of HIF-dependent regulation of cellular metabolism on T cell differentiation and function is less clear than its regulation of glycolytic genes. However, recent work has demonstrated several novel roles for HIF regulation of T cell differentiation and effector function. *In vitro* experiments with CD4$^+$ T cells demonstrated that inhibiting glycolytic metabolism directly impacted effector function [16]. More specifically, *in vitro* activation of CD4$^+$ T cells in glucose-free media supplemented with galactose prevented glycolytic metabolism and reduced IFNγ production, dependent on the ability of GAPDH to bind the 3' UTR of IFNγ mRNA. When the binding site of the 3' UTR was mutated, inhibition of glycolysis in CD4$^+$ T cells no longer affected the production of IFNγ. These data show that usage of glycolytic metabolism in CD4$^+$ T cells occupies GAPDH, preventing interaction with cytokine mRNA and potentiating effector function [16]. These data provide a potential link between HIF regulation of T cell glycolysis and effector function that warrants further study.

HIF-1α and the T_H17/iT_{reg} balance

Recent reports examining the T cell intrinsic role of HIF-1α on CD4$^+$ T cell differentiation demonstrated that deletion of HIF-1α resulted in reduced capacity for differentiation of T_H17 CD4$^+$ T cells *in vitro* and reduced induction of experimental autoimmune encephalitis, a T_H17-driven murine model of autoimmunity *in vivo* [23,89]. The reduction in differentiation of T_H17 CD4$^+$ T cells in HIF-1α-deficient CD4$^+$ T cells was concomitant with an increase in differentiation of iT_{reg} cells. These initial reports suggested that HIF-1α was critical for expression of IL-17A [23, 89]. However, two distinct mechanisms were proposed to explain how HIF-1α promoted T_H17 differentiation and suppressed iT_{reg} formation. One study

argued that HIF-1α deficiency primarily perturbed T_H17 differentiation through a reduction in glycolytic metabolism and demonstrated *in vitro* that suppression of glycolysis in wildtype $CD4^+$ T cells by addition of 2-deoxyglucose to T_H17 culture conditions inhibited T_H17 differentiation and promoted iT_{reg} differentiation [89]. In contrast, Dang *et al* proposed several novel regulatory functions and targets for HIF-1α [23]. *In vitro* activation of $CD4^+$ T cells in T_H17 polarizing conditions resulted in STAT3 signaling which drove expression of HIF-1α mRNA and HIF-1α protein stabilization. HIF-1α in turn drove expression of RORγt, and in cooperation with RORγt, promoted expression of IL-17A. HIF-1α deficiency resulted in loss of RORγt expression and therefore a loss of IL-17A expression [23]. In addition to driving expression of T_H17 signature genes, HIF-1α also inhibited differentiation of iT_{reg} by binding to Foxp3 through a n-terminal domain resulting in proteosomal degradation of Foxp3 in spite of TGF-β signaling present in *in vitro* T_H17 culture conditions [23]. Additional studies by several groups in human and murine models, have both supported and contradicted aspects of these initial reports [3, 7, 21, 24, 35–37]. It is clear that HIF-1α can play a role in the differentiation of T_H17 versus iT_{reg} cells. However, the functional effects may be subject to experimental context. Indeed, data from recent reports suggest that HIF-1α may play a role in supporting thymic T_{reg} function in addition to regulating differentiation of T_H17/iT_{reg} cells [3, 21, 35].

In addition, as demonstrated in $CD8^+$ T cells, HIFα isoforms may have compensatory regulation that occurs in cytokine-dependent fashion [26, Chapter 1]. Therefore, the complicated results seen in various models of T_H17 and T_{reg} differentiation may result from differential HIFα expression/stabilization. Careful dissection of the relative contribution of HIFα subunits will be necessary to truly understand the contributions of hypoxia, glycolytic metabolism, and HIFα activity on $CD4^+$ T cell differentiation given that T_H differentiation is cytokine dependent.

Regulation of CD8$^+$ T cell metabolism

Multiple lines of evidence also support a role for HIF regulation of metabolism in CD8$^+$ T cells. A recent study prevented formation of functional HIFα transcriptional complexes through conditional deletion of HIF-1β in T cells. CD8$^+$ T cells deficient in HIF-1β activated in response to TCR signaling and costimulation were unable to sustain levels of glucose uptake and lactate production following extended culture *in vitro* [28]. Experiments examining VHL-null CD8$^+$ T cells, which have constitutive stabilization of HIFα subunits, further support a role for HIFα in regulating CD8$^+$ T cell metabolism. Microarray analysis of adoptively transferred P14 TCR transgenic VHL-null CD8$^+$ T cells demonstrated significantly higher expression of glycolysis-associated genes compared to wildtype [26, Chapter 1]. Most directly, *in vitro* activated VHL-null CD8$^+$ T cells have significantly increased glycolytic throughput as well as suppression of oxidative phosphorylation in line with HIF regulation of cellular metabolism seen in other tissues [26, Chapter 1].

Impact on CD8$^+$ T cell differentiation and function

Similar to CD4$^+$ T cells, recent work has established important novel roles for HIFs in the regulation of CD8$^+$ T cell differentiation and function [26, 28, Chapter 1]. In addition to preventing the maintenance of glycolytic metabolism in activated CD8$^+$ T cells, conditional deletion of HIF-1β from T cells resulted in a reduction in gene expression of perforin and some granzymes following *in vitro* activation and culture with IL-2. Increased stabilization of HIF-1α produced the opposite effect of HIF-1β deletion, as culture of wildtype cells in 1% O_2 drove increased stabilization of HIF-1α and resulted in increased perforin expression [28]. Chromatin immunoprecipitation of RNA Polymerase II (RNAPol II) at the transcription start site or distal exon of perforin showed no difference between wildtype or HIF-1β-deficient cells in RNAPol II binding suggesting that increases in perforin mRNA and protein are indirectly affected by functional HIF transcriptional complexes [28]. In

addition to alterations in cytotoxic molecules, HIF-1β deletion results in significant increases in mRNA expression for chemokine receptors and trafficking molecules including CCR7, CXCR3, and CD62L [28]. Interestingly, increased gene expression was not restricted to either secondary lymphoid organ homing-receptors or those of inflammatory chemokines, thereby making it difficult to predict whether HIF-1β-deficient $CD8^+$ T cells may traffic preferentially to certain tissues *in vivo* following infection. However, *in vitro* generated wildtype and HIF-1β-deficient CTLs mixed in equal proportions, labeled, and transferred into naive hosts yielded increased recovery of HIF-1β-deficient $CD8^+$ T cells from lymph nodes suggesting HIF signaling favored effector $CD8^+$ T cell trafficking to non-lymphoid tissues [28]. In order to assess whether the increased expression of secondary lymphoid homing receptors such as CD62L on HIF-1β-deficient CTL is a direct transcriptional effect or an indirect result of metabolic perturbation the authors examined CD62L expression on *in vitro* activated wildtype $CD8^+$ T cells cultured in IL-2 and low levels of glucose and found that limiting levels of glucose prevented downregulation of CD62L following activation, arguing that the increased expression of CD62L in HIF-1β-deficient cells is the result of reduced glucose uptake due to lower *glut1* expression. However, these data do not rule out a direct role for HIF transcriptional activity in regulating T cell trafficking molecules as well [28].

Data regarding loss of HIF-1β are complimented by studies examining $CD8^+$ T cells following conditional deletion of *Vhl*, which resulted in constitutive stabilization of HIFα subunits, responding to chronic viral infection [26, Chapter 1]. VHL-deficient CTL exhibited increased expression of effector molecules such as GranzymeB as well as resistance to T cell exhaustion in the face of chronic antigen exposure. The resistance of VHL-deficient CTL to exhaustion resulted in reduced viral titers following chronic LCMV clone 13 infection in comparison to wildtype, however, this was accompanied by host death resulting from severe immunopathology [26, Chapter 1]. Microarray analysis of VHL-deficient $CD8^+$ T

cells responding to either chronic or acute infection demonstrated that constitutive HIF activity drives significant alterations in a variety of effector molecules (*GzmB*), secreted factors (*Ifng*), costimulatory receptors (*Tnfrsf9*), exhaustion/activation associated genes (*Lag3*), and key $CD8^+$ T cell differentiation transcription factors (*Prdm1*, *Tbx21*, *Eomes*) exhibited significantly altered expression. Many of these changes in gene expression were confirmed at the protein level and analysis of $CD8^+$ T cell subsets during the course of response to viral infections showed a dramatic HIFα-dependent loss of shorter-lived effectors ($KLRG1^{hi}CD127^{lo}$) in both acute and chronic viral infection [26, Chapter 1]. Deletion of both HIF-1α and HIF-2α in addition to VHL rescued host death by eliminating immunopathology and restoring expression of many altered genes to wildtype levels following chronic LCMV infection, demonstrating HIFα dependence of enhanced effector responses to persistent antigen [26, Chapter 1]. While it is clear from these data that HIF-1α and HIF-2α play a role in the regulation of $CD8^+$ T cell exhaustion in the face of persistent antigen, the impact each subunit has on both the metabolic phenotype and the functional phenotype will be important topics to address.

Together these data demonstrate a role for HIFs in the differentiation and function of $CD8^+$ T cells during the response to viral infections. HIFs appear to play a significant role in the modulation of effector molecule expression during the course of an infectious response and likely serve to modulate antigen-dependent responses as constitutive HIFα signaling drives resistance to T cell exhaustion signals. This model is supported by studies of tumor infiltrating $CD8^+$ T cells where HIF-1α increased 4-1BB (*Tnfrsf9*) expression, a costimulatory receptor, which when ligated has been shown to drive proliferation, IL-2 production, and increased cytolytic activity in response to tumors and chronic viral infections [72, 96]. VHL deletion ultimately impacts both receptor expression and likely signaling events downstream of the receptors as well. Further work exploring how HIFs crosstalk with these signaling pathways will be highly informative and present novel therapeutic approaches. It is important to note

that these studies have focused on effector responses and early $CD8^+$ T cell differentiation events. Whether HIFs regulate the formation of T cell memory remains unknown and experiments exploring HIF's control of both transcriptional and metabolic targets will be helpful for parsing out the individual contributions of transcription factors and cellular metabolism to the differentiation of protective memory T cells and explored in part in this dissertation [Chapter 2 and Chapter 3].

Control of memory $CD8^+$ T cell differentiation

As mentioned above, recent studies have observed significant metabolic transitions upon T cell activation that coincide with T cell fate decisions [16,22,45,56,61,69,89,91,94,95, 102]. Further examination of $CD8^+$ T cell metabolism following activation found that memory $CD8^+$ T cells exhibited an increase in spare respiratory capacity (SRC) fueled by fatty acid oxidation (FAO) which have been argued to be essential for the differentiation of memory $CD8^+$ T cell populations and rapid recall responses upon secondary challenge [69, 94, 95]. While these studies demonstrate a significant correlation between metabolic transitions and $CD8^+$ T cell fate, they do not demonstrate that the usage of a particular metabolic pathway drives fate determination. These studies have posited that upon inhibition of FAO driven SRC, memory $CD8^+$ T cells fail to survive and thus, usage of this specific metabolic pathway is essential for the differentiation and survival of memory $CD8^+$ T cells. An alternative interpretation of these data would be that energy generation is essential for the differentiation and survival of memory $CD8^+$ T cells and that ultimately memory $CD8^+$ T cell differentiation is a metabolic pathway-agnostic process. In order to test this hypothesis, in this dissertation [Chapter 2] we leverage HIF's ability to regulate both glycolysis and oxidative phosphorylation to alter the metabolic pathway $CD8^+$ T cells rely upon during differentiation and ascertain whether metabolic pathway usage inherently limits potential cell fates following acute viral infection.

The following chapters in this dissertation will cover some of the findings discussed above [26, Chapter 1], utilize the unique control of cellular metabolic pathways by HIF to interrogate the necessity of metabolic pathway choice in $CD8^+$ T cell memory differentiation [Chapter 2], and determine the direct impact of HIFα-deficiency on $CD8^+$ T cell responses to acute viral infection [Chapter 3]. Finally, this dissertation will conclude with an examination of future directions to be explored with regards to the impact of HIFs on $CD8^+$ T cell differentiation and function and how these questions may be helpful in understanding how microenvironmental inputs may regulate immunity and thus inform choices in the development of novel therapeutic strategies.

The introduction, in part, has been published in *Molecular Immunology.* "Hypoxia-inducible factors regulate T cell metabolism and function." Anthony T Phan; Ananda W Goldrath. *Molecular Immunology* (2015). The author of this dissertation was the primary author of this review.

Chapter 1

Hypoxia-inducible factors enhance the effector responses of CD8$^+$ T cells to persistent antigen

1.1 Introduction

The CD8$^+$ T cell response to intracellular pathogens such as bacteria, viruses and protozoans is critical to host resistance to infection. Naive CD8$^+$ T cells respond to infection by rapidly expanding and differentiating into effector and memory precursor populations while traversing to various host tissues from secondary lymphoid organs to fight the infection. During infection host tissues present unique microenvironments with distinct challenges, including but not limited to, restricted access to nutrients such as oxygen. Clearance of the infection results in a contraction of the CD8$^+$ T cell pool resulting in the emergence of a stable memory cell population capable of providing long-lived immunity. The rapid expansion and contraction of CD8$^+$ T cell number are accompanied by metabolic transitions, such as a dramatic increase in reliance on glycolytic metabolism upon T cell activation

and subsequent return to usage of fatty acid fueled oxidative phosphorylation in long-lived memory $CD8^+$ T cells. These events occur in a diverse set of tissue environments, including secondary lymphoid tissues, suggesting that microenvironmental signals such as oxygen tension may play a significant role in regulating $CD8^+$ T cell function and differentiation following infection.

In the following chapter, adapted from [26], a novel role for HIF signaling in the regulation of $CD8^+$ T cell differentiation, effector function, and metabolic activity following chronic viral infection is described. T cell receptor signaling, oxygen, and cytokines regulate HIFα stability resulting in alteration of $CD8^+$ T cell differentiation and function. Constitutive HIFα activity, due to deletion of VHL, in $CD8^+$ T cells resulted in dramatic alteration of expression of several critical transcription factors previously shown to regulate $CD8^+$ T cell fate and function. Additionally, chronic viral infection by LCMV strain clone 13 in mice with $CD8^+$ T cells deficient in VHL resulted in severe immunopathology leading to host death. The immunopathology was seen most prominently in the lung and was the direct result of improved effector function by $CD8^+$ T cells and resistance to persistent viral infection-driven T cell exhaustion. Improved effector capacity and resistance to exhaustion was driven by HIF-1α and HIF-2α expression as deletion of both along with VHL rescued host death following chronic LCMV infection. Additionally, constitutive HIFα activity in *in vitro* activated $CD8^+$ T cells promoted enhanced glycolytic metabolism. Intriguingly, pharmacologic inhibition of glycolytic metabolism altered expression of effector and activation-associated receptors by *in vitro* cultured $CD8^+$ T cells supporting a role for cellular metabolism in regulating effector function. These results demonstrated the impact of environmental sensors, like HIF, in the regulation of effector function and differentiation of $CD8^+$ T cells during the response to infection and provided a unique model by which future studies could be completed to dissect the role of cellular metabolic pathway choice on $CD8^+$ T cell differentiation and function.

1.2 Results

HIF-1α and HIF-2α regulate effector, activation/inhibitory, and differentiation markers in CD8$^+$ T cells.

Effector CD8$^+$ T cells responding to infection travel through a range of host tissues, including those with low oxygen tension. Oxygen availability has been demonstrated to regulate a wide range of biological processes including the responses to tissue damage, infection, and tumor growth in numerous cell types [65, 71, 85]. In order to assess the role oxygen tension may play in regulating CD8$^+$ T cell differentiation and function we utilized an *in vitro* system to assess the impact of HIF-1α, HIF-2α, and cytokines on critical effector, activation/inhibitory, and differentiation-associated proteins in CD8$^+$ T cells. We enriched CD8$^+$ T cells from spleens of uninfected wildtype (WT), *Hif1a*$^{fl/fl}$*Cd4*-Cre (HIF1-KO), *Epas1*$^{fl/fl}$*Tie2*-Cre (HIF2-KO), or *Vhl*$^{fl/fl}$*dLck*-Cre (VHL-KO) mice, activated *in vitro* with plate bound anti-CD3 and soluble anti-CD28, expanded the activated CD8$^+$ T cells in medium containing IL-2, and then incubated in normoxic (21% O_2) or hypoxic (1% O_2) conditions.

Immunoblot analysis of nuclear extracts isolated from these activated cytotoxic lymphocytes (CTL) revealed the accumulation of moderate amounts of HIF-1α protein in activated WT cells under normoxic conditions, which was increased by hypoxic incubation (Figure 1.1A). As expected, both HIF-1α and HIF-2α were found at higher levels in VHL-KO cells compared to WT in normoxia (Figure 1.1A). Examination of several critical CD8$^+$ T cell effector, activation/inhibitory, and differentiation-associated markers by flow cytometry revealed significant increases in expression of granzyme B (GzmB), an essential CTL effector molecule, 4-1BB, GITR, and OX40, activation-associated costimulatory receptors, as well as LAG-3 and CTLA-4, inhibitory receptors, in WT cells exposed to hypoxia compared to WT cells cultured in normoxia (Figure 1.1B). The upregulation of these markers was

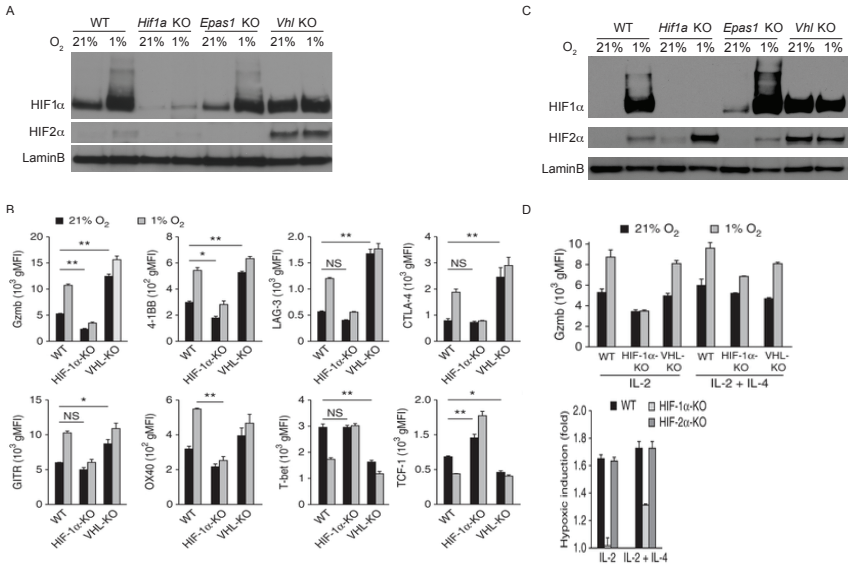

Figure 1.1: **HIF-1α and HIF-2α differentially regulate essential effector, activation/inhibitory, and differentiation-associated molecules.**(A) Immunoblot analysis of HIF-1α, HIF-2α and lamin B in nuclear extracts of WT, *Hif1a*$^{fl/fl}$*Cd4-Cre*, *Epas1*$^{fl/fl}$*Tie2-Cre* and VHL-KO CD8$^+$ T cells activated *in vitro* with anti-CD3 plus anti-CD28, followed by population expansion for 96 hrs in IL-2 and incubation for 6 hrs in normoxia (ambient air; ∼21% O_2) or hypoxia (1% oxygen).(B) Geometric mean fluorescence intensity (gMFI) from flow cytometry analysis of cell-surface and intracellular proteins in WT, *Hif1a*$^{fl/fl}$*Cd4-Cre*, and VHL-KO cells (n = 3 mice per genotype). * $p < 0.01$ and ** $p < 0.001$ (two-way analysis of variance with Bonferroni's post-hoc test).(C) (D)

HIF1α-dependent in these culture conditions, as all of these molecules were poorly induced in HIF1α-KO cells exposed to hypoxia (Figure 1.1B). Intriguingly, expression of T-bet and TCF1, transcription factors known to regulate CD8$^+$ T cell differentiation, was decreased in WT cells following exposure to hypoxia, mirroring the expression level of both in VHL-KO cells (Figure 1.1B). The decrease in T-bet and TCF1 expression was also rescued in HIF-1KO cells, demonstrating that oxygen tension, through a HIF-1α-dependent mechanism, was capable of modulating the expression of critical regulators of CD8$^+$ T cell function and differentiation. Moreover, carefully examining the expression of TCF-1 and GzmB in WT cells in comparison to HIF1-KO cells in normoxia demonstrated HIF-1α-dependent surpression of TCF1 and promotion of GzmB expression irrespective of oxygen tension, consistent with previous findings of HIF-1α accumulation following TCR and cytokine

signaling in normoxia [12, 50, 51, 64, 89].

The induction of HIF-1α or HIF-2α can be regulated by cytokines in macrophages [92] and given the diverse microenvironmental signals CTL likely encounter in host tissues we explored whether accumulation of HIF-1α and HIF-2α could also be regulated by cytokines in $CD8^+$ T cells. We once again performed immunoblot analysis of cells treated as described above (Figure 1.1A and 1.1B) but with the addition of IL-4, which drives HIF-2α accumulation in macrophages [92]. As expected, addition of IL-4 was sufficient to induce increased accumulation of HIF-2α along with HIF-1α in WT CTLs exposed to hypoxia (Figure 1.1C). The addition of IL-4 not only altered the accumulation of HIF-2α, but also influenced whether HIF-1α or HIF-2α drove expression of particular molecules such as GzmB. While hypoxic induction of GzmB expression in WT cells cultured with IL-2 was entirely HIF-1α-dependent, *in vitro* expansion with both IL-2 and IL-4 shifted the regulation of GzmB expression to both HIF-1α and HIF-2α (Figure 1.1D). Thus, the cytokine milieu and abundance of individual HIFα subunits determined the contribution of HIF-1α or HIF-2α in the regulation $CD8^+$ T cell differentiation and function. Together these data showed that HIFs can serve as modulators of multiple aspects of $CD8^+$ T cell function that are influenced by TCR signaling, cytokines, and oxygen tension.

Constitutive HIFα activity in $CD8^+$ T cells results in immunopathology-dependent host death following LCMV Cl13 infection.

To further explore the role of HIFα signaling on $CD8^+$ T cell function *in vivo*, we generated VHL-KO mice expressing the P14 transgene, which encodes for a T cell receptor specific for a peptide fragment of LCMV glycoprotein (gp33) presented by major histocompatibility complex molecule H-$2D^b$. We then adoptively transferred WT or VHL-KO P14 $CD8^+$ T cells, which have constitutive stabilization of HIFα subunits (Figure 1.1), into wildtype hosts and infected the host mice with either the acute (Armstrong) or chronic

(clone 13) strain of LCMV and observed the outcome. A significant proportion of mice which received VHL-KO P14 $CD8^+$ T cells (∼80%) succumbed to clone 13 infection, unlike those which received WT cells (∼5%) (Figure 1.2A). Neither WT or VHL-KO P14 $CD8^+$ T cells caused host death following acute infection, suggesting that VHL-dependent control of HIF activity is relevant for the $CD8^+$ T cell response to chronic viral infection (Figure 1.2A). Both strains of LCMV do not normally result in host death and tissue damage is caused by the host immune response rather than direct virus-mediated cell death. Histological analysis of host tissues revealed that LCMV clone 13-infected hosts that received VHL-KO cells exhibited lungs with substantial edema and evidence of significant immunopathology, in contrast to mice which received WT cells or were uninfected, indicating substantial $CD8^+$ T cell-mediated immunopathology.

Given the significant accumulation of HIF-1α and HIF-2α in VHL-KO $CD8^+$ T cells and the milieu dependent regulation of $CD8^+$ T cell differentiation seen *in vitro* we generated mice with T cell specific deletion of *Vhl*, *Hif1a*, and *Epas1* driven by *dLck*-Cre and expression of the P14 TCR in order to determine whether host-mortality was HIF-1α and HIF-2α-dependent (Figure 1.1). Following adoptive transfer of WT or VHL-HIF1α-HIF2α-KO P14 $CD8^+$ T cells and infection with LCMV clone 13 no increased mortality was observed despite the ample expansion of antigen-specific VHL-HIF1α-HIF2α-KO cells (Figure 1.2C and [26]).

Notably, previous studies have demonstrated that LCMV clone 13 infection drives markedly compromised CTL responses by day 9 after infection [add refs]. Therefore, the death of host mice receiving VHL-KO cells suggests that $CD8^+$ T cell-mediated immunopathology driven by constitutive HIFα signaling maybe the result of augmented effector function by VHL-KO $CD8^+$ T cells in response to chronic infection (Figure 1.2).

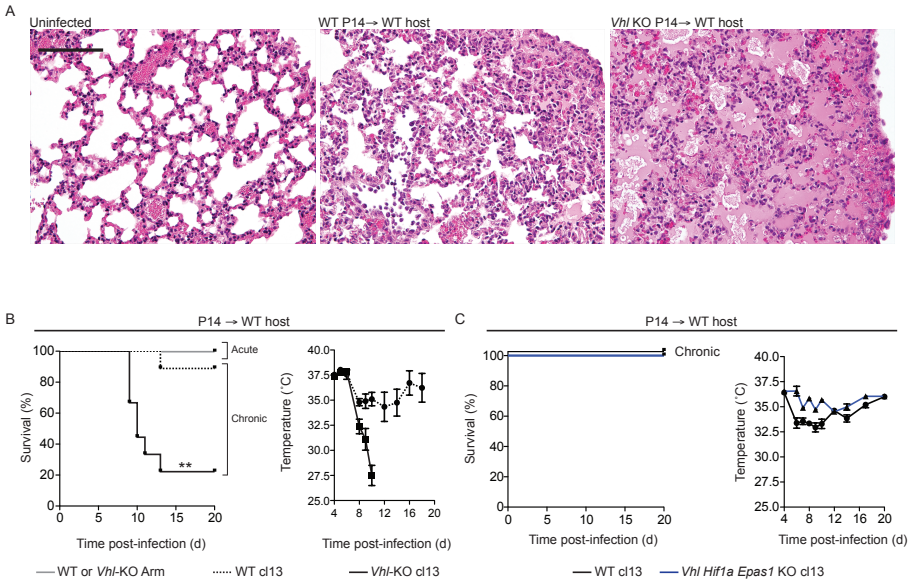

Figure 1.2: **Constitutive HIF-1α and HIF-2α activity in CD8$^+$ T cells mediates host death during persistent viral infection.**(A) Perimortal lung pathology in wild-type host mice (n = 4) given no cells and left uninfected or given 1 x 10^4 WT P14 CD8$^+$ T cells (WT P14 → WT) or VHL-KO P14 CD8$^+$ T cells (VHL-KO P14 → WT), then infected 1 day later with LCMV clone 13 and assessed 9 day later. Scale bar, 100 μm. (B) Survival of and core body temperature of wild-type host mice given 1 x 10^4 WT or VHL-KO P14 CD8$^+$ T cells (P14 → WT), then infected 1 day later. Pooled sample size survival, n = 9 (WT P14, dashed line) or 9 (VHL-KO P14, black line); body temperature, n ≥ 3 per time point. * p = 0.003 and ** p < 0.001, WT versus VHL-KO during chronic infection (log-rank (Mantel-Cox) test). (C) Survival and core body temperature as in (A) in wild-type hosts after transfer of WT or VHL-HIF-1α-HIF-2α-deficient P14 cells (P14 → WT). Pooled sample size (mice): survival, n = 5 (WT P14, black line) or 8 (VHL-HIF-1α-HIF-2α-KO P14, blue line); body temperature, n ≥ 3 mice per time point. Data are pooled from three experiments (b,c; error bars, s.e.m.) or are representative of two experiments.

Sustained HIFα activity enhances effector function and drives resistance to T cell exhaustion

The dramatic immunopathology seen following clone 13 infection in host mice that received VHL-KO P14 CD8$^+$ T cells presented many intriguing questions about why the infection was no longer tolerated when HIF signaling was sustained in CD8$^+$ T cells (Figure 1.2). In order to further dissect the differences between WT and VHL-KO cells, we once again performed adoptive transfers of WT or VHL-KO P14 CD8$^+$ T cells into wildtype hosts followed by infection with LCMV clone 13. Prior to significant core temperature loss

we harvested tissues from infected mice and determined viral titers by quantitative PCR analysis of the mRNA encoding LCMV glycoprotein (LCMV gp) from mice receiving WT or VHL-KO cells. In both spleen and liver we found a significant reduction in LCMV gp mRNA by day 7 post infection (Figure 1.3A). The reduction in viral mRNA correlated with a reduction in viral antigen detected by immunofluorescent staining of liver sections with an antibody specific to LCMV antigen (Figure 1.3B). These data were particularly intriguing as LCMV clone 13 infection has been shown to drive progressive loss of antigen-specific $CD8^+$ T cell function [add ref]. Similar to *in vitro* generated CTL (Figure 1.1) examining GzmB and TNF production by WT and VHL-KO cells that had been cotransferred into wildtype hosts followed by clone 13 infection demonstrated that VHL-KO cells maintained higher GzmB and TNF expression over the course of infection relative to WT cells while experiencing the same inflammatory environment (Figure 1.3C and 1.3D). These data suggest the sustained expression of effector molecules was the result of cell-intrinsic alterations in $CD8^+$ differentiation following VHL-deletion rather than the product of cell-extrinsic differences.

A hallmark of T cell exhaustion is the loss of the ability of CTLs to produce multiple cytokines such as TNF and IFNγ, an effect which progressively worsens as antigen persists. To determine the polyfunctionality of VHL-KO cells, we analyzed surviving mice after adoptive transfer of WT or VHL-KO P14 $CD8^+$ T cells (at day 21 following infection, Figure 1.3E) or mixed transfer of WT and VHL-KO P14 $CD8^+$ T cells (day 17 following infection, Figure 1.3F). We found that VHL-KO P14 $CD8^+$ T cells were resistent to exhaustion, with a significant increase in the proportion of cells that maintained the ability to produce both TNF and IFNγ in both single and mixed-transfer experiments (Figure 1.3E and 1.3F). Consistent with enhanced effector function and resistance to exhaustion VHL-KO cells expressed GzmB at higher levels than WT cells and were cytolytic on a per cell basis than WT cells in an *in vivo* cytotoxicity assay (Figure 1.3E-1.3G). These data suggest that *in vivo* HIF activity plays a key role in modulating effector function during the response to

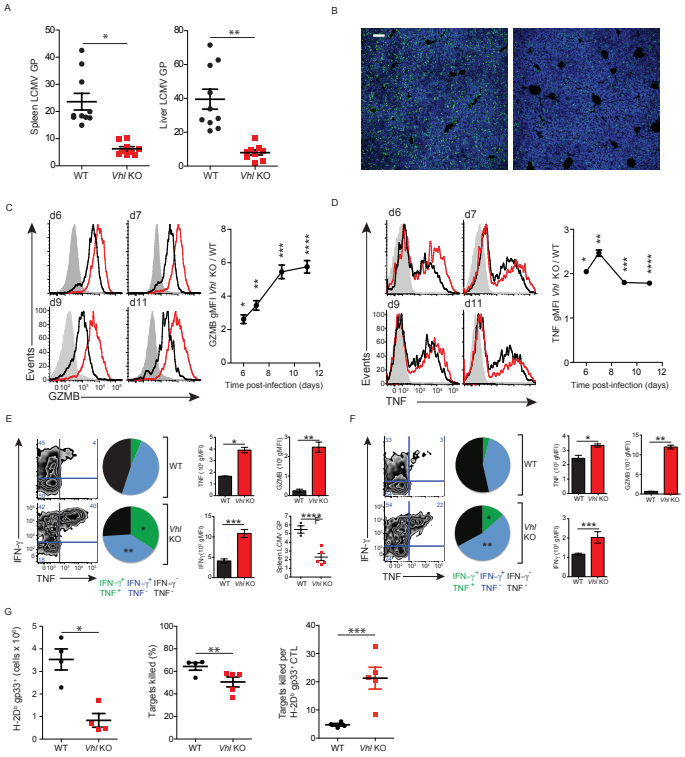

Figure 1.3: **VHL-deficient CD8$^+$ T cells sustain expression of effector molecules, resist exhaustion, and demonstrate improved clearance of persistent viral infection.**(A) Quantitative PCR analysis of the abundance of mRNA encoding LCMV glycoprotein (LCMV gp) in spleen and liver tissue from B6 hosts receiving 1 x 10^4 WT or VHL-deficient P14 CD8$^+$ T cells followed by infection with LCMV clone 13 and analysis 7 days later. LCMV gp expression relative to mRNA encoding HPRT. WT, n = 10 mice and VHL-KO, n = 9 mice. (B) Immunofluorescence microscopy of LCMV antigen (green) and DAPI (blue) in liver sections of host mice as in (A). n = 5 mice, scale bar = 100 μm (C,D) Expression of GzmB (C) and TNF (D) of WT and VHL-KO P14 CD8$^+$ T cells following cotransfer of 2 x 10^4 (1 x 10^4 of each genotype) cells into B6 hosts followed by infection with LCMV clone 13 and flow cytometry analysis (left) on days 6-11 of infection. Relative expression of VHL-KO cells compared to WT summarized (right). (E,F) Expression of IFNγ and TNF of WT or VHL-KO P14 CD8$^+$ T cells single-transferred (E) or cotransferred (F) followed by infection with LCMV clone 13 and analyzed day 21 post infection (E) or day 17 post infection (F) by flow cytometry following restimulation with LCMV gp33-41 peptide (left). (E,F middle) Summarized proportion of cells producing IFNγ and or TNF. (E,F right) Summarized expression (gMFI) of IFNγ, TNF, and GzmB. (E, bottom right) Abundance of viral RNA in spleen from mice receiving WT or VHL-KO P14 CD8$^+$ T cells. (G) *In vivo* cytotoxicity assay: WT or VHL-KO P14 T cells were transferred into B6 hosts and infected with LCMV clone 13. Whole splenocytes coated with or without gp33 peptide, 2.5 10^6 gp33 peptide coated cells per mouse were injected i.v. 16 days after infection. The absolute number of cells binding H-2D^b gp33 tetramer (left), the % killing of target cells (middle) and the approximate killing per antigen-specific CD8$^+$ T cell (right) are shown. * p = 0.003, ** p = 0.05 and *** p = 0.007 (Student's unpaired t-test). Error bars indicate s.e.m. for all plots.

infection in CD8$^+$ T cells.

HIF signaling modulates CD8$^+$ T cell metabolism impacting expression of effector molecules, cell surface receptors, and transcription factors.

Previous studies have demonstrated that naive T cells significantly increase their usage of glycolytic metabolism following activation, which correlates with effector function, however the role of HIFs in this process following infection is unknown [98, 99]. Gene expression analysis of VHL-KO P14 CD8$^+$ T cells in comparison to WT P14 CD8$^+$ T cells sorted from mice that received a mixed transfer of WT and VHL-KO P14 CD8$^+$ T cells followed by infection with LCMV clone 13 revealed a potent induction of transcripts involved in glycolytic metabolism and those involved in suppressing oxidative phosphorylation in VHL-KO cells (Figure 1.4A). Upregulation of these targets is in accordance with known target genes of HIF [79]. Extracellular flux analysis of *in vitro* activated WT and VHL-KO CD8$^+$ T cells demonstrated that glycolytic metabolism of VHL-KO cells was enhanced in comparison to WT cells (Figure 1.4B). Measurement of oxygen consumption and proton-production rate, proxies for oxidative phosphorylation and glycolytic metabolism respectively, revealed that VHL-KO cells consumed oxygen (OCR) at a significantly lower rate and conversely generated protons (ECAR) at a significantly higher rate than WT cells (Figure 1.4B). Examining the ratio of these two measures confirms that VHL-KO cells skew towards the usage of glycolytic metabolism more heavily than WT cells (Figure 1.4B). These data suggest that a potential mechanism by which constitutive HIF signaling mediates alterations in CD8$^+$ T cell differentiation and function is through modulation of cellular metabolism. In support of this hypothesis, culture of *in vitro* activated WT CTLs with the glucose analog 2-deoxy-D-glucose, a competitive inhibitor of hexokinase, mitigated hypoxia-mediated alterations in expression of multiple effector- and activation-associated molecules (Figure 1.4C).

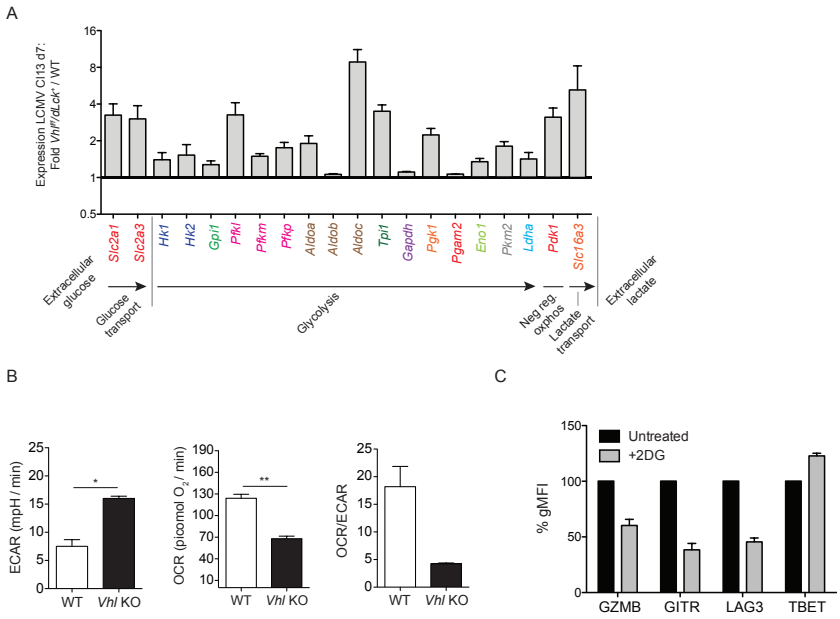

Figure 1.4: **HIF signaling regulates cellular metabolism in CD8$^+$ T cells.** (A) Expression of transcripts involved in the glycolytic pathway and negative regulation of oxidative phosphorylation (Neg reg oxphos) in VHL-KO cells relative to their expression in WT cells on day 7 following infection with LCMV clone 13 (B) Extracellular flux analysis of *in vitro* activated WT and VHL-KO CD8$^+$ T cells. Proton production (ECAR), oxygen consumption (OCR) and OCR/ECAR ratio of WT and VHL-KO CD8$^+$ T cells (n = 4 mice per genotype) activated with anti-CD3 and anti-CD28 and then incubated with IL-2. * p = 0.0005 and ** p <0.0001 (Student's unpaired t-test). (C) Expression of GzmB, GITR, LAG-3 and T-bet in WT CD8$^+$ splenocyte populations (n = 3 mice) activated and expanded as in Figure 1.1, then incubated for 48 hrs in 1% oxygen with 1 mM 2-deoxy-D-glucose (grey), presented relative to vehicle treated cells (black).

1.3 Discussion

Host defense is dependent on numerous cell types producing coordinated responses to invading pathogens in unique microenvironments. Tissue-specific challenges, such as the availability of nutrients or cofactors such as oxygen, vary throughout the host and require infiltrating immune cells to remain flexible and adapt to microenvironment-specific requirements. Oxygen has been demonstrated to play a critical role in regulating differentiation and effector function of immune cells, such as macrophages [6, 78, 92], suggesting that other immune cells may sense oxygen tension to modulate differentiation or effector function.

In this chapter, adapted from [26], we demonstrated that HIFα subunits are regulated

by TCR signaling, oxygen tension, and cytokines resulting in alterations in expression of effector molecules, activation/inhibitory receptors, and transcription factors in $CD8^+$ T cells. These effects suggested that HIF-signaling may play a significant role in effector responses to infection. Following chronic viral infection, constitutive HIF signaling in antigen-specific $CD8^+$ T cells drives enhanced effector function and substantial resistance to immune exhaustion in a HIF-1α- and HIF-2α-dependent fashion. Surprisingly, enhanced antigen-specific $CD8^+$ T cell responses drove dramatic immunopathology and significantly increased host mortality. The increase in host mortality highlights the importance of tolerance generating mechanisms in the context of persistent viral infections as continued effector responses may ultimately be detrimental to the survival of the host. While the increased immunopathology and host mortality are clearly HIF-1α- and HIF-2α-dependent the direct mechanism driving sustained effector function in the face of numerous pro-exhaustion signals following LCMV clone 13 infection remains unclear. A potential mechanism may be the enhanced glycolytic metabolism by enforced by constitutive HIF activity. Intriguingly, when glycolytic metabolism was inhibited pharmacologically in WT CTL, this suppressed HIFα-dependent alterations in expression of effector molecules, activation/inhibitory receptors, and critical $CD8^+$ T cell transcription factors. These results suggest an intriguing role for HIF signaling in modulating $CD8^+$ T cell effector function either through direct transcriptional regulation of activation-associated receptors (i.e. 4-1BB and GITR) or by sustaining effector function-promoting glycolytic metabolism in antigen-specific $CD8^+$ T cells to adapt the level of effector activity to the conditions of the microenvironment.

Previous studies have demonstrated that HIF-1α expression can be driven by TCR-signaling in T cells regardless of oxygen tension and in line with those reports we demonstrated that *in vitro* culture of wildtype $CD8^+$ T cells can drive stabilization of HIF-1α even in normoxia [12, 50, 51, 64, 89]. Importantly, our data expand on these findings by additionally demonstrating that, in addition to TCR signaling, the level of HIFα protein stabilization in

$CD8^+$ T cells can be further regulated by oxygen tension and cytokines. The differential regulation of HIF-1α and HIF-2α subunit stabilization by cytokines appears similar to that reported for macrophages and suggests an intriguing flexibility in HIF-pathway mediated regulation of $CD8^+$ T cell responses [92]. Stabilization of both HIF-1α and HIF-2α subunits by TCR signaling, oxygen, and cytokines drove alterations in expression of a variety of molecules important for $CD8^+$ differentiation and function. Future studies exploring whether cytokine-dependent stabilization of HIFs serve as an additional context-specific mechanism for modulating T cell responses may provide valuable insight into how individual cytokines affect different aspects of T cell immunity. Moreover, while we demonstrated that constitutive HIF signaling drives augmented glycolytic metabolism, further examination of the contribution of individual HIFα subunits on the regulation of glycolysis will clarify how each HIFα subunit impacts $CD8^+$ T cell differentiation and function.

In sum, these data suggest a role for the microenvironment in regulating effector function. For example, when activated CTLs traffic to host tissues and encounter pathogen-infected cells, localized hypoxia, due to either tissue damage or the inflammatory environment, may provide an additional instructional cue for CTL by increasing stabilization of HIF-1α and HIF-2α thereby supporting expression of effector molecules *in situ*. Clearance of the infection, coinciding with a resolution of inflammatory signaling or possibly improved local oxygenation as tissue repair begins, could then drive the inverse process promoting degradation of HIFα-subunits and a dampening of effector functions which are no longer necessary. Furthermore, the local cytokine milieu would provide additional flexibility in modulating CTL differentiation and function. Future studies utilizing localized infection models could provide tremendous insight into this potential regulatory loop. Finally, mechanistic studies to dissect the direct and indirect mechanisms by which HIF signaling modulates effector function would be of significant interest as this may identify novel targets for modulating immunotherapy strategies.

1.4 Materials and Methods

Mice and experimental design.

Mice were bred and housed in specific pathogenfree conditions in accordance with the Institutional Animal Care and Use Guidelines of the University of California San Diego. The following mice have been described: $Vhl^{fl/fl}$ mice [33], $Hif1a^{fl/fl}$ mice [84], and $Epas1^{fl/fl}$ mice [30]. Deletion of those loxP-flanked genes in T cells was achieved by crossing of mice with loxP-flanked alleles to dLck mice [103], mice expressing Cre from the T cell-specific CD4 promoter [49] or Tie2-Cre mice to obtain mice with homozygous loxP-flanked alleles without Cre or hemizygous for Cre. P14 mice [80], which recognize an immunodominant epitope of the LCMV glycoprotein common to LCMV Armstrong strain and clone 13 were bred to the appropriate lines with loxP-flanked alleles. All mice were backcrossed over ten generations to the B6 background. Randomization and steps to reduce experimental bias were done as follows: after initial identification and selection of wild-type and mutant mice, processing of samples and was routinely done by identification code rather than genotype until data-acquisition stages.

Infection and cell transfer.

Mice were infected with LCMV Armstrong strain (2 x 10^5 plaque-forming units, injected intraperitoneally) or LCMV clone 13 (2 x 10^6 plaque-forming units, injected intravenously). For adoptive transfer, 1 x 10^4 to 5 x 10^4 Vα2$^+$CD8$^+$ P14 cells were injected intravenously into B6 recipient mice, which were then infected with the relevant pathogen 1 day later.

In vitro and hypoxic incubation.

Samples from mouse spleens were enriched for $CD8^+$ T cells by negative selection. Incubation with biotinylated anti-B220 (RA3-6B2), anti-CD19 (1D3), anti-Ter-119 (Ter119), anti-CD4 (GK1.5), anti-NK1.1 (PK136) and anti-CD11b (M1/70; all from eBiosceince) was followed by incubation with streptavidin-labeled magnetic beads and depletion on a MACS column. Purified $CD8^+$ T cells were then activated for 48-72 hrs with plate bound anti-CD3 (145-2C11; University of California, San Francisco, Antibody Core) and soluble anti-CD28 (37.51; University of California, San Francisco, Antibody Core) in RPMI-1640 medium containing 10% FCS, 25 mM HEPES, pH 7.2, 1% penicillin-streptomycin-glutamine and 55 μM β-mercaptoethanol. Cells were then pelleted and replated in fresh medium supplemented with 100 U/ml human IL-2 alone or additionally with 20 ng/ml mouse IL-4, followed by incubation for 24-48 hrs. Cells were then split into normoxic conditions (standard incubator) or 1% oxygen (Thermo Scientific-Hera Cell incubator equipped to replace oxygen with nitrogen), followed by incubation for indicated times. Flasks were then removed, placed immediately on ice and processed for flow cytometry or immunoblot analysis. For analysis of cellular metabolism, $CD8^+$ T cells were purified on columns, activated and cultured in IL-2 as described above and then were plated and analyzed on a Seahorse XF24-3 according to the manufacturer's instructions. 1 mM 2-deoxy-D-glucose (Sigma) was added to the medium when indicated.

Immunoblot analysis.

Nuclear extracts were isolated (NE-PER kit; Pierce) and ∼15 μg protein was loaded in each lane (Novex). HIF-1α and HIF-2α were detected with NB100-449 and NB100-122, respectively (Novus). Lamin B was used as a loading control (sc-6217; Santa Cruz Biotechnology).

Flow cytometry and sorting.

Cells were immunostained and acquired on a BD FACSCalibur or Fortessa, and were sorted (where needed) on a BD FACSAria. In addition to the antibody clones noted above for depletion, the following fluorophore-conjugated antibodies were used for flow cytometry and sorting: anti-4-1BB (17B5), anti-CD8 (53-6.7), anti-CD25 (PC61.5), anti-CD44 (IM7), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD62L (MEL-14), anti-CD69 (H1.2F3), anti-CD127 (A7R34), anti-CD244 (244F4), anti-GITR (DTA-1), anti-Eomes (Dan11mag), anti-IFN-γ (XMG1.2), anti-KLRG1 (2F1), anti-LAG-3 (C9B7W), anti-OX40 (OX86), anti-T-bet (4B10), anti-TNF (MPG-XT22), anti-PD-1 (J43), anti-perforin (OMAK-D), anti-TIM-3 (RMT3-23) and anti-V2 (B20.1; all from eBioscience); anti-CTLA-4 (UC10-4F10-11; BD), anti-granzyme B (MHGB05; Life Technologies); and anti-TCF1 (C63D9; Cell Signaling Technologies). Phycoerythrin-conjugated H-2D^{b} tetramers loaded with a peptide with the sequence KAVYNFATC were from Beckman Coulter.

Statistical analysis.

Two-group comparisons were assessed with an unpaired Student's t test, survival data were assessed with the log-rank (Mantel-Cox) test, and grouped data were assessed by two-way analysis of variance followed by Bonferroni post-test (to adjust for multiple comparisons). P values of less than 0.05 were considered significant. No data-point-exclusion criteria were used, and the normality and variance of the distribution of the data was not assessed.

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Chapter 2

Constitutive Glycolytic Metabolism Supports $CD8^+$ T cell Effector Memory

2.1 Summary

Extensive metabolic changes accompany T cell activation including a switch to glycolytic energy production and increased biosynthesis. Recent studies suggest that a subsequent return to reliance on fatty acid oxidation to fuel oxidative phosphorylation and increasing spare respiratory capacity are essential for the differentiation of memory $CD8^+$ T cells. Strikingly, we find that constitutive glycolytic metabolism and suppression of oxidative phosphorylation, due to HIF transcriptional activity, accelerates $CD8^+$ memory cell differentiation during infection. In spite of HIF-driven glycolysis, $CD8^+$ memory cells emerge that upregulate IL-7-receptor and key transcription factors, as well as show a heightened response to secondary challenge. Importantly, increased glycolysis favors formation of long-lived effector-memory $CD8^+$ T cells. These data redefine the role of cellular metabolism

in memory cell differentiation, showing that constitutive reliance on glycolytic metabolism does not hinder formation of a protective memory population and highlight complexity in the relationship between metabolic activity and $CD8^+$ T cell immunity.

2.2 Introduction

Memory $CD8^+$ T cells can provide enduring protection against intracellular pathogens and tumors [43]. As such, eliciting memory T cells is a primary objective of vaccination strategies. Memory $CD8^+$ T cells are a heterogeneous population consisting of multiple subsets: central memory (T_{CM}) cells, that reside in secondary lymphoid tissues and have high proliferative potential upon secondary infection; effector memory (T_{EM}) cells, that patrol peripheral tissues and provide rapid effector responses; and tissue resident memory (T_{RM}) cells, that permanently reside in non-lymphoid tissues, provide localized defense, and aid in rapid recruitment of adaptive and innate immune cells to sites of infection [17, 41, 43, 63].

Rapamycin-mediated inhibition of mTOR signaling [2] or metformin-induced activation of AMPK signaling [76] can enhance the production of memory $CD8^+$ T cells, suggesting that $CD8^+$ differentiation can be manipulated by altering cellular metabolism [69,75,82,91,94]. It has been hypothesized that reliance on fatty acid oxidation (FAO) and a concomitant increase in spare respiratory capacity (SRC) support both memory cell survival and the ability of these cells to respond rapidly to reinfection [69, 94, 95]. These data demonstrate that metabolic pathway usage can be correlated with fate determination in $CD8^+$ T cells, leading to the suggestion that metabolic pathway choice drives memory $CD8^+$ T cell differentiation [17, 52, 75]. However, the degree to which memory formation is controlled by metabolism and how metabolic flux integrates with transcriptional control of effector function and differentiation is currently unknown. Critically, the differential metabolic states of *in vivo* differentiated memory $CD8^+$ T cell subsets have not been determined

either in terms of the requirement for SRC or the role of oxidative phosphorylation in generation of protective cells. A number of transcription factors have been implicated in the regulation of T cell metabolism following activation such as c-myc, mTOR, FOXO1, and the Hypoxia-Inducible Factor (HIF) [17, 75]. The HIF family of transcription factors (HIFs) are the central sensors of oxygen tension and adaptation to low oxygen tensions in all cells, including T cells [33, 59, 65, 79]. Post-translational regulation by the von Hippel Lindau tumor suppressor protein (VHL), an E3 ubiquitin ligase, drives degradation of HIFα subunits in normal oxygen tensions [59, 65, 79]. HIF drives oxygen conservation through the upregulation of glycolytic metabolism and direct suppression of oxygen consumption by mitochondria [65]. Suppression of oxygen consuming mitochondrial respiration is the result of increased expression of nearly all glycolytic enzymes in particular lactate dehydrogenase a (LDHA), which potentiates increased glycolytic throughput, while simultaneously suppressing mitochondrial respiration by preventing the shunting of pyruvate into the citric acid cycle through inhibition of pyruvate dehydrogenase by pyruvate dehydrogenase kinase 1 (PDK1) [46, 79]. Thus, HIF-dependent enhancement of glycolytic metabolism and suppression of cellular respiration presents a unique model by which to interrogate the relationship between metabolic pathway choice and $CD8^+$ T cell differentiation.

To determine the necessity of enhanced SRC and oxidative phosphorylation in memory $CD8^+$ T cell formation, we altered the source of cellular energy production during $CD8^+$ T cell differentiation *in vivo*. This was accomplished through conditional deletion of VHL in mature T cells by expression of the Cre recombinase driven by the distal Lck promoter, resulting in constitutive stabilization of HIF transcription factors [33]. Previously, we demonstrated that deletion of VHL and constitutive HIF activity drives a differentiation program resistant to T cell exhaustion following chronic viral infection [26]. Constitutive HIF activity additionally altered the cellular metabolism of $CD8^+$ T cells *in vitro* and pharmacological inhibition of glycolytic metabolism following *in vitro* activation and culture

suggested that effector function and co-stimulatory and inhibitory receptor expression may be impacted by maintaining heightened glycolytic metabolism [26]. Therefore, we reasoned that modulation of glycolysis and oxidative phosphorylation by HIF provides a powerful *in vivo* model for assessing the role of cellular metabolism on $CD8^+$ T cell differentiation and function without eliminating critical mitochondrial transporters or enzymes. Using this model, we tested the impact of constitutive glycolytic metabolism on $CD8^+$ T cell differentiation to the memory state during the response to acute infection and found that generation of increased SRC and reliance on oxidative phosphorylation are not essential for the generation of long-lived $CD8^+$ T cells. VHL-deficient $CD8^+$ T cells formed fully functional long-lived memory cells that maintain reliance on glycolytic metabolism and respond with improved kinetics to secondary challenge compared to primary challenge despite altered cellular metabolism. Furthermore, *ex vivo* measurement of metabolism of wildtype memory cell subsets showed that T_{CM} exhibited greater SRC than T_{EM}, mirroring the transcriptional heterogeneity found in memory $CD8^+$ T cell subsets, suggesting a link between metabolic pathway usage and memory T cell subset heterogeneity.

2.3 Results

Deletion of VHL does not impair formation or survival of memory $CD8^+$ T cells

We previously demonstrated that *in vitro* activation of VHL-deficient $CD8^+$ T cells results in elevated glycolytic metabolism while suppressing oxidative phosphorylation in comparison to wildtype $CD8^+$ T cells [26]. Thus, we asked whether the enhanced glycolytic metabolism that characterizes these cells impairs formation of memory $CD8^+$ T cells *in vivo*. VHL-deficient P14 $CD8^+$ T cells (VHL-KO) or wildtype P14 $CD8^+$ T cells (WT) were adoptively transferred into naive host mice that were infected one day later with

Lymphocytic Choriomeningitis Virus (LCMV) Armstrong, resulting in a rapidly cleared acute viral infection (Figure 2.1A). Counterintuitively, deletion of VHL and constitutive HIF activity did not impair the survival of VHL-KO cells in secondary lymphoid tissues, or alter expression of CD127 at memory time points (>60 days post infection, Figure 2.1B). Long-lived VHL-KO cells expressed similar levels of key transcription factors relative to WT memory CD8$^+$ T cells, albeit with subtle differences: lower levels of T-bet, and TCF1 expression, and higher levels of FOXO1 (Figure 2.1C). Thus, long-lived memory CD8$^+$ T cells were formed and maintained at similar numbers compared to WT regardless of constitutive HIF activity (Figure 2.1).

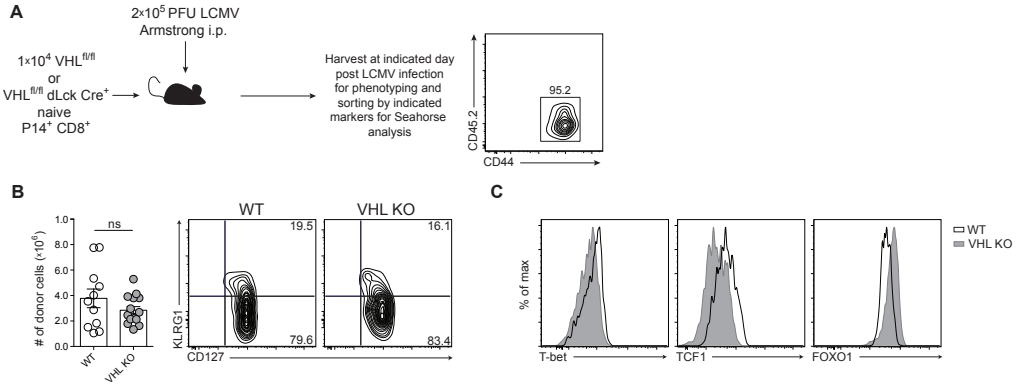

Figure 2.1: **VHL-deficient CD8$^+$ T cells form long-lived memory CD8$^+$ T cells.** (A) Schematic of experimental design; 1 x 10^4 WT or VHL-KO cells were adoptively transferred i.v. into naive hosts with differential expression of congenic CD45 alleles followed by infection one day later with LCMV Armstrong. (B) Representative KLRG1 and CD127 surface phenotype of memory WT and VHL-KO cells (n = 3-5 per 5 independent experiments) and absolute numbers from spleen of host mice (cumulative from 4 independent experiments, n = 26). (C) Representative flow cytometric quantitation of transcription factors; total donor WT (open black histogram) or VHL-KO (filled grey histogram) cells from spleen (n = 3-5 per 5 independent experiments). (B) Numbers represent percentage of cells in respective gates. Data in (B) show mean ± SEM with Student's t test, ns not significant.

Memory VHL-KO CD8$^+$ T cells maintain reliance on glycolytic metabolism

Given that memory cell formation has been closely correlated with reliance on oxidative phosphorylation [69, 94], we investigated the possibility that long-lived VHL-KO

cells bypassed HIF-mediated suppression of oxidative phosphorylation and generated SRC for differentiation and survival. We measured ex vivo glycolytic and oxidative metabolism of resting long-lived VHL-KO and WT cells via extracellular flux analysis of extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) respectively, 60+ days following infection (Figure 2.2). Long-lived VHL-KO cells continued to exhibit significantly higher basal and maximal glycolytic rates (>5-fold) and lower basal and maximal oxidative phosphorylation rates compared to their WT counterparts (Figures 2.2A and 2.2B). Suppression of cellular respiration in VHL-KO cells responding to infection is consistent with our analysis of in vitro activated cells, and is an expected result of HIF-driven induction of PDK1, a canonical target gene. This is indicative of sustained HIF-transcriptional activity and continued suppression of oxidative phosphorylation in VHL-KO cells. This resulted in a complete lack of SRC and a dramatic skewing towards glycolytic metabolism (Figures 2.2C and 2.2D). Importantly, enhanced glycolytic metabolism, suppression of oxidative phosphorylation, and lack of SRC did not prevent differentiation to a memory phenotype or survival of VHL-KO cells in secondary lymphoid tissues (Figures 2.1 and 2.2).

VHL-KO memory CD8$^+$ T cells function as *bona fide* memory CD8$^+$ T cells

A principal characteristic of immunological memory is the capacity to respond with faster kinetics to a pathogen upon rechallenge. To measure the capacity of long-lived VHL-KO cells to respond to secondary infection, VHL-KO or WT memory CD8$^+$ T cells were harvested from spleen and lymph nodes, and re-transferred into naive hosts that were then infected with LCMV Armstrong (Figures 2.2E and 2.2F). Both WT and VHL-KO secondary effector cells respond robustly to infection and clear pathogen efficiently (data not shown). VHL-KO secondary effector cells accumulate at lower numbers in the spleen than WT (Figure 2.2E). However, secondary VHL-KO effector cells showed significantly

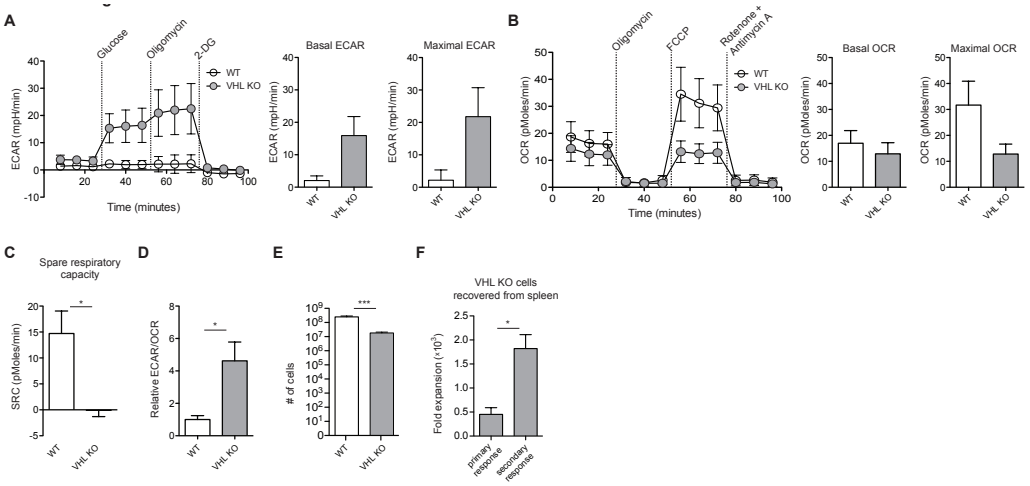

Figure 2.2: **Memory VHL-KO CD8$^+$ T cells rely on glycolytic metabolism and are functional secondary effectors.**(A-D) Experimental design as in Figure 2.1A, (n = 3-5), WT or VHL-KO donor cells were sorted from pooled spleens and lymph nodes and assayed directly *ex vivo* with the Seahorse Extracellular Flux XF-96 analyzer under basal conditions and following addition of indicated metabolic inhibitors. Data from 3 independent experiments with rate measurements normalized to 1.25x10^5 cells/sample. (A) Extracellular Acidification Rate (ECAR) and (B) Oxygen Consumption Rate (OCR) of WT and VHL-KO cells at day >60 following acute viral infection measured over time after addition of metabolic inhibitors (left). Basal and maximal (A) ECAR or (B) OCR (right). (C) SRC of WT and VHL-KO cells calculated from (B). (D) Ratio of basal ECAR to basal OCR of WT and VHL-KO cells relative to ratio of WT cells from measurements in (B). Long-lived WT or VHL-KO donor cells were harvested from secondary lymphoid tissues, sort purified, and 1 x 10^4 were re-transferred into congenically distinct naive host mice followed by infection with LCMV Armstrong one day later. Representative absolute numbers (E) of donor WT or VHL-KO cells on day 5 of secondary challenge (n = 4-5 per 2 experiments) and comparative fold expansion (F) of VHL-KO effector cells following primary (day 6) versus secondary (day 5) LCMV Armstrong challenge from spleen. Data in (A-E) show mean ± SEM with Student's *t* test, ns $p > 0.15$, * $p < 0.05$, *** $p < 0.001$.

higher fold expansion than primary VHL-KO effector cells, a hallmark of memory responses (Figure 2.2F), further supporting the finding that these long-lived VHL-KO cells are *bona fide* memory CD8$^+$ T cells in spite of their altered cellular metabolism (Figures 2.1 and 2.2).

VHL-KO memory-precursor cells sustain elevated glycolytic metabolism and suppress oxidative phosphorylation

While *ex vivo* measurement of resting metabolic rates of VHL-KO and WT cells demonstrated significantly altered metabolic programming in VHL-KO cells that suggests

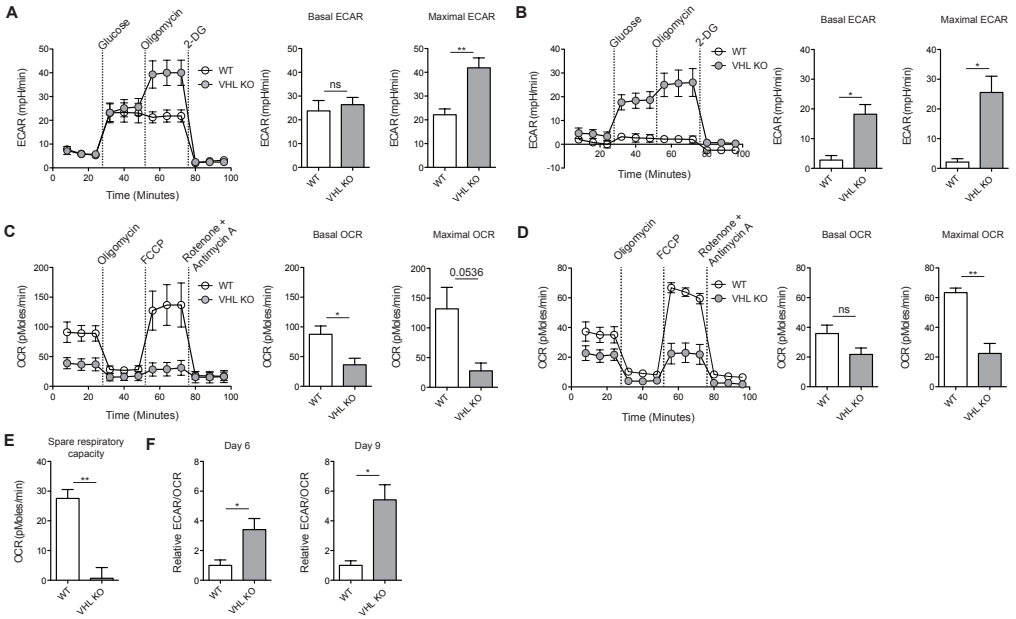

Figure 2.3: **Sustained HIF activity drives glycolysis and suppresses oxidative phosphorylation during the effector response.**(A-F) ECAR and OCR of KLRG1^{lo} CD44^{hi} WT and VHL-KO cells on (A,C) day 6 and (B,D) day 9 of infection (left). Summarized metabolic measures of basal and maximal ECAR or OCR of WT and VHL-KO cells (right). (E) Spare respiratory capacity (SRC) of WT and VHL-KO cells on day 9 of infection. (F) Ratio of basal ECAR to basal OCR of WT and VHL-KO cells relative to ratio of WT cells at 6 and 9 days following infection. Data in (A-F) are mean ± SEM with two-tailed Student's t test. ns $p < 0.05$, * $p < 0.05$, ** $p < 0.01$.

SRC and reliance on oxidative phosphorylation are dispensable for survival of memory CD8$^+$ T cells. We next examined whether HIF-driven glycolytic metabolism was maintained during expansion and contraction phases of the immune response when CD8$^+$ T cell fate is specified. Therefore, we measured *ex vivo* glycolytic and oxidative metabolism of VHL-KO and WT cells responding to acute viral infection. Effector cells were sort purified from infected hosts during the expansion phase (day 6 of infection, Figures 2.3A, 2.3C and 2.3F) and contraction phase (day 9 of infection, Figures 2.3B, 2.3D-2.3F) of LCMV infection, and metabolic activity was measured by extracellular flux analysis (Figure 2.3). *Ex vivo* measurement of glycolytic rates reveal that VHL-KO cells are capable of sustaining significantly higher maximal glycolytic rates during expansion (nearly 2-fold) and contraction (>10-fold) in comparison to WT cells; however, basal glycolytic rates are significantly higher only during

the contraction phase (>5-fold) when WT cells significantly reduce glycolytic activity as the need for effector function wanes (Figures 2.3A and 2.3B). These data correlate well with recent reports that demonstrate the importance of glycolytic metabolism in $CD8^+$ T cell effector function [16, 45, 91, 98] and may support the sustained effector capacity of VHL-KO $CD8^+$ T cells following chronic viral infection [26]. Conversely, the rate of oxidative phosphorylation in VHL-KO cells is substantially lower at basal levels during expansion and contraction. It is also lower throughout the response to infection following treatment with the ionophore FCCP, an agent that uncouples mitochondria and induces maximal oxygen consumption (Figures 2.3C and 2.3D).

During contraction, when WT cells exhibit increased SRC relative to naive and effector $CD8^+$ T cells (proposed as a key feature of memory formation [94]), VHL-KO cells surprisingly fail to generate significant SRC (Figure 2.3E). Furthermore, examining the ratio of glycolytic metabolism to cellular respiration reveals that VHL-KO cells skew towards increased reliance towards glycolytic metabolism during expansion (nearly 4-fold) and contraction (5-fold) phases of the effector response. VHL-KO cells are thus resistant to suppression of proliferation following inhibition of mitochondrial ATP synthase activity with oligomycin treatment in vitro (Figure 2.S1A). Additionally, analysis of relevant metabolites of *in vitro* cultured WT and VHL-KO cells supports *ex vivo* measurements of extracellular flux as VHL-KO cells exhibited significantly higher levels of lactate (nearly 2-fold), indicative of high glycolytic activity, as well as a significant buildup of citrate (2-fold), representative of reduced TCA cycle flux (Figure 2.S1B). These results demonstrate that elevated HIF activity drives a dramatic skewing towards glycolytic metabolism that is sustained throughout the $CD8^+$ T cell effector response to acute infection, which the prevailing model would should argue suppress memory formation (Figure 2.3).

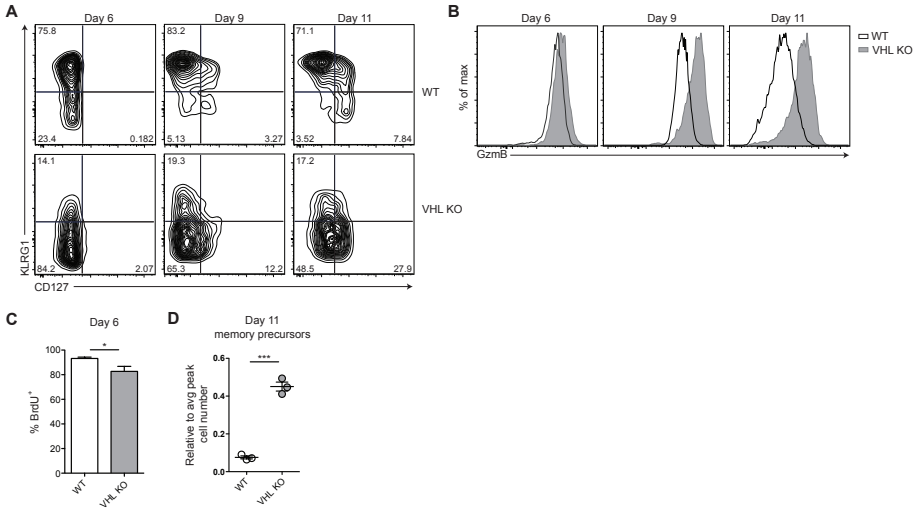

Figure 2.4: **Constitutive HIF activity enhances CD8$^+$ memory cell formation.**(A) Representative surface phenotype of donor WT (top) or VHL-KO (bottom) cells at indicated time points following acute viral infection from spleen (n = 3-5 per 3 independent experiments). Numbers represent percentage of cells in respective gates. (B) Representative intracellular staining of Granzyme B (GzmB) expression of donor WT (open black histogram) or VHL-KO (filled grey histogram) cells from spleen at indicated time points (n = 3-5 per 3 independent experiments) (C) Percent of indicated donor cells incorporating BrdU on day 6 of LCMV infection. (D) Efficiency of memory cell generation: To normalize VHL-KO and WT responses to their absolute number of memory-precursor WT or VHL-KO cells on day 11 of infection were divided by their respective peak number of responding donor WT or VHL-KO cells from day 9 of infection. (C,D) Representative data, n = 3-4 from 2 independent experiments. Data show mean ± SEM with Student's *t* test, * $p < 0.05$, *** $p < 0.001$.

Sustained HIF activity accelerates memory-precursor cell emergence

To examine the contraction phase, when memory precursors can be first followed, we examined differentiation of VHL-KO and WT effector CD8$^+$ T cell subsets (Figure 2.4A). We previously reported that VHL-KO CD8$^+$ T cells become activated and clear both chronic and acute infections with heightened effector function [26]. Notably, we observe that VHL-KO CD8$^+$ T cells show impaired formation of terminally-differentiated effector cells (Figure 2.4A and [26]). Instead, VHL-KO cells form a population of KLRG1^{lo}CD127^{hi} memory-precursor cells at a higher frequency than WT cells, which is seen by day 9 of infection (Figure 2.4A). This coincides with increased expression of Granzyme B (GzmB) throughout the response to acute viral infection (Figure 2.4B). The accelerated appearance of memory-precursors

by VHL-KO cells also occurs when VHL-KO and WT cells are transferred into the same host, thus confirming constitutive HIF activity alters $CD8^+$ T cell differentiation in a cell intrinsic manner and that this is also partially independent of alterations in inflammation or antigen load (Figure 2.S2). Microarray analysis reveals that while VHL-KO cells express genes associated with effector function [26], they also exhibit enriched expression of genes associated with memory differentiation (Figure 2.S3A). This expression pattern is distinct from that seen in WT $KLRG1^{hi}$ effector cells, suggesting VHL-KO cells undergo altered differentiation towards the memory fate, rather than a failure to upregulate KLRG1 itself (Figure 2.S3B and [26]).

Analysis of the number of memory-precursor cells following contraction reveals that while VHL-KO cells proliferate at a slightly reduced rate compared to WT cells, the proportion of cells which form memory-precursors compared to the peak number of donor effector cells is 5-fold greater for VHL-KO than WT cells (Figures 2.4C and 2.4D). Surprisingly, despite a lack of SRC, VHL-KO cells both maintain increased effector molecule expression and demonstrate enhanced memory differentiation (Figures 2.3 and 2.4). The enhancement in memory differentiation by VHL-KO cells demonstrates that a metabolic shift towards reliance on oxidative phosphorylation is not a requirement for differentiation of memory $CD8^+$ T cells (Figures 2.2-2.4).

Sustained glycolytic metabolism yields sufficient ATP for memory formation

While secondary metabolites may be essential for specific cellular functions or fate decisions, adenosine triphosphate (ATP) produced by both glycolysis and oxidative phosphorylation may be central to survival during contraction and throughout the memory phase [22]. We examined whether enhanced glycolytic metabolism and suppressed oxidative phosphorylation of VHL-KO cells negatively impacts ATP production during contraction.

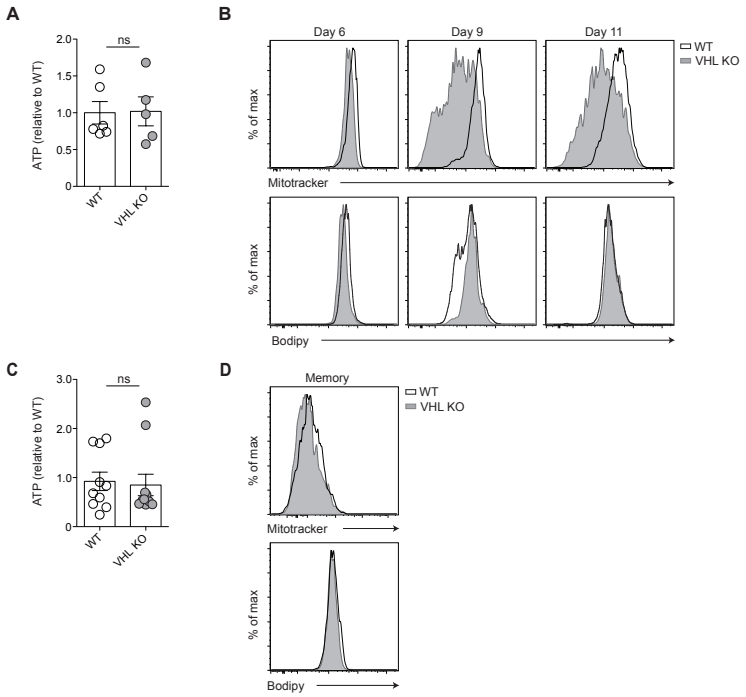

Figure 2.5: **Glycolytic metabolism does not impair ATP production and compensates for suppressed oxidative phosphorylation.** (A,C) Cellular ATP extracted from WT and VHL-KO donor cells (per 10^4 cells) sorted from spleen and lymph nodes of individual host mice (A) post peak of $CD8^+$ response and (C) >60 days following infection. Data are relative to average WT cell ATP levels. Cumulative data (n = 6 mice) from two independent experiments. (B,D) Representative flow cytometric analysis of total donor WT cells (open black histogram) or VHL-KO (filled grey histogram) for analysis of mitochondrial mass and free fatty acid levels at indicated time point following infection, splenocytes (B, n = 3-4 mice per 2 independent experiments D, n = 3-5 mice per 3 independent experiments). Data in (A,C) show mean ± SEM with two-tailed Student's *t* test, ns $p < 0.8$.

VHL-KO or WT memory-precursor cells ($KLRG1^{lo}CD127^{hi}$) from individual mice were sort purified during the contraction phase, cellular ATP was extracted, and ATP levels were then measured by luciferase assay (Figure 2.5A). Surprisingly, the dramatic skewing of VHL-KO cell metabolism towards glycolysis during the effector response did not alter the levels of ATP in VHL-KO memory-precursor cells when compared to WT memory-precursor cells. This suggests that a primary threshold for $CD8^+$ T cells to differentiate into a memory population is sufficient production of ATP, rather than the usage of specific metabolic pathways (Figure 2.5A). Further, we find that VHL-KO cells do not generate additional ATP through a compensatory increase in mitochondrial abundance or through increased fatty acid

stores. Flow cytometric analysis of cells by Mitotracker and Bodipy, stains of mitochondrial content and free fatty acids respectively [69,94], showed a reduction in mitochondrial content during the effector response, but similar free fatty acid levels in VHL-KO cells relative to WT cells (Figure 2.5B). In addition, VHL-KO and WT resting memory cells have similar amounts of ATP (Figure 2.5C), and also maintain similar mitochondrial content and fatty acid stores, suggesting that any compensatory energy production is generated through the glycolytic pathway (Figure 2.5D).

$CD8^+$ memory cells exhibit heterogeneous transcription factor expression and metabolic activity

Our data suggest that generation of SRC and reliance on oxidative phosphorylation is not essential for survival or differentiation of memory $CD8^+$ T cells. However, differential metabolic pathway usage could still play a role in specification of memory subset heterogeneity [44]; thus, we examined memory populations in more detail following contraction (Figure 2.6). Here, we find fewer VHL-KO cells in secondary lymphoid tissues re-expressed L-selectin (CD62L), a marker of T_{CM}. These cells remain $CD62L^{lo}$, a characteristic of T_{EM}, and form approximately 4-fold fewer T_{CM} compared to WT cells (Figure 2.6A). Similarly, the VHL-KO memory population show a higher proportion of $CD27^{lo}CD43^{lo}$ cells (1.5-fold), which correlates with an effector-like memory phenotype [68] (Figure 2.6B). A comparison of key transcription factor expression for WT memory subsets by flow cytometry shows that T_{EM} display significantly lower levels of T-bet, TCF1, Eomes and FOXO1 than T_{CM} (Figure 2.6C). Interestingly, VHL-KO memory cells express transcription factors at levels similar to WT T_{EM} (Figure 2.6C), with the exception of FOXO1, which is higher than WT T_{CM} and T_{EM}. An elevation in FOXO1 (an essential mediator of T_{CM} [62]) by VHL-KO memory cells makes the resemblance to T_{EM} by other criteria even more striking.

Given that WT T_{CM} and T_{EM} express key transcription factors at distinct levels,

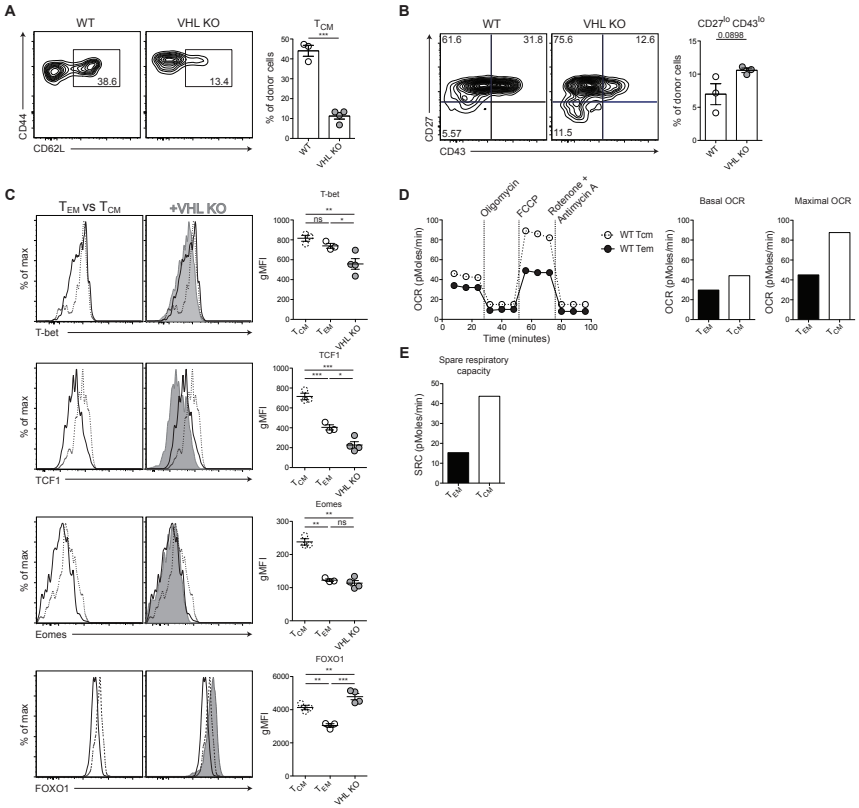

Figure 2.6: **Glycolytic metabolism correlates with differentiation of effector-memory CD8$^+$ T cells.** (A-B) Representative flow cytometric analysis of splenic WT or VHL-KO cells at memory time points (A) for effector memory and central memory CD8$^+$ T cell subsets (n = 3-5 per 4 independent experiments) with summarized frequency of central memory cells. (B) CD27 and CD43 expression and summarized frequency of CD27^{lo}CD43^{lo} memory cells (n = 3 per 2 independent experiments). (C) Representative expression of transcription factors and cytokine receptors of WT central memory (T_{CM}, dashed line) and effector memory cells (T_{EM}, black line, left histograms) and with total VHL-KO memory cell (grey filled) expression overlaid for comparison (right histograms). Representative staining of n = 3-5 per 4 independent experiments. Geometric mean fluorescence intensity (gMFI) of transcription factor expression for WT T_{CM} and T_{EM} compared to total VHL-KO memory cells. (D) Representative OCR of WT T_{CM} (dotted line) and T_{EM} (solid line) measured as in Figure 2.2 (left) with basal and maximal OCR (right) of 2 independent experiments. (E) SRC of WT T_{CM} and T_{EM} from (D). Data show mean ± SEM: (A-B) Student's t test, ns $p > 0.05$, *** $p < 0.0005$; (C) one-way ANOVA followed by Tukeys Multiple Comparison Test, ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

we hypothesized that metabolic pathways may be differentially utilized as well. Thus, we sort purified WT T_{CM} and T_{EM} (CD44^{hi}CD62L^{hi} and CD44^{hi}CD62L^{lo}, respectively) from mice previously infected with LCMV at least 60 days prior and measured ECAR and OCR via extracellular flux analysis (Figure 2.6D). Interestingly, WT T_{EM} maintain substantially

lower levels of SRC and a reduced basal rate of cellular respiration compared to WT T_{CM}; these levels are similar to those seen in VHL-KO memory cells cells (Figures 2.1A-2.1B and 2.6D-2.6E). In contrast, WT T_{CM} exhibit a slightly higher basal rate of oxidative phosphorylation, but develop higher SRC (Figures 2.6D and 2.6E). These data likely support previous studies regarding SRC of WT cells where no discrimination between T_{CM} and T_{EM} was used; thus the predominance of T_{CM} in WT memory cell populations may have biased the metabolic measurements observed [94].

2.4 Discussion

Recent studies have demonstrated that skewing of metabolic pathways in $CD8^+$ T cells correlates with formation of memory $CD8^+$ T cells and pharmacological inhibition of critical metabolic sensors can perturb $CD8^+$ T cell differentiation [2, 76, 94]. Furthermore, deletion of molecules critical for various metabolic processes can have both deleterious or beneficial effects on effector and memory cell generation and survival [18,22,67,69,82,83,88,90]. These data support a role for cellular metabolism in $CD8^+$ T cell differentiation and function; however, the critical question of whether metabolic pathway choice is the driving force behind memory cell differentiation is not yet resolved. By enhancing glycolytic metabolism throughout the effector response to acute infection, we demonstrate that the generation of SRC and a shift towards reliance on oxidative phosphorylation are not essential for the generation of functional long-lived memory $CD8^+$ T cells. Most importantly, our study finds that constitutive glycolytic metabolism does not hinder differentiation of memory $CD8^+$ T cells, but may promote differentiation of T_{EM} cells, skewing the memory pool and impacting functional immunity.

Through manipulation of metabolite transporters [22, 94] or enzymes [5, 69, 76, 83], several studies have demonstrated that inhibition of FAO and oxidative phosphorylation yield

defects in $CD8^+$ memory cell differentiation or survival. Knockdown of carnitine palmitoyl transferase 1 (CPT1a) or deletion of aquaporin 9 (AQP9) starve memory cells of sufficient fatty acids, and deletion of AMPK prevents induction of oxidative phosphorylation in $CD8^+$ T cells, supporting a model in which provision of cellular energy through mitochondrial respiration is critical for memory survival and function [22, 83, 94]. These findings support early reports where memory cell formation was enhanced by promoting mitochondrial fatty acid oxidation and suppressing glycolytic metabolism [2, 76]. While the strong correlation between memory differentiation and the reliance of memory $CD8^+$ T cells on increased mitochondrial biogenesis and fatty acid fueled oxidative phosphorylation lead to the inference that memory differentiation is driven by mitochondrial respiration.

An alternative explanation of these observations is that: promoting or reducing total energy production in $CD8^+$ T cells can improve or inhibit differentiation of memory irrespective of metabolic pathway. We demonstrate that elevated glycolytic metabolism, as a result of VHL deletion, results in production of similar levels of ATP in VHL-KO cells relative to WT cells, thereby allowing survival of contracting effector cells and potentiates differentiation of memory cells. These results argue that it is "the fuel, not the refinery" which is paramount in memory cell differentiation, and that generation of sufficient ATP by $CD8^+$ T cells, regardless of the source, may allow for differentiation of memory cells. This suggests that fate determination in $CD8^+$ T cells can accommodate a range of cellular adaptations as long as basic conditions are met, such as basal energy levels. Further study of the impact of alterations in cellular energy stores on fate determination will be necessary to clearly define how cellular metabolism affects T cell differentiation.

Moreover, while it is clear that cellular metabolism is essential for numerous processes within T cells, our data and those of others do not demonstrate that specific metabolic pathways can inherently drive specific differentiation programs. In light of this, our interpretation of our findings and that of the current literature suggest that T cells are highly flexible

in their reliance on metabolic pathways for differentiation. Function, on the other hand, may be uniquely dependent on environmental conditions, nutrients, and metabolic pathways utilized, in a context specific fashion. Additionally, our data fit well with the inherent migratory differences of T_{CM} and T_{EM} subsets as T_{CM} primarily reside in secondary lymphoid tissues with minimal migration, while T_{EM} likely encounter a wide range of environmental conditions, and therefore, nutrient levels which may drive a necessity for metabolic flexibility. Further work carefully dissecting metabolic requirements for differentiating and sustaining all memory subsets (T_{CM}, T_{EM}, and T_{RM}) and enabling the distinct functional roles of each will be necessary to determine whether manipulating cellular metabolism may sustain or prevent particular cellular fates following T cell activation.

In further support of this model, our examination of metabolic activity by memory $CD8^+$ T cell subsets confirms that SRC and oxidative phosphorylation are elevated in T_{CM} similar to the total $CD8^+$ memory pool as previously described [52, 69, 94]. However, we also find T_{EM} exhibit lower levels of SRC which may reflect the differential functional roles and localization of T_{CM} and T_{EM}. These data allow for a context-specific role for specific pathways or metabolites in particular memory $CD8^+$ T cell subsets and suggest a cell-intrinsic role for metabolic pathway choice in promoting heterogeneity in the memory pool. While the apparent metabolic flexibility demonstrated by memory $CD8^+$ T cells following acute viral infection suggests a direct role for metabolic regulation of $CD8^+$ T cell fate, the significant differences in transcription factor expression between T_{CM} and T_{EM} cells emphasize the need to dissect whether specific metabolic pathways drive differential transcriptional programs or simply correlate with subset diversity. These data parallel studies in $CD4^+$ T cells where particular T_H subsets, that express distinct master transcription factors, exhibit differential reliance on metabolic pathways for differentiation and function [23, 25, 56, 61, 89]; however, in many cases the contribution of differential metabolic regulation towards T_H subset differentiation has yet to be absolutely defined. Our studies and those of others,

highlight the difficulty and importance of discerning direct impacts of manipulating cellular metabolism *in vivo* through modulation of critical cellular sensors such as HIF [26, 28, 89], mTOR [48, 82, 90], and AMPK [5, 76, 83]. Our work does not negate a role for cellular metabolism in specifying T cell fate, but demonstrates that $CD8^+$ T cell differentiation makes use of specific pathways in a much more nuanced fashion than previously appreciated.

In light of the relationship between metabolism and memory cell subsets, modulation of metabolic pathways holds promise as a means to increase the efficacy of T-cell mediated clinical therapies, including vaccination and adoptive cell transfers. Alteration of cellular metabolism by T cells is clearly essential for the functional adjustments and environmental adaptations that occur during the response to infection. Our studies suggest that $CD8^+$ T cells can make use of glycolysis and oxidative phosphorylation for the specification of memory fate and raise the total energy supply in a context-dependent fashion. Importantly, emphasis on specific metabolic characteristics (e.g., SRC) may drive diversification of the $CD8^+$ T cell memory pool. It remains to be determined whether the metabolic differences between T_{CM} and T_{EM} are primary or secondary to the unique transcriptional and migratory circumstances of each subset, highlighting important outstanding questions relating to the generation of a population of protective $CD8^+$ memory cells.

2.5 Materials and Methods

Mice and experimental design

Mice were bred and housed in specific pathogen-free conditions in accordance with the Institutional Animal Care and Use Guidelines of the University of California San Diego. $Vhl^{fl/fl}$ mice have been described [33]. Deletion of LoxP-flanked VHL in T cells was achieved by crossing $Vhl^{fl/fl}$ mice to mice hemizygous for dLck Cre [103]. P14 mice, which express a transgenic TCR that recognizes an immunodominant epitope of the LCMV glycoprotein

expressed by LCMV Armstrong were bred to $Vhl^{fl/fl}$ dLck lines to generate mice with WT or VHL-KO P14 $CD8^+$ T cells. All mice were backcrossed over ten generations to the C57BL/6 background.

Infection and cell transfer

$V\alpha2^+V\beta8.1.2^+$ $CD8^+$ cells were injected intravenously with $1x10^4$ per host for all adoptive transfer experiments except for post peak ATP analysis where $1x10^6$ $V\alpha2^+$ $V\beta8.1.2^+$ $CD8^+$ cells were transferred and mice were infected with LCMV Armstrong ($2x10^5$ plaque-forming units, injected intraperitoneally).

Flow cytometry and sorting

Cells were immunostained and analyzed on a BD Fortessa or Fortessa X-20 or sort purified on a BD FACSAria IIu. All antibodies from EBiosciences unless indicated. The following biotin-conjugated antibodies were used for depletion of unwanted cells prior to sorting: anti-B220 (RA3-6B2), anti-CD4 (GK1.5), anti-Ter119 (TER-119), anti-MHCII (M5/114.15.2), anti-NK1.1 (PK136). Fluorophore-conjugated antibodies used for flow cytometry analysis are as follows: anti-CD8α (53-6.7), anti-KLRG1 (2F1), anti-CD127 (A7R34), anti-Vα2 (B20.1), anti-Vβ8.1.2 (KJ16-133), anti-CD44 (IM7), anti-CD62L (MEL-14), anti-CD27 (LG.7F9), anti-CD43 (1B11), anti-T-bet (4B10), anti-TCF1 (Cell Signaling Technology, C63D9), anti-Eomes (Dan11Mag), and anti-Foxo1 (Cell Signaling Technology, C29H4). Mitotracker Deep Red FM (ThermoFisher, M22426) was used for mitochondrial staining at 25 nM according to manufacturers instructions. For staining of neutral lipids Bodipy 493/503 (ThermoFisher, D-2191) was used at 500 ng/mL according to manufacturers instructions.

Metabolism assays

Indicated numbers of sort purified cells were plated in buffer free, glucose free media (Seahorse Biosciences or Sigma-Aldrich) with glutamine (2 mM) and ± glucose (11mM) for OCR and ECAR measurements which were made under basal conditions and following addition of oligomycin (1 μM), FCCP (1 μM), Rotenone (1 μM) and Antimycin A (1 μM), 2-Deoxyglucose (100 mM) at indicated time points and recorded on a Seahorse XF-96. All compounds from Sigma-Aldrich. Basal ECAR and maximal ECAR calculated from average of three measurements following addition of glucose and oligomycin respectively. Basal OCR and maximal OCR calculated from average of three measurements before addition of oligomycin and following addition of FCCP respectively. Cellular ATP was measured with the ATP Determination Kit (ThermoFisher, A22066) according to manufacturer's instructions.

Statistical analysis

Two-group comparisons were assessed with an unpaired two-tailed Student's t test and multi-group comparisons were assessed by one-way analysis of variance followed by Tukey's Multiple Comparison test.

2.6 Supplemental Information

Metabolomics

Metabolomic experiments were performed by Metabolon Inc. using GC/MS and LC/MS/MS platforms for determination of intracellular metabolites. Samples were normalized for protein concentration measured by the Bradford assay and rescaled to set the median to 1. The minimum value was substituted for missing metabolite values.

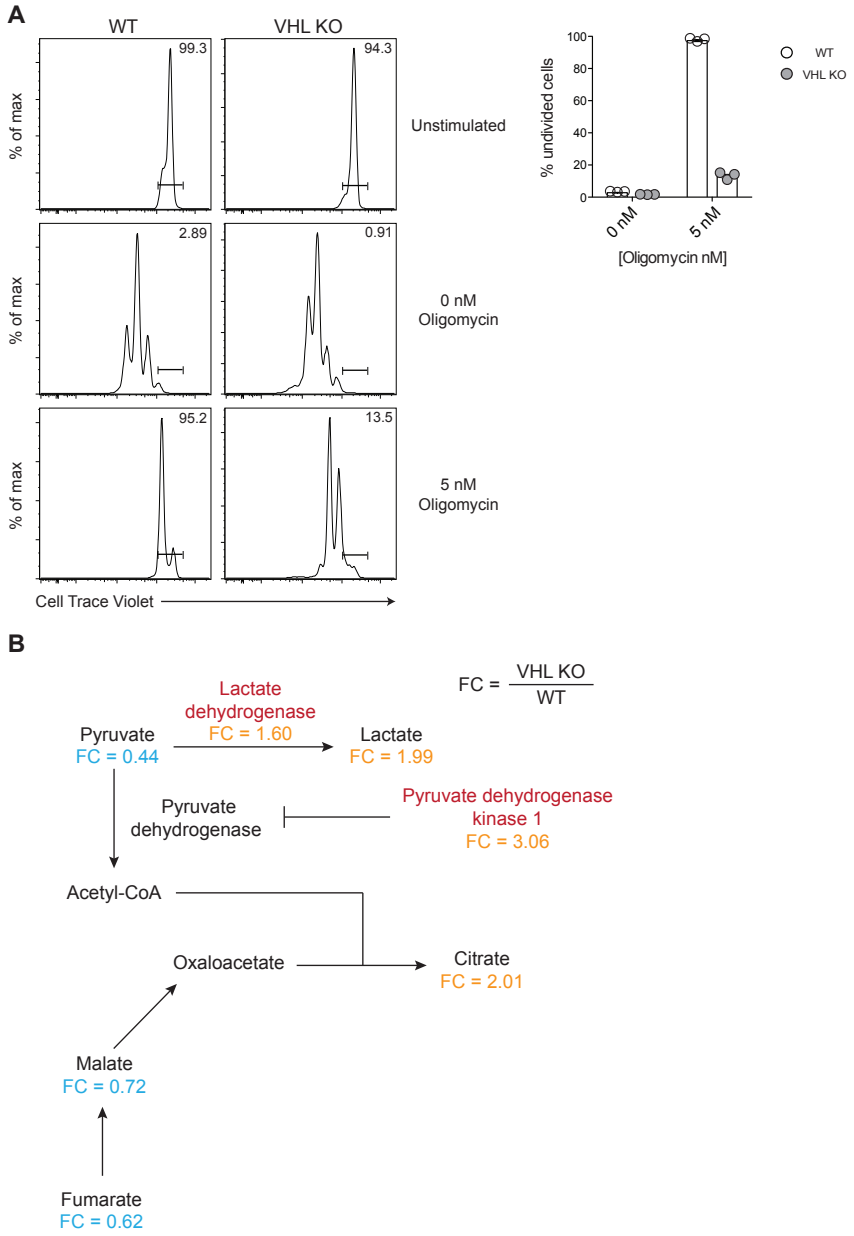

Figure 2.S1: **VHL-KO cell proliferation following activation is resistant to inhibition of oxidative phosphorylation.** (A) Analysis of proliferation 48 hrs following *in vitro* activation with anti-CD3 and anti-CD28 of enriched polyclonal WT and VHL-KO $CD8^+$ T cells from spleen by dilution of Cell Trace Violet. (B) Relevant intracellular metabolites of in vitro activated and expanded VHL-KO and WT $CD8^+$ T cells analyzed by metabolomics performed by Metabolon. Fold change (FC) is comparison of VHL KO over WT ($p \pm 0.05$ for all FC indicated). Critical metabolic enzymes regulating pyruvate usage following glycolysis listed (HIF regulated in red with FC from gene expression analysis).

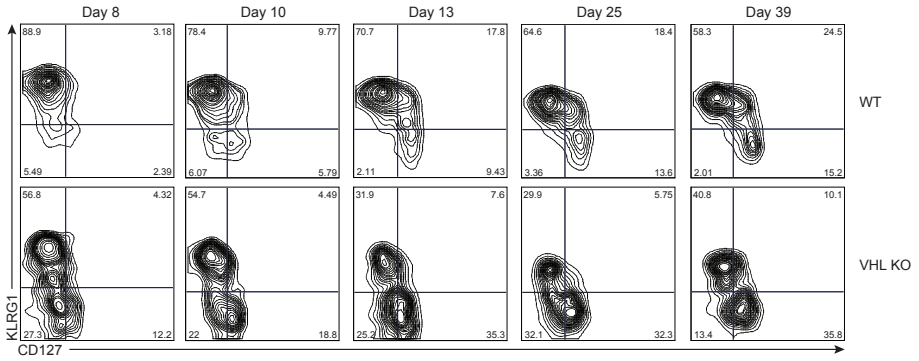

Figure 2.S2: **Accelerated differentiation of VHL-KO cells is primarily cell intrinsic.** Kinetic analysis of VHL-KO and WT donor P14 CD8+ T cells from PBL following mixed transfer (1:1) of 10^4 cells into congenically distinct hosts and subsequent infection with LCMV by flow cytometry for expression of KLRG1 and CD127.

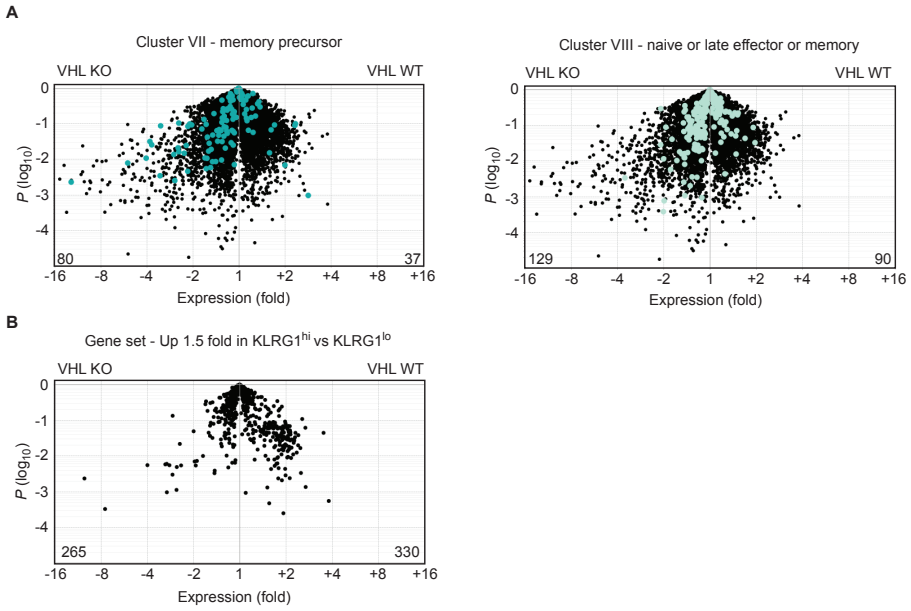

Figure 2.S3: **Gene-expression analysis of WT and VHL-KO KLRG1^{lo} cells at day 7 following infection.**'Volcano plots of the comparison of WT versus VHL-KO cells. Showing clusters of genes identified from [4]. Numbers in bottom left and right corners indicate number of genes in that region. (A) We previously identified temporally regulated gene expression patterns of pathogen-reactive CD8$^+$ T cells that were parsed into 10 unbiased groups [4]. Showing cluster VII (highlighted in green, left) and VIII (highlighted in light green, right) -specific genes overlaid on comparison of total WT and VHL-KO gene-expression. (B) Fold changes versus p value for the cluster of genes expressed 1.5-fold higher in WT KLRG1^{hi} effector cells compared to WT KLRG1^{lo} cells shows a loss of expression of KLRG1^{lo} associated genes by VHL-KO cells.

2.7 Acknowledgements

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Chapter 2, in full, has been submitted for review. *Phan, Anthony T*; Doedens, Andrew L; Palazon, Asis; Tyrakis, Petros A; Cheung, Kitty P; Johnson, Randall S; Goldrath, Ananda W. "Constitutive Glycolytic Metabolism Supports $CD8^+$ T cell Effector Memory." The author of this dissertation was the primary investigator and author of this paper.

Chapter 3

HIF-1α and HIF-2α deficiency limits glycolytic metabolism and skews differentiation of CD8$^+$ T cells following acute viral infection

3.1 Introduction

The previous chapters in this dissertation have defined the impact constitutive HIF signaling has on CD8$^+$ T cell responses to chronic viral infection and determined whether restricting CD8$^+$ T cells to glycolytic metabolism inhibits the differentiation of protective memory CD8$^+$ T cells. In Chapter 1, we demonstrated that HIF signaling enhances effector function and can provide resistance to immune exhaustion in CD8$^+$ T cells following chronic viral infection [26]. In addition, we demonstrated that a potential mechanism by which HIF signaling drives resistance to exhaustion and improved effector function maybe through enhancing glycolytic metabolism in CD8$^+$ T cells [26, Chapter 1]. Additionally, constitutive

HIF signaling accelerated differentiation of memory precursor $CD8^+$ T cells following acute viral infection resulting in a protective memory population predominantly composed of effector memory $CD8^+$ T cells (Chapter 2). These data expanded our understanding of T cell biology by defining novel roles for HIF signaling in regulating $CD8^+$ T cell immunity, and present further questions regarding the necessity of HIF signaling in $CD8^+$ T cell differentiation and function.

In order to further define the molecular pathways driving the dramatic alterations in $CD8^+$ T cell responses following VHL deletion we sought to evaluate the contribution of HIFα subunits to $CD8^+$ T cell immunity. Therefore, we generated mice with mature T cells deficient for *Hif1a* and *Epas1* by expression of Cre recombinase driven by the distal Lck promoter [30, 84, 103]. These mice were then crossed to P14 mice which express a transgenic TCR specific to an immunodominant epitope of the LCMV glycoprotein. Examining mice deficient in HIF-1α and HIF-2α without deletion of VHL allowed us eliminate potential VHL-dependent, HIF-1α- and HIF-2α-independent effects on $CD8^+$ T cell responses and restrict our analysis directly to HIF-mediated effects. Analysis of donor cells following adoptive transfer of wildtype (WT) or HIF-1α- and HIF-2α-deficient (HIF1HIF2 KO) P14 $CD8^+$ T cells surprisingly demonstrated that HIFα-deficiency results in a reduction in glycolytic metabolism as well as accelerated accumulation of $KLRG1^{lo}CD127^{hi}$ memory precursor cells over time following acute LCMV infection. However, no other significant alterations in effector molecule or transcription factor expression by HIF1HIF2 KO cells in comparison to WT cells was observed.

3.2 Results

HIF-1αHIF-2α-deficient CD8$^+$ T cells expand and differentiate similarly to WT CD8$^+$ T cells following acute viral infection.

Recent work has demonstrated that effector function in T cells is reliant on glycolytic metabolism and that blockade of glycolysis can reduce cytokine production [16]. Previous studies by we and others have also demonstrated that HIF signaling regulates differentiation, effector function, and glycolytic metabolism in CD8$^+$ T cells [26, 28, Chapter 1]. Thus, we asked whether HIF-1α and HIF-2α deletion would result in increased differentiation of terminally differentiated effector cells and reduced effector function in antigen-specific CD8$^+$ T cells. To determine whether HIF-1α- and HIF-2α-deficiency would alter differentiation and negatively impact effector responses to acute viral infection we adoptively transferred WT or HIF1HIF2 KO P14 CD8$^+$ T cells into naive host mice that were infected one day later with Lymphocytic Choriomeningitis Virus (LCMV) Armstrong and examined the responding donor cells isolated from spleen by flow cytometry prior to (day 6 post infection) and after the peak (day 9 post infection) of the cellular response (Figure 3.1). Surprisingly, analysis of expression of KLRG1 and CD127 of responding donor cells did not demonstrate any significant differences in differentiation of effector HIF1HIF2 KO cells in comparison to WT (Figure 3.1A and 3.1C). Moreover, there was no difference in the expansion of HIF1HIF2 KO cells as the absolute number of donor cells was similar to WT cells (Figure 3.1B). Given that previous studies have demonstrated the expression of Granzyme B (GzmB) to be HIF-signaling dependent, we anticipated that HIF1HIF2 KO cells would exhibit lower expression of GzmB in comparison to WT cells [26,28]. However, in line with the unperturbed expansion and differentiation of HIF1HIF2 KO cells, GzmB expression was identical in HIF1HIF2 KO donor cells in comparison to WT donor cells (Figure 3.1D). The similarity in differentiation of HIF1HIF2 KO cells to WT cells was reflected in the expression by donor cells of critical

$CD8^+$ T cell transcription factors which was predominantly similar (T-bet, TCF1, Foxo1) in comparison to WT cells, except in the case of Eomes where HIF1HIF2 KO cells appear to maintain slightly higher Eomes expression relative to WT cells (Figure 3.1D).

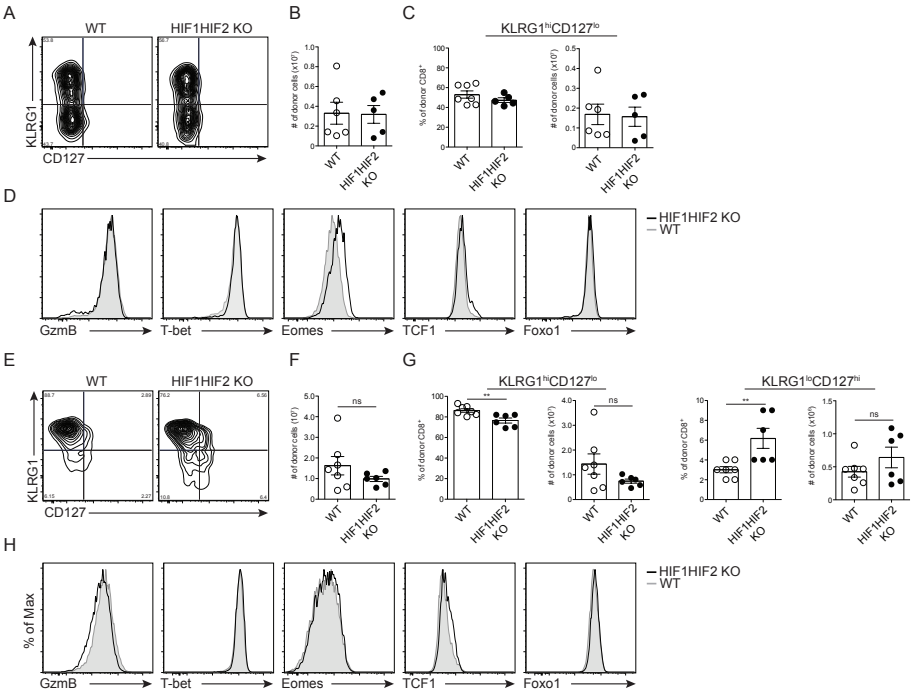

Figure 3.1: **HIF-1α- and HIF-2α-deficient $CD8^+$ T cells do not exhibit defects in effector function following acute viral infection.**(A-F) Flow cytometry analysis of spleens isolated from wildtype host mice that received adoptive transfer of 1 x 10^4 WT or HIF1HIF2 KO P14 $CD8^+$ T cells 6 days (A-D) or 9 days (E-H) following acute viral infection. (A and E) Representative flow cytometry analysis of KLRG1 and CD127 expression of WT and HIF1HIF2 KO P14 $CD8^+$ T cells 6 days following infection (A) or 9 days following infection (E). (B and F) Cell number of responding donor cells (C and G) frequency and cell number of indicated KLRG1 versus CD127 effector cell subset 6 days post infection (C) or 9 days post infection (G). (D and H) Representative histograms showing expression of indicated molecules by WT (filled grey) or HIF1HIF2 KO (open black) P14 $CD8^+$ T cells 6 days (D) or 9 days (H) following infection. (A-H) Cumulative data from 2 independent experiments, n = 5-6 mice. (B-C and F-G) Data show mean ± SEM: Student's *t* test, ns $p > 0.05$, ** $p < 0.01$.

Interestingly, while HIF1HIF2 KO cells exhibited no significant differences in expansion, differentiation, effector molecule, and transcription factor expression prior to the peak of the cellular response, analysis of HIF1HIF2 KO and WT cells from splenocytes isolated 9 days following infection, as the effector $CD8^+$ T cell pool begins to contract, revealed a subtle difference in differentiation beginning to emerge (Figure 3.1E and 3.1G). While there is not

a significant difference in number of donor cells or differentiating subsets of effector $CD8^+$ T cells ($KLRG1^{hi}CD127^{lo}$ terminally-differentiated effector (TE) and $KLRG1^{lo}CD127^{hi}$ memory precursor (MP) cells) between HIF1HIF2 KO and WT donor cells, a significant reduction in the frequency of TE HIF1HIF2 KO donor cells in comparison to WT donor cells is seen following the peak of the response (Figure 3.1F and 3.1G). Moreover, this results in a significant increase in frequency of MP HIF1HIF2 KO cells in comparison to WT cells. While the frequency of TE and MP cells is beginning to shift in HIF1HIF2 KO cells in comparison to WT cells, the expression of GzmB and key transcription factors remain nearly identical to the expression in WT cells (Figure 3.1H).

HIF-1α- and HIF-2α-deficiency accelerates accumulation of memory precursor $CD8^+$ T cells.

Surprisingly, HIF-1α and HIF-2α deficiency did not appear to significantly impact the effector $CD8^+$ T cell response at early time points following acute infection (Figure 3.1). However, following the peak of the cellular response to the infection a significant skewing in the proportion of TE and MP cells was seen in HIF1HIF2 KO cells raising the possibility that more significant differences may be seen at later time points following infection. In order to determine whether the differences in frequency of TE and MP cells seen at the beginning of the contraction phase by HIF1HIF2 KO cells may increase over time we performed a kinetic analysis of KLRG1 and CD127 expression of HIF1HIF2 KO and WT donor cells isolated from peripheral blood following adoptive transfer of HIF1HIF2 KO or WT cells at indicated time points following acute infection (Figure 3.2A and 3.2B). Analysis of donor cells as a percentage of host $CD8^+$ T cells showed no difference between HIF1HIF2 KO and WT cells found in peripheral blood up to 4 weeks following infection (Figure 3.2A). However, following the peak of the cellular response HIF1HIF2 KO cells exhibit accelerated accumulation of MP cells in comparison to WT cells when MP cells were analyzed as a

proportion of donor cells (Figure 3.2B).

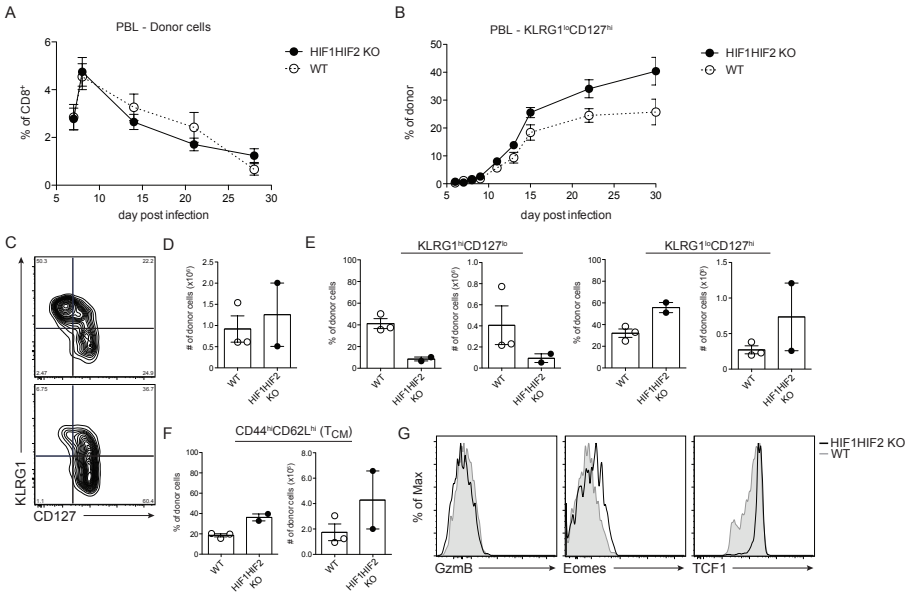

Figure 3.2: **Deficiency in HIFα signaling accelerates accumulation of memory precursor cells.**(A and B) Kinetics of donor WT and HIF1HIF2 KO P14 $CD8^+$ T cells from peripheral blood lymphocytes analyzed by flow cytometry. (A) Donor cells as a percent of $CD8^+$ T cells in PBL at indicated time points. (B) Frequency of $KLRG1^{lo}CD127hi$ memory precursor cells of donor cells in PBL at indicated time points. (C) Representative flow cytometry analysis of KLRG1 and CD127 expression of donor WT (top) and HIF1HIF2 KO (bottom) P14 $CD8^+$ T cells from host spleens isolated 27 days following acute viral infection. (D) Summarized cell number of donor WT and HIF1HIF2 KO P14 $CD8^+$ T cells. (E) Frequency of donor cells and cell numbers of indicated KLRG1 and CD127 expressing subsets. (F) Frequency and cell number of $CD44^{hi}CD62L^{hi}$ donor cells. (G) Representative histograms of expression of indicated molecules by WT (filled grey) and HIF1HIF2 KO (open black) P14 $CD8^+$ T cells.

In order to determine whether the increased proportion of HIF1HIF2 KO MP cells in blood equated to increased differentiation of MP cells in secondary lymphoid tissues we harvested spleens at day 27 post infection from mice that received adoptively transferred HIF1HIF2 KO or WT cells as in Figure 3.1. Intriguingly, HIF1HIF2KO cells trend towards increased differentiation of MP cells in comparison to WT cells, in contrast to our hypothesis that HIFα-deficiency would drive increased TE cell differentiation (Figure 3.2C-3.2E). Additionally, the accelerated emergence of MP cells by HIF1HIF2 KO cells coincides with accelerated re-expression of CD62L (a marker of central memory cells) on

HIF1HIF2 KO cells in comparison to WT cells (Figure 3.2F). Expression of GzmB is similar between HIF1HIF2 KO and WT donor cells, while the increased proportion of MP cells in the donor HIF1HIF2 KO cell population corresponds with the slightly higher levels of Eomes and TCF1 expression in comparison to WT cells suggesting that the expression of fate determining transcription factors by HIF1HIF2 KO cells remains similar to WT cells at later time points (Figure 3.2G). These data suggest that the accelerated differentiation of MP cells by HIF1HIF2 KO cells is not dependent on known transcriptional regulators of memory $CD8^+$ T cell differentiation or the result of cell-extrinsic factors (Figure 3.2C-3.2G). Many cell-intrinsic factors may play a role in the skewing of HIF1HIF2 KO cell differentiation, including HIF-1α- and HIF-2α-deficiency itself, however further studies will need to be performed in order to determine the mechanism driving accelerated MP differentiation in HIF1HIF2 KO donor cells.

HIF-1α and HIF-2α regulate glycolytic metabolism in $CD8^+$ T cells.

We have previously demonstrated that constitutive HIF signaling drives enhanced glycolytic metabolism in $CD8^+$ T cells, suggesting that loss of HIF signaling would result in lower glycolytic metabolism in $CD8^+$ T cells [26, Chapter 2]. Thus, in order to determine the contribution of HIF-1α and HIF-2α-deficiency on glycolytic throughput in $CD8_+$ T cells responding to acute viral infection we performed an adoptive transfer of HIF1HIF2 KO or WT cells as in Figure 3.1 into host mice followed by acute viral infection. HIF1HIF2 KO and WT donor cells were then sort purified at day 6 post infection and the rate of extracellular acidification (ECAR) was measured by extracellular flux analysis directly *ex vivo* (Figure 3.3). As expected, HIF1HIF2 KO cells exhibit reduced basal and maximal glycolytic metabolism (∼75% of the ECAR of WT cells) indicating compensatory mechanisms, such as c-myc [98], are sufficient to induce glycolytic metabolism in HIF1HIF2 KO cells however not to maximal levels (Figure 3.3A). Despite the reduction in glycolytic metabolism, ATP production is

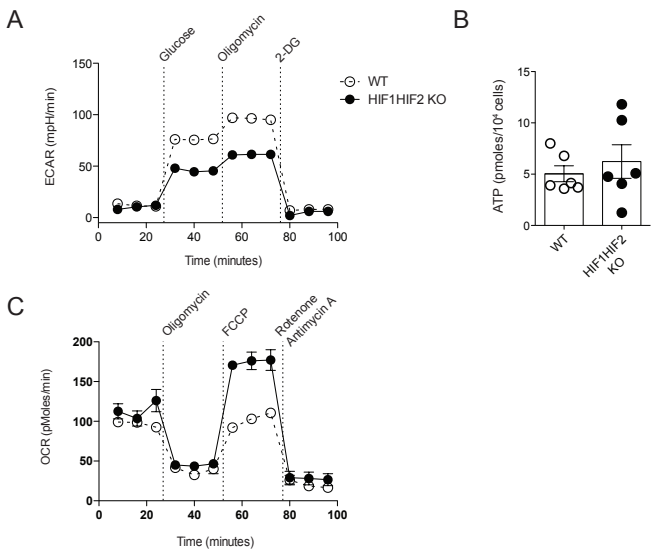

Figure 3.3: **Loss of HIF-1α and HIF-2α reduces glycolytic metabolism in CD8$^+$ T cells without perturbing ATP production.** (A) Measurement of extracellular acidification rate by extracellular flux analysis of *in vitro* activated wildtype, HIF1a KO, and HIF2a KO CD8$^+$ T cells over time and following addition of indicated metabolic inhibitors (left). Summarized basal and maximal glycolytic rates (right). (B) Measurement of glycolytic metabolism as in (A) from WT or HIF1HIF2 KO P14 CD8$^+$ sorted day 6 following acute viral infection. (C) ATP levels of WT and HIF1HIF2 KO memory precursor CD8$^+$ T cells post peak of cellular response to acute viral infection. (D) Oxygen consumption rate of *in vitro* activated WT and HIF1HIF2 KO CD8$^+$ T cells over time and following addition of indicated metabolic inhibitors. Data in (A and D) are representative of two independent experiments, n = 2-3 replicates per genotype, with mean ± SEM. (B) Data are representative of 2 independent experiments. (C) Cumulative data from 2 independent experiments, n = 6 mice per genotype, with mean ± SEM.

unperturbed in HIF1HIF2 KO cells in comparison to WT cells when ATP levels are measured from cells sort purified during the contraction phase of the effector response (Figure 3.3B). This is likely due to a compensatory increase in maximal respiratory capacity by HIF1HIF2 KO cells in comparison to WT cells when oxygen consumption rate, a proxy for the rate of cellular respiration, is measured 60 hours after *in vitro* activation (Figure 3.3C).

Additional analysis of the rate of oxidative phosphorylation being performed by HIF1HIF2 KO cells following acute infection in comparison to WT cells will need to be performed in order to make direct conclusions regarding the metabolic state of HIF1HIF2 KO effector cells, however these data suggest that a possible mechanism allowing accelerated

MP differentiation in HIF1HIF2 KO cells is a shift in cellular metabolism towards oxidative phosphorylation (Figure 3.3). These data would also support previous findings in which inhibition of glycolytic metabolism improved the differentiation of memory $CD8^+$ T cell populations [2, 76].

3.3 Discussion

In an effort to carefully dissect the molecular pathways regulated by HIF-1α and HIF-2α during $CD8^+$ responses to acute viral infection we evaluated the differentiation and expression of effector molecules by HIF1HIF2 KO cells following infection. Surprisingly, in spite of HIF-1α- and HIF-2α-deficiency and reduced glycolytic metabolism, which has been linked to effector molecule expression, HIF1HIF2 KO cells expressed GzmB at identical levels to WT cells throughout the response to infection. This suggests that the enhanced GzmB expression seen in VHL KO $CD8^+$ T cells from previous studies [26, Chapter1, Chapter 2] was regulated by an indirect mechanism that was not sufficiently perturbed by deletion of HIF-1α and HIF-2α. A potential explanation that would fit this hypothesis is that the relatively small reduction in glycolytic metabolism (∼25%) is insufficient drive a reduction in GzmB expression. Alternatively, it's possible that inflammatory signals following acute LCMV infection are so high, they drive a high basal expression of GzmB that compensates for the loss of HIF-1α and HIF-2α. Similarly, the inflammatory milieu may explain why an expected increase in TE cell differentiation was not observed in contrast to the the failure to form TE cells by VHL KO cells (Chapter 2). The high proportion of WT cells that differentiate into TE following LCMV Armstrong infection, may make it unlikely to discern an increase in TE differentiation in HIF1HIF2 KO cells. Future experiments will be performed with different acute infections that drive lower levels of TE cell differentiation in order to assess whether the inflammatory milieu compensates for HIF-1α- and HIF-2α-deficiency. These

results emphasize the complexity in regulation of $CD8^+$ T cell differentiation and function.

In addition to utilizing infection models that drive differing levels of inflammation, it is also possible that HIF-1α and HIF-2α signaling is less relevant in systemic responses analyzed from secondary lymphoid tissues and thus, deletion of both HIF-1α and HIF-2α does not perturb systemic $CD8^+$ T cell responses as dramatically as constitutive stabilization of HIFα subunits following VHL deletion [26, Chapter 1, Chapter 2]. Localized infection models, in particular in tissues that are hypoxic at steady state, may be the optimal models for demonstrating a dramatic phenotype following HIF-1α and HIF-2α loss in $CD8^+$ T cells.

Interestingly, despite the lack of obvious alterations in effector function or differentiation during the early phase of the effector response, tracking the differentiation of HIF1HIF2 KO cells up to 4 weeks following infection revealed that HIF-1α- and HIF-2α-deficiency results in an accelerated accumulation of MP cells in peripheral blood and spleen. Unfortunately, due to issues with rejection of our adoptively transferred cells we have been unable to repeat these experiments as of yet, however we hope to validate these findings and attempt to dissect whether this effect is the direct result of inhibition of glycolytic metabolism or a novel role for HIF-1α and HIF-2α in $CD8^+$ T cells. It is important to note, numerous factors such as the number of cell divisions as well as the rate of apoptosis of TE versus MP HIF1HIF2 KO cells maybe influencing the perceived accelerated differentiation of MP HIF1HIF2 KO cells [17, 41, 43]. Thus, care must be taken in future experiments to clearly demonstrate accelerated differentiation and survival of HIF1HIF2 KO MP cells. Moreover, we must determine whether these putative MP cells result in production of a protective memory $CD8^+$ T cell pool. Secondary challenge of putative memory HIF1HIF2 KO $CD8^+$ T cells will need to be performed and analyzed in comparison to WT memory $CD8^+$ cells. If HIF1HIF2 KO cells do exhibit accelerated memory $CD8^+$ T cell differentiation it will be intriguing to see if modulation of their metabolism, will affect the fate of HIF1HIF2 KO cells.

While HIF-1α- and HIF-2α-deficiency did not reveal dramatic short term defects in

$CD8^+$ effector T cell differentiation and function, these results suggests that the impact of HIF signaling on $CD8^+$ T cell immunity may be much more context-specific than the dramatic phenotypes driven by VHL-deletion suggested. Additional study of HIF-1α and HIF-2α deletion in $CD8^+$ T cells responding to different infectious models may yield important insight into tissue- and inflammation-dependent regulation of HIF activity which could provide novel avenues for manipulation of $CD8^+$ T cell immunity.

3.4 Materials and Methods

Mice and experimental design.

Mice were bred and housed in specific pathogenfree conditions in accordance with the Institutional Animal Care and Use Guidelines of the University of California San Diego. The following mice have been described: *Hif1α*$^{fl/fl}$ mice [84] and *Epas1*$^{fl/fl}$ mice [30]. Deletion of those loxP-flanked genes in T cells was achieved by crossing of mice with loxP-flanked alleles to distal Lck promoter-driven Cre recombinase mice [103] to obtain mice with homozygous loxP-flanked alleles without Cre or hemizygous for Cre. P14 mice, which recognize an immunodominant epitope of the LCMV glycoprotein common to LCMV Armstrong [80] were bred to the appropriate lines with loxP-flanked alleles.

Flow cytometry and sorting

Cells were immunostained and analyzed on a BD Fortessa. All antibodies from EBiosciences unless indicated. Fluorophore-conjugated antibodies used for flow cytometry analysis are as follows: anti-CD8α (53-6.7), anti-KLRG1 (2F1), anti-CD127 (A7R34), anti-Vα2 (B20.1), anti-Vβ8.1.2 (KJ16-133), anti-CD44 (IM7), anti-CD62L (MEL-14), anti-T-bet (4B10), anti-TCF1 (Cell Signaling Technology, C63D9), anti-Eomes (Dan11Mag), and anti-Foxo1 (Cell Signaling Technology, C29H4).

Metabolism assays

Indicated numbers of sort purified cells were plated in buffer free, glucose free media (Seahorse Biosciences or Sigma-Aldrich) with glutamine (2 mM) and ± glucose (11mM) for OCR and ECAR measurements which were made under basal conditions and following addition of oligomycin (1 μM), FCCP (1 μM), Rotenone (1 μM) and Antimycin A (1 μM), 2-Deoxyglucose (100 mM) at indicated time points and recorded on a Seahorse XF-96. All compounds from Sigma-Aldrich. Basal ECAR and maximal ECAR calculated from average of three measurements following addition of glucose and oligomycin respectively. Basal OCR and maximal OCR calculated from average of three measurements before addition of oligomycin and following addition of FCCP respectively. Cellular ATP was measured with the ATP Determination Kit (ThermoFisher, A22066) according to manufacturers instructions.

Infection and cell transfer.

Mice were infected with LCMV Armstrong strain (2 10^5 plaque-forming units, injected intraperitoneally). For adoptive transfer, 1 10^4 Vα2$^+$Vβ8.1.2$^+$CD8$^+$ congenically distinct P14 cells were injected intravenously into B6 recipient mice, which were then infected with LCMV Armstrong 1 day later.

Statistical analysis.

Two-group comparisons were assessed with an unpaired Student's t test. P values of less than 0.05 were considered significant. No data-point-exclusion criteria were used, and the normality and variance of the distribution of the data was not assessed.

Perspectives

Recent studies have highlighted the importance of tissue specific lymphocyte populations [17,41]. Our understanding of T cell differentiation and effector function have primarily focusing on T cells responding to systemic infections in secondary lymphoid organs. The work in this dissertation began in an effort to define when and how microenvironmental conditions, such as oxygen tension, impact T cell immunity. During the course of our studies we were able to define a role for HIF in regulating $CD8^+$ T cell immunity and metabolism. Importantly, however is the observation that while HIF is best known for it's ability to regulate cellular responses to oxygen tension the many oxygen-independent regulatory mechanisms controlling HIF in T cells suggest that T cells have in some respects co-opted this evolutionarily conserved sensor of oxygen-levels to drive unique metabolic and effector programs.

By modulating the HIF pathway in $CD8^+$ T cells via genetic models we were able to dissect how oxygen-sensing impacted $CD8^+$ T cell function in both chronic and acute viral infection. By deleting the primary negative regulator of HIF subunits, VHL, specifically in $CD8^+$ T cells we generated antigen-specific $CD8^+$ T cells which maintained constitutively stabilized HIFα subunits driving an evolutionarily conserved hypoxia-response program. Intriguingly, constitutive HIF signaling following persistent viral infection resulted in enhanced effector function and resistance to immune exhaustion in $CD8^+$ T cells (Chapter 1). The enhanced effector response resulted in dramatic immunopathology and increased

host mortality suggesting that low oxygen-tension may be a signal for increasing effector capacity as inflammatory environments and wounds often exhibit dramatically lower oxygen levels [21, 100]. However, it is unclear whether the impact of HIF signaling seen in our systemic chronic infection experiments relate to localized infection and whether the *in situ* availability of oxygen modulates T cell function. Additionally, whether hypoxic signaling ultimately impacts clearance of infection or pathology is unknown [21, 100]. These data suggest that determining whether localized hypoxia can impact T cell responses will be critical in understanding the signals that drive T cell fate decisions *in vivo*.

Constitutive HIF signaling in $CD8^+$ T cells drives an evolutionarily conserved program designed to limit oxygen consumption, which relies on shifting cellular metabolism away from oxygen-intensive oxidative phosphorylation towards a reliance on glycolytic metabolism. The dramatic skewing of $CD8^+$ T cells towards glycolytic metabolism, produced by VHL deletion, provided a powerful model to dissect whether metabolic pathway choice impacts $CD8^+$ T cell differentiation. Contrary to previous studies, we found that constitutive reliance on glycolytic metabolism by $CD8^+$ T cells does not impair the formation of protective long-lived memory $CD8^+$ T cells (Chapter 2). Intriguingly, following acute viral infection VHL-KO $CD8^+$ T cells exhibited accelerated differentiation to memory precursor cells and ultimately formed predominantly effector memory $CD8^+$ T cells in comparison to wildtype. As discussed in Chapter 2, these data revise the current model of metabolic pathway choice by demonstrating that the generation of SRC and reliance on FAO can correlate with memory formation, but are not causative. These results support an alternative model in which differentiation of memory can occur irrespective of metabolic pathway and that provision of sufficient cellular energy (ATP) is all that is necessary for the differentiation of long-lived memory cells. Importantly, this does not rule out a role for specific metabolites or pathways to impact T cell function or heterogeneity of memory subsets as our measurement of metabolism of central and effector memory $CD8^+$ T cell subsets demonstrated metabolic heterogeneity

within these wildtype memory cell subsets. Interestingly, the metabolic heterogeneity seen between effector and central memory $CD8^+$ T cells, suggests that cellular metabolism may be a readout of the environment that these respective memory populations predominantly reside or traffic through in the host. Determining whether specific metabolites or metabolic pathway choice can drive differentiation of particular memory $CD8^+$ T cell subsets warrants further study and study of T_{RM} populations continues, assessing whether unique tissue-dependent metabolic requirements drive T_{RM} would provide additional insight into the level of metabolic flexiblity that is allowable for memory formation and maintenance.

More detailed analyses of metabolic pathways will be necessary to elucidate the metabolic requirements of T cell function and fate decisions. Careful examination of cellular metabolomics in conjunction with genetic models impacting cellular metabolism, in addition to the HIF pathway, could identify important metabolites for the maintenance of T cell function or fate, thereby adding direct evidence for the necessity of metabolic transitions in T cell biology and explain the functional consequences of shifts in metabolic pathway usage. Moreover, as the field carefully dissects the metabolic requirements of individual memory cell subsets it is likely that roles for specific metabolites, beyond energy provision, will be revealed that impact cell fate decisions and effector function. Discovery of these molecules may provide unique strategies by which to improve vaccinations and immunotherapy.

Our study of HIF-1α and HIF-2α knockout $CD8^+$ T cells following acute viral infection has surprisingly shown that loss of HIF signaling following systemic infection does not dramatically alter expansion, differentiation, or expression of effector molecules in antigen-specific $CD8^+$ T cells early following acute viral infection. This is despite a significant reduction in glycolytic metabolism in $CD8^+$ T cells-deficient for HIF-1α and HIF-2α. As discussed in Chapter 3 glycolytic metabolism has been linked to effector function in T cells [16] and in light of our published findings that HIF signaling can drive enhanced effector function it was surprising to see expression of GzmB in $CD8^+$ T cells was unaffected

by HIF-1α and HIF-2α deletion. While unexpected these data do not rule out a physiological role for oxygen-dependent HIF signaling, or HIF signaling in general, in regulating $CD8^+$ T cell responses. It is possible that these results primarily reflect the role of HIF in acute systemic infections is minimal due to lower levels of HIF stabilization than compared to our VHL-deficient models and that further examination of localized infection models may reveal tissue-specific roles for HIF signaling (i.e. hypoxic tissues such as skin). Additionally, it is becoming evident that the regulation of HIFs occurs on multiple levels. While oxygen plays an important role, depending on the context, oxygen availability may be irrelevant to HIF function. Thus, experiments to assess novel regulatory factors of HIF stability and, more importantly, activity will need to be performed. Expanding the model of HIF regulation in T cells will provide a clearer understanding of the scenarios in which HIFs play a role in T cell function and provide mechanistic insight into how T cells translate signals into cell fate decisions. These data highlight the importance of *in situ* signals in regulating $CD8^+$ T cell responses following infection and point to a necessity for studying T cell responses in additional contexts beyond secondary lymphoid tissues and systemic infections.

Interestingly, when we examined HIF-1α and HIF-2α knockout $CD8^+$ T cells at later time points following infection we observed accelerated differentiation towards memory precursor cells. Surprisingly, there continued to be no striking differences in expression of memory associated transcription factors in these HIF1HIF2 KO $CD8^+$ T cells suggesting that a potential mechanism to explain the accelerated differentiation of memory precursor cells would be a reduction in glycolytic metabolism by HIFα-deletion. These results, which require further studies to validate (discussed in Chapter 3), would support prior studies in which inhibition of glycolytic metabolism promoted differentiation of memory $CD8^+$ T cells [2, 76]. Careful examination of the functional capacity and characteristics of HIF1HIF2 KO memory $CD8^+$ T cells will be necessary to draw significant conclusions about the impact HIFα-deficiency has on memory $CD8^+$ T cell differentiation. Given that T_{RM} reside

primarily in tissues, examination of HIF1HIF2 KO and VHL KO $CD8^+$ T_{RM} populations in hypoxic tissues such as skin or gut in comparison to well oxygenated tissues following infection may provide unique insights into the impact microenvironmental conditions have on memory T cell differentiation.

Tremendous progress has been made in recent years to define the molecular mechanisms regulating $CD8^+$ T cell differentiation. This dissertation has focused on studying the Hypoxia-Inducible Factors, transcription factors known to regulate immunity and cellular metabolism in order to better understand $CD8^+$ T cell biology. Through the course of this work we have defined a unique role for Hypoxia-Inducible Factors in $CD8^+$ T cell differentiation and function, as well as modified the prevailing model for cellular metabolism's impact on memory $CD8^+$ T cell differentiation. Hopefully, these findings provide a new perspective by which to examine $CD8^+$ T cell differentiation and function and present novel avenues to pursue in future studies of $CD8^+$ T cell biology.

Bibliography

[1] R. J. Appelhoff, Y.-M. Tian, R. R. Raval, H. Turley, A. L. Harris, C. W. Pugh, P. J. Ratcliffe, and J. M. Gleadle. Differential Function of the Prolyl Hydroxylases PHD1, PHD2, and PHD3 in the Regulation of Hypoxia-inducible Factor. *Journal of Biological Chemistry*, 279:38458–38465, 2004.

[2] K. Araki, A. P. Turner, V. O. Shaffer, S. Gangappa, S. A. Keller, M. F. Bachmann, C. P. Larsen, and R. Ahmed. mTOR regulates memory CD8 T-cell differentiation. *Nature*, 460:108–112, 2009.

[3] J. Ben-Shoshan, S. Maysel-Auslender, A. Mor, G. Keren, and J. George. Hypoxia controls CD4+CD25+ regulatory T-cell homeostasis via hypoxia-inducible factor-1. *European Journal of Immunology*, 38:2412–2418, 2008.

[4] J. A. Best, D. A. Blair, J. Knell, E. Yang, V. Mayya, A. Doedens, M. L. Dustin, A. W. Goldrath, and T. I. G. P. Consortium. Transcriptional insights into the CD8+ T cell response to infection and memory T cell formation. *Nature Immunology*, 14:404–412, 2013.

[5] J. Blagih, F. Coulombe, E. E. Vincent, F. Dupuy, G. Galicia-Vázquez, E. Yurchenko, T. C. Raissi, G. J. W. van der Windt, B. Viollet, E. L. Pearce, J. Pelletier, C. A. Piccirillo, C. M. Krawczyk, M. Divangahi, and R. G. Jones. The Energy Sensor AMPK Regulates T Cell Metabolic Adaptation and Effector Responses In Vivo. *Immunity*, 42:41–54, 2015.

[6] C. C. Blouin, E. L. Pagé, G. M. Soucy, and D. E. Richard. Hypoxic gene activation by lipopolysaccharide in macrophages: implication of hypoxia-inducible factor 1. *Blood*, 103:1124–1130, 2004.

[7] T. Bollinger, S. Gies, J. Naujoks, L. Feldhoff, A. Bollinger, W. Solbach, and J. Rupp. HIF-1- and hypoxia-dependent immune responses in human CD4+CD25high T cells and T helper 17 cells. *Journal of Leukocyte Biology*, 96:305–312, 2014.

[8] C. P. Bracken, M. L. Whitelaw, and D. J. Peet. The hypoxia-inducible factors: key transcriptional regulators of hypoxic responses. *Cellular and Molecular Life Sciences CMLS*, 60:1376–1393, 2003.

[9] R. K. Bruick and S. L. McKnight. A Conserved Family of Prolyl-4-Hydroxylases That Modify HIF. *Science*, 294:1337–1340, 2001.

[10] J. K. Brunelle, E. L. Bell, N. M. Quesada, K. Vercauteren, V. Tiranti, M. Zeviani, R. C. Scarpulla, and N. S. Chandel. Oxygen sensing requires mitochondrial ROS but not oxidative phosphorylation. *Cell Metabolism*, 1:409–414, 2005.

[11] U. Bruning, L. Cerone, Z. Neufeld, S. F. Fitzpatrick, A. Cheong, C. C. Scholz, D. A. Simpson, M. O. Leonard, M. M. Tambuwala, E. P. Cummins, and C. T. Taylor. MicroRNA-155 Promotes Resolution of Hypoxia-Inducible Factor 1 Activity during Prolonged Hypoxia. *Molecular and Cellular Biology*, 31:4087–4096, 2011.

[12] C. C. Caldwell, H. Kojima, D. Lukashev, J. Armstrong, M. Farber, S. G. Apasov, and M. V. Sitkovsky. Differential Effects of Physiologically Relevant Hypoxic Conditions on T Lymphocyte Development and Effector Functions. *The Journal of Immunology*, 167:6140–6149, 2001.

[13] Y. Cao, J. C. Rathmell, and A. N. Macintyre. Metabolic Reprogramming towards Aerobic Glycolysis Correlates with Greater Proliferative Ability and Resistance to Metabolic Inhibition in CD8 versus CD4 T Cells. *PLoS ONE*, 9:e104104, 2014.

[14] N. S. Chandel, E. Maltepe, E. Goldwasser, C. E. Mathieu, M. C. Simon, and P. T. Schumacker. Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. *Proceedings of the National Academy of Sciences*, 95:11715–11720, 1998.

[15] N. S. Chandel, D. S. McClintock, C. E. Feliciano, T. M. Wood, J. A. Melendez, A. M. Rodriguez, and P. T. Schumacker. Reactive Oxygen Species Generated at Mitochondrial Complex III Stabilize Hypoxia-inducible Factor-1 during Hypoxia A MECHANISM OF O2 SENSING. *Journal of Biological Chemistry*, 275:25130–25138, 2000.

[16] C.-H. Chang, J. D. Curtis, L. B. Maggi, B. Faubert, A. V. Villarino, D. O'Sullivan, S. C.-C. Huang, G. J. van der Windt, J. Blagih, J. Qiu, J. D. Weber, E. J. Pearce, R. G. Jones, and E. L. Pearce. Posttranscriptional Control of T Cell Effector Function by Aerobic Glycolysis. *Cell*, 153:1239–1251, 2013.

[17] J. T. Chang, E. J. Wherry, and A. W. Goldrath. Molecular regulation of effector and memory T cell differentiation. *Nature Immunology*, 15:1104–1115, 2014.

[18] N. Chaoul, C. Fayolle, B. Desrues, M. Oberkampf, A. Tang, D. Ladant, and C. Leclerc. Rapamycin Impairs Antitumor CD8+ T-cell Responses and Vaccine-Induced Tumor Eradication. *Cancer Research*, 75:3279–3291, 2015.

[19] J. Cheng, A. Montecalvo, and L. P. Kane. Regulation of NF-B induction by TCR/CD28. *Immunologic research*, 50:113–117, 2011.

[20] Y. L. Chua, E. Dufour, E. P. Dassa, P. Rustin, H. T. Jacobs, C. T. Taylor, and T. Hagen. Stabilization of Hypoxia-inducible Factor-1 Protein in Hypoxia Occurs Independently of Mitochondrial Reactive Oxygen Species Production. *The Journal of Biological Chemistry*, 285:31277–31284, 2010.

[21] E. T. Clambey, E. N. McNamee, J. A. Westrich, L. E. Glover, E. L. Campbell, P. Jedlicka, E. F. d. Zoeten, J. C. Cambier, K. R. Stenmark, S. P. Colgan, and H. K.

Eltzschig. Hypoxia-inducible factor-1 alpha–dependent induction of FoxP3 drives regulatory T-cell abundance and function during inflammatory hypoxia of the mucosa. *Proceedings of the National Academy of Sciences*, 109:E2784–E2793, 2012.

[22] G. Cui, M. M. Staron, S. M. Gray, P.-C. Ho, R. A. Amezquita, J. Wu, and S. M. Kaech. IL-7-Induced Glycerol Transport and TAG Synthesis Promotes Memory CD8+ T Cell Longevity. *Cell*, 161:750–761, 2015.

[23] E. V. Dang, J. Barbi, H.-Y. Yang, D. Jinasena, H. Yu, Y. Zheng, Z. Bordman, J. Fu, Y. Kim, H.-R. Yen, W. Luo, K. Zeller, L. Shimoda, S. L. Topalian, G. L. Semenza, C. V. Dang, D. M. Pardoll, and F. Pan. Control of TH17/Treg Balance by Hypoxia-inducible Factor 1. *Cell*, 146:772–784, 2011.

[24] J. Darce, D. Rudra, L. Li, J. Nishio, D. Cipolletta, A. Y. Rudensky, D. Mathis, and C. Benoist. An N-terminal mutation of the Foxp3 transcription factor alleviates arthritis but exacerbate diabetes. *Immunity*, 36:731–741, 2012.

[25] V. De Rosa, M. Galgani, A. Porcellini, A. Colamatteo, M. Santopaolo, C. Zuchegna, A. Romano, S. D. Simone, C. Procaccini, C. L. Rocca, P. B. Carrieri, G. T. Maniscalco, M. Salvetti, M. C. Buscarinu, A. Franzese, E. Mozzillo, A. La Cava, and G. Matarese. Glycolysis controls the induction of human regulatory T cells by modulating the expression of FOXP3 exon 2 splicing variants. *Nature Immunology*, 16:1174–1184, 2015.

[26] A. L. Doedens, A. T. Phan, M. H. Stradner, J. K. Fujimoto, J. V. Nguyen, E. Yang, R. S. Johnson, and A. W. Goldrath. Hypoxia-inducible factors enhance the effector responses of CD8+ T cells to persistent antigen. *Nature Immunology*, 14:1173–1182, 2013.

[27] A. C. R. Epstein, J. M. Gleadle, L. A. McNeill, K. S. Hewitson, J. O'Rourke, D. R. Mole, M. Mukherji, E. Metzen, M. I. Wilson, A. Dhanda, Y.-M. Tian, N. Masson, D. L. Hamilton, P. Jaakkola, R. Barstead, J. Hodgkin, P. H. Maxwell, C. W. Pugh, C. J. Schofield, and P. J. Ratcliffe. C. elegans EGL-9 and Mammalian Homologs Define a Family of Dioxygenases that Regulate HIF by Prolyl Hydroxylation. *Cell*, 107:43–54, 2001.

[28] D. K. Finlay, E. Rosenzweig, L. V. Sinclair, C. Feijoo-Carnero, J. L. Hukelmann, J. Rolf, A. A. Panteleyev, K. Okkenhaug, and D. A. Cantrell. PDK1 regulation of mTOR and hypoxia-inducible factor 1 integrate metabolism and migration of CD8+ T cells. *The Journal of Experimental Medicine*, 209:2441–2453, 2012.

[29] J. D. Firth, B. L. Ebert, C. W. Pugh, and P. J. Ratcliffe. Oxygen-regulated control elements in the phosphoglycerate kinase 1 and lactate dehydrogenase A genes: similarities with the erythropoietin 3' enhancer. *Proceedings of the National Academy of Sciences*, 91:6496–6500, 1994.

[30] M. Gruber, C.-J. Hu, R. S. Johnson, E. J. Brown, B. Keith, and M. C. Simon. Acute postnatal ablation of Hif-2 results in anemia. *Proceedings of the National Academy of Sciences*, 104:2301–2306, 2007.

[31] P. M. Gubser, G. R. Bantug, L. Razik, M. Fischer, S. Dimeloe, G. Hoenger, B. Durovic, A. Jauch, and C. Hess. Rapid effector function of memory CD8+ T cells requires an immediate-early glycolytic switch. *Nature Immunology*, 14:1064–1072, 2013.

[32] R. D. Guzy, B. Hoyos, E. Robin, H. Chen, L. Liu, K. D. Mansfield, M. C. Simon, U. Hammerling, and P. T. Schumacker. Mitochondrial complex III is required for hypoxia-induced ROS production and cellular oxygen sensing. *Cell Metabolism*, 1:401–408, 2005.

[33] V. H. Haase, J. N. Glickman, M. Socolovsky, and R. Jaenisch. Vascular tumors in livers with targeted inactivation of the von Hippel–Lindau tumor suppressor. *Proceedings of the National Academy of Sciences*, 98:1583–1588, 2001.

[34] T. S. P. Heng, M. W. Painter, K. Elpek, V. Lukacs-Kornek, N. Mauermann, S. J. Turley, D. Koller, F. S. Kim, A. J. Wagers, N. Asinovski, S. Davis, M. Fassett, M. Feuerer, D. H. D. Gray, S. Haxhinasto, J. A. Hill, G. Hyatt, C. Laplace, K. Leatherbee, D. Mathis, C. Benoist, R. Jianu, D. H. Laidlaw, J. A. Best, J. Knell, A. W. Goldrath, J. Jarjoura, J. C. Sun, Y. Zhu, L. L. Lanier, A. Ergun, Z. Li, J. J. Collins, S. A. Shinton, R. R. Hardy, R. Friedline, K. Sylvia, and J. Kang. The Immunological Genome Project: networks of gene expression in immune cells. *Nature Immunology*, 9:1091–1094, 2008.

[35] M. Higashiyama, R. Hokari, H. Hozumi, C. Kurihara, T. Ueda, C. Watanabe, K. Tomita, M. Nakamura, S. Komoto, Y. Okada, A. Kawaguchi, S. Nagao, M. Suematsu, N. Goda, and S. Miura. HIF-1 in T cells ameliorated dextran sodium sulfate-induced murine colitis. *Journal of Leukocyte Biology*, 91:901–909, 2012.

[36] H.-W. Hsiao, T.-S. Hsu, W.-H. Liu, W.-C. Hsieh, T.-F. Chou, Y.-J. Wu, S.-T. Jiang, and M.-Z. Lai. Deltex1 antagonizes HIF-1 and sustains the stability of regulatory T cells in vivo. *Nature Communications*, 6, 2015.

[37] A. Ikejiri, S. Nagai, N. Goda, Y. Kurebayashi, M. Osada-Oka, K. Takubo, T. Suda, and S. Koyasu. Dynamic regulation of Th17 differentiation by oxygen concentrations. *International Immunology*, 24:137–146, 2012.

[38] J. S. Isaacs, Y. J. Jung, D. R. Mole, S. Lee, C. Torres-Cabala, Y.-L. Chung, M. Merino, J. Trepel, B. Zbar, J. Toro, P. J. Ratcliffe, W. M. Linehan, and L. Neckers. HIF overexpression correlates with biallelic loss of fumarate hydratase in renal cancer: Novel role of fumarate in regulation of HIF stability. *Cancer Cell*, 8:143–153, 2005.

[39] M. Ivan, K. Kondo, H. Yang, W. Kim, J. Valiando, M. Ohh, A. Salic, J. M. Asara, W. S. Lane, and W. G. K. Jr. HIF Targeted for VHL-Mediated Destruction by Proline Hydroxylation: Implications for O2 Sensing. *Science*, 292:464–468, 2001.

[40] P. Jaakkola, D. R. Mole, Y.-M. Tian, M. I. Wilson, J. Gielbert, S. J. Gaskell, A. v. Kriegsheim, H. F. Hebestreit, M. Mukherji, C. J. Schofield, P. H. Maxwell, . C. W. Pugh, and . P. J. Ratcliffe. Targeting of HIF- to the von Hippel-Lindau Ubiquitylation Complex by O2-Regulated Prolyl Hydroxylation. *Science*, 292:468–472, 2001.

[41] S. C. Jameson and D. Masopust. Diversity in T Cell Memory: An Embarrassment of Riches. *Immunity*, 31:859–871, 2009.

[42] L. T. Jeker and J. A. Bluestone. microRNA regulation of T-cell differentiation and function. *Immunological reviews*, 253:65–81, 2013.

[43] S. M. Kaech and E. J. Wherry. Heterogeneity and Cell-Fate Decisions in Effector and Memory CD8+ T Cell Differentiation during Viral Infection. *Immunity*, 27:393–405, 2007.

[44] O. U. Kawalekar, R. S. O'Connor, J. A. Fraietta, L. Guo, S. E. McGettigan, J. Posey, A. D., P. R. Patel, S. Guedan, J. Scholler, B. Keith, N. Snyder, I. Blair, M. C. Milone, and C. H. June. Distinct Signaling of Coreceptors Regulates Specific Metabolism Pathways and Impacts Memory Development in CAR T Cells. *Immunity*, 44(2):380–90, 2016.

[45] Y. Kidani, H. Elsaesser, M. B. Hock, L. Vergnes, K. J. Williams, J. P. Argus, B. N. Marbois, E. Komisopoulou, E. B. Wilson, T. F. Osborne, T. G. Graeber, K. Reue, D. G. Brooks, and S. J. Bensinger. Sterol regulatory element–binding proteins are essential for the metabolic programming of effector T cells and adaptive immunity. *Nature Immunology*, 14:489–499, 2013.

[46] J.-w. Kim, I. Tchernyshyov, G. L. Semenza, and C. V. Dang. HIF-1-mediated expression of pyruvate dehydrogenase kinase: A metabolic switch required for cellular adaptation to hypoxia. *Cell Metabolism*, 3:177–185, 2006.

[47] D. Lando, D. J. Peet, D. A. Whelan, J. J. Gorman, and M. L. Whitelaw. Asparagine Hydroxylation of the HIF Transactivation Domain: A Hypoxic Switch. *Science*, 295:858–861, 2002.

[48] K. Lee, P. Gudapati, S. Dragovic, C. Spencer, S. Joyce, N. Killeen, M. A. Magnuson, and M. Boothby. Mammalian Target of Rapamycin Protein Complex 2 Regulates Differentiation of Th1 and Th2 Cell Subsets via Distinct Signaling Pathways. *Immunity*, 32:743–753, 2010.

[49] P. P. Lee, D. R. Fitzpatrick, C. Beard, H. K. Jessup, S. Lehar, K. W. Makar, M. Pérez-Melgosa, M. T. Sweetser, M. S. Schlissel, S. Nguyen, S. R. Cherry, J. H. Tsai, S. M. Tucker, W. M. Weaver, A. Kelso, R. Jaenisch, and C. B. Wilson. A Critical Role for Dnmt1 and {DNA} Methylation in T Cell Development, Function, and Survival. *Immunity*, 15(5):763 – 774, 2001.

[50] D. Lukashev, C. Caldwell, A. Ohta, P. Chen, and M. Sitkovsky. Differential Regulation of Two Alternatively Spliced Isoforms of Hypoxia-inducible Factor-1 in Activated T Lymphocytes. *Journal of Biological Chemistry*, 276:48754–48763, 2001.

[51] D. Lukashev, B. Klebanov, H. Kojima, A. Grinberg, A. Ohta, L. Berenfeld, R. H. Wenger, A. Ohta, and M. Sitkovsky. Cutting Edge: Hypoxia-Inducible Factor 1 and Its Activation-Inducible Short Isoform I.1 Negatively Regulate Functions of CD4+ and CD8+ T Lymphocytes. *The Journal of Immunology*, 177:4962–4965, 2006.

[52] N. J. MacIver, R. D. Michalek, and J. C. Rathmell. Metabolic Regulation of T Lymphocytes. *Annual Review of Immunology*, 31:259–283, 2013.

[53] P. C. Mahon, K. Hirota, and G. L. Semenza. FIH-1: a novel protein that interacts with HIF-1 and VHL to mediate repression of HIF-1 transcriptional activity. *Genes & Development*, 15:2675–2686, 2001.

[54] Y. Makino, A. Kanopka, W. J. Wilson, H. Tanaka, and L. Poellinger. Inhibitory PAS Domain Protein (IPAS) Is a Hypoxia-inducible Splicing Variant of the Hypoxia-inducible Factor-3 Locus. *Journal of Biological Chemistry*, 277:32405–32408, 2002.

[55] J. H. Marxsen, P. Stengel, K. Doege, P. Heikkinen, T. Jokilehto, T. Wagner, W. Jelkmann, P. Jaakkola, and E. Metzen. Hypoxia-inducible factor-1 (HIF-1) promotes its degradation by induction of HIF--prolyl-4-hydroxylases. *Biochemical Journal*, 381:761–767, 2004.

[56] I. D. Mascanfroni, M. C. Takenaka, A. Yeste, B. Patel, Y. Wu, J. E. Kenison, S. Siddiqui, A. S. Basso, L. E. Otterbein, D. M. Pardoll, F. Pan, A. Priel, C. B. Clish, S. C. Robson, and F. J. Quintana. Metabolic control of type 1 regulatory T cell differentiation by AHR and HIF1-. *Nature Medicine*, advance online publication, 2015.

[57] M. A. Maynard, H. Qi, J. Chung, E. H. L. Lee, Y. Kondo, S. Hara, R. C. Conaway, J. W. Conaway, and M. Ohh. Multiple Splice Variants of the Human HIF-3 Locus Are Targets of the von Hippel-Lindau E3 Ubiquitin Ligase Complex. *Journal of Biological Chemistry*, 278:11032–11040, 2003.

[58] S. McMahon, M. Charbonneau, S. Grandmont, D. E. Richard, and C. M. Dubois. Transforming Growth Factor 1 Induces Hypoxia-inducible Factor-1 Stabilization through Selective Inhibition of PHD2 Expression. *Journal of Biological Chemistry*, 281:24171–24181, 2006.

[59] E. N. McNamee, D. Korns Johnson, D. Homann, and E. T. Clambey. Hypoxia and hypoxia-inducible factors as regulators of T cell development, differentiation, and function. *Immunol Res*, 55(1-3):58–70, 2013.

[60] E. Metzen, D. P. Stiehl, K. Doege, J. H. Marxsen, T. Hellwig-Bürgel, and W. Jelkmann. Regulation of the prolyl hydroxylase domain protein 2 (phd2/egln-1) gene: identification of a functional hypoxia-responsive element. *Biochemical Journal*, 387:711–717, 2005.

[61] R. D. Michalek, V. A. Gerriets, S. R. Jacobs, A. N. Macintyre, N. J. MacIver, E. F. Mason, S. A. Sullivan, A. G. Nichols, and J. C. Rathmell. Cutting Edge: Distinct Glycolytic and Lipid Oxidative Metabolic Programs Are Essential for Effector and Regulatory CD4+ T Cell Subsets. *The Journal of Immunology*, 186:3299–3303, 2011.

[62] R. H. Michelini, A. L. Doedens, A. W. Goldrath, and S. M. Hedrick. Differentiation of CD8 memory T cells depends on Foxo1. *The Journal of Experimental Medicine*, 210:1189–1200, 2013.

[63] S. N. Mueller, T. Gebhardt, F. R. Carbone, and W. R. Heath. Memory T Cell Subsets, Migration Patterns, and Tissue Residence. *Annual Review of Immunology*, 31:137–161, 2013.

[64] H. Nakamura, Y. Makino, K. Okamoto, L. Poellinger, K. Ohnuma, C. Morimoto, and H. Tanaka. TCR Engagement Increases Hypoxia-Inducible Factor-1 Protein Synthesis via Rapamycin-Sensitive Pathway under Hypoxic Conditions in Human Peripheral T Cells. *The Journal of Immunology*, 174:7592–7599, 2005.

[65] V. Nizet and R. S. Johnson. Interdependence of hypoxic and innate immune responses. *Nature Reviews Immunology*, 9:609–617, 2009.

[66] K. J. Oestreich, K. A. Read, S. E. Gilbertson, K. P. Hough, P. W. McDonald, V. Krishnamoorthy, and A. S. Weinmann. Bcl-6 directly represses the gene program of the glycolysis pathway. *Nature Immunology*, 15:957–964, 2014.

[67] I. Okoye, L. Wang, K. Pallmer, K. Richter, T. Ichimura, R. Haas, J. Crouse, O. Choi, D. Heathcote, E. Lovo, C. Mauro, R. Abdi, A. Oxenius, S. Rutschmann, and P. G. Ashton-Rickardt. The protein LEM promotes CD8+ T cell immunity through effects on mitochondrial respiration. *Science*, 348:995–1001, 2015.

[68] J. A. Olson, C. McDonald-Hyman, S. C. Jameson, and S. E. Hamilton. Effector-like CD8+ T Cells in the Memory Population Mediate Potent Protective Immunity. *Immunity*, 38:1250–1260, 2013.

[69] D. O'Sullivan, G. J. W. van der Windt, S. C.-C. Huang, J. D. Curtis, C.-H. Chang, M. D. Buck, J. Qiu, A. M. Smith, W. Y. Lam, L. M. DiPlato, F.-F. Hsu, M. J. Birnbaum, E. J. Pearce, and E. L. Pearce. Memory CD8+ T Cells Use Cell-Intrinsic Lipolysis to Support the Metabolic Programming Necessary for Development. *Immunity*, 41:75–88, 2014.

[70] M. Pagani, G. Rossetti, I. Panzeri, P. de Candia, R. J. P. Bonnal, R. L. Rossi, J. Geginat, and S. Abrignani. Role of microRNAs and long-non-coding RNAs in CD4+ T-cell differentiation. *Immunological Reviews*, 253:82–96, 2013.

[71] A. Palazon, A. W. Goldrath, V. Nizet, and R. S. Johnson. HIF Transcription Factors, Inflammation, and Immunity. *Immunity*, 41:518–528, 2014.

[72] A. Palazón, I. Martínez-Forero, A. Teijeira, A. Morales-Kastresana, C. Alfaro, M. F. Sanmamed, J. L. Perez-Gracia, I. Peñuelas, S. Hervás-Stubbs, A. Rouzaut, M. O. d. Landázuri, M. Jure-Kunkel, J. Aragonés, and I. Melero. The HIF-1 Hypoxia Response in Tumor-Infiltrating T Lymphocytes Induces Functional CD137 (4-1BB) for Immunotherapy. *Cancer Discovery*, 2:608–623, 2012.

[73] K. C. Pang, M. E. Dinger, T. R. Mercer, L. Malquori, S. M. Grimmond, W. Chen, and J. S. Mattick. Genome-Wide Identification of Long Noncoding RNAs in CD8+ T Cells. *The Journal of Immunology*, 182:7738–7748, 2009.

[74] S. Paul and B. C. Schaefer. A new look at TCR signaling to NF-B. *Trends in immunology*, 34:269–281, 2013.

[75] E. L. Pearce, M. C. Poffenberger, C.-H. Chang, and R. G. Jones. Fueling Immunity: Insights into Metabolism and Lymphocyte Function. *Science*, 342:1242454–1242454, 2013.

[76] E. L. Pearce, M. C. Walsh, P. J. Cejas, G. M. Harms, H. Shen, L.-S. Wang, R. G. Jones, and Y. Choi. Enhancing CD8 T-cell memory by modulating fatty acid metabolism. *Nature*, 460:103–107, 2009.

[77] N. Pescador, Y. Cuevas, S. Naranjo, M. Alcaide, D. Villar, M. O. Landázuri, and L. del Peso. Identification of a functional hypoxia-responsive element that regulates the expression of the egl nine homologue 3 (egln3/phd3) gene. *Biochemical Journal*, 390:189–197, 2005.

[78] C. Peyssonnaux, V. Datta, T. Cramer, A. Doedens, E. A. Theodorakis, R. L. Gallo, N. Hurtado-Ziola, V. Nizet, and R. S. Johnson. HIF-1 expression regulates the bactericidal capacity of phagocytes. *Journal of Clinical Investigation*, 115:1806–1815, 2005.

[79] A. T. Phan and A. W. Goldrath. Hypoxia-inducible factors regulate T cell metabolism and function. *Mol Immunol*, 68(2 Pt C):527–35, 2015.

[80] H. Pircher, K. Burki, R. Lang, H. Hengartner, and R. M. Zinkernagel. Tolerance induction in double specific T-cell receptor transgenic mice varies with antigen. *Nature*, 342(6249):559–561, 11 1989.

[81] K. N. Pollizzi and J. D. Powell. Integrating canonical and metabolic signalling programmes in the regulation of T cell responses. *Nature reviews. Immunology*, 14:435–446, 2014.

[82] R. R. Rao, Q. Li, K. Odunsi, and P. A. Shrikant. The mTOR Kinase Determines Effector versus Memory CD8+ T Cell Fate by Regulating the Expression of Transcription Factors T-bet and Eomesodermin. *Immunity*, 32:67–78, 2010.

[83] J. Rolf, M. Zarrouk, D. K. Finlay, M. Foretz, B. Viollet, and D. A. Cantrell. AMPK1: A glucose sensor that controls CD8 T-cell memory. *European Journal of Immunology*, 43:889–896, 2013.

[84] H. E. Ryan, M. Poloni, W. McNulty, D. Elson, M. Gassmann, J. M. Arbeit, and R. S. Johnson. Hypoxia-inducible Factor-1 Is a Positive Factor in Solid Tumor Growth. *Cancer Research*, 60:4010–4015, 2000.

[85] R. S. Schwartz, H. K. Eltzschig, and P. Carmeliet. Hypoxia and Inflammation. *New England Journal of Medicine*, 364:656–665, 2011.

[86] M. A. Selak, S. M. Armour, E. D. MacKenzie, H. Boulahbel, D. G. Watson, K. D. Mansfield, Y. Pan, M. C. Simon, C. B. Thompson, and E. Gottlieb. Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF- prolyl hydroxylase. *Cancer Cell*, 7:77–85, 2005.

[87] G. L. Semenza. Oxygen Sensing, Hypoxia-Inducible Factors, and Disease Pathophysiology. *Annual Review of Pathology: Mechanisms of Disease*, 9:47–71, 2014.

[88] L. A. Sena, S. Li, A. Jairaman, M. Prakriya, T. Ezponda, D. A. Hildeman, C.-R. Wang, P. T. Schumacker, J. D. Licht, H. Perlman, P. J. Bryce, and N. S. Chandel. Mitochondria Are Required for Antigen-Specific T Cell Activation through Reactive Oxygen Species Signaling. *Immunity*, 38:225–236, 2013.

[89] L. Z. Shi, R. Wang, G. Huang, P. Vogel, G. Neale, D. R. Green, and H. Chi. HIF1-dependent glycolytic pathway orchestrates a metabolic checkpoint for the differentiation of TH17 and Treg cells. *Journal of Experimental Medicine*, 208:1367–1376, 2011.

[90] S. Shrestha, K. Yang, J. Wei, P. W. F. Karmaus, G. Neale, and H. Chi. Tsc1 promotes the differentiation of memory CD8+ T cells via orchestrating the transcriptional and metabolic programs. *Proceedings of the National Academy of Sciences*, 111:14858–14863, 2014.

[91] M. Sukumar, J. Liu, Y. Ji, M. Subramanian, J. G. Crompton, Z. Yu, R. Roychoudhuri, D. C. Palmer, P. Muranski, E. D. Karoly, R. P. Mohney, C. A. Klebanoff, A. Lal, T. Finkel, N. P. Restifo, and L. Gattinoni. Inhibiting glycolytic metabolism enhances CD8+ T cell memory and antitumor function. *The Journal of Clinical Investigation*, 123:4479–4488, 2013.

[92] N. Takeda, E. L. O'Dea, A. Doedens, J.-w. Kim, A. Weidemann, C. Stockmann, M. Asagiri, M. C. Simon, A. Hoffmann, and R. S. Johnson. Differential activation and antagonistic function of HIF- isoforms in macrophages are essential for NO homeostasis. *Genes & Development*, 24:491–501, 2010.

[93] C. T. Taylor. Mitochondria and cellular oxygen sensing in the HIF pathway. *Biochemical Journal*, 409:19, 2008.

[94] G. J. van der Windt, B. Everts, C.-H. Chang, J. D. Curtis, T. C. Freitas, E. Amiel, E. J. Pearce, and E. L. Pearce. Mitochondrial Respiratory Capacity Is a Critical Regulator of $CD8^+$ T Cell Memory Development. *Immunity*, 36:68–78, 2012.

[95] G. J. W. van der Windt, D. O'Sullivan, B. Everts, S. C.-C. Huang, M. D. Buck, J. D. Curtis, C.-H. Chang, A. M. Smith, T. Ai, B. Faubert, R. G. Jones, E. J. Pearce, and E. L. Pearce. CD8 memory T cells have a bioenergetic advantage that underlies their rapid recall ability. *Proceedings of the National Academy of Sciences*, 110:14336–14341, 2013.

[96] V. Vezys, P. Penaloza-MacMaster, D. L. Barber, S.-J. Ha, B. Konieczny, G. J. Freeman, R. S. Mittler, and R. Ahmed. 4-1BB Signaling Synergizes with Programmed Death Ligand 1 Blockade To Augment CD8 T Cell Responses during Chronic Viral Infection. *The Journal of Immunology*, 187:1634–1642, 2011.

[97] H. Wang, H. Flach, M. Onizawa, L. Wei, M. T. McManus, and A. Weiss. Negative regulation of Hif1a expression and TH17 differentiation by the hypoxia-regulated microRNA miR-210. *Nature Immunology*, 15:393–401, 2014.

[98] R. Wang, C. P. Dillon, L. Z. Shi, S. Milasta, R. Carter, D. Finkelstein, L. L. McCormick, P. Fitzgerald, H. Chi, J. Munger, and D. R. Green. The transcription factor Myc controls metabolic reprogramming upon T lymphocyte activation. *Immunity*, 35:871–882, 2011.

[99] T. Wang, C. Marquardt, and J. Foker. Aerobic glycolysis during lymphocyte proliferation. *Nature*, 261:702–705, 1976.

[100] N. Werth, C. Beerlage, C. Rosenberger, A. S. Yazdi, M. Edelmann, A. Amr, W. Bernhardt, C. von Eiff, K. Becker, A. Schäfer, A. Peschel, and V. A. J. Kempf. Activation of Hypoxia Inducible Factor 1 Is a General Phenomenon in Infections with Human Pathogens. *PLoS ONE*, 5:e11576, 2010.

[101] F. Yang, H. Zhang, Y. Mei, and M. Wu. Reciprocal Regulation of HIF-1 and LincRNA-p21 Modulates the Warburg Effect. *Molecular Cell*, 53:88–100, 2014.

[102] Y. Yin, S.-C. Choi, Z. Xu, D. J. Perry, H. Seay, B. P. Croker, E. S. Sobel, T. M. Brusko, and L. Morel. Normalization of CD4+ T cell metabolism reverses lupus. *Science Translational Medicine*, 7:274ra18–274ra18, 2015.

[103] D. J. Zhang, Q. Wang, J. Wei, G. Baimukanova, F. Buchholz, A. F. Stewart, X. Mao, and N. Killeen. Selective Expression of the Cre Recombinase in Late-Stage Thymocytes Using the Distal Promoter of the Lck Gene. *The Journal of Immunology*, 174:6725–6731, 2005.