Suffocated CD8 T Cells in the Hypoxic Tumor Microenvironment

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<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>[x]</td>
<td>Concentration of substance &quot;x&quot;</td>
</tr>
<tr>
<td>2-ME</td>
<td>Beta-mercaptoethanol</td>
</tr>
<tr>
<td>aCD28</td>
<td>anti-CD28</td>
</tr>
<tr>
<td>aCD3</td>
<td>anti-CD3</td>
</tr>
<tr>
<td>ACT</td>
<td>Adoptive cell transfer</td>
</tr>
<tr>
<td>ADAM17</td>
<td>ADAM Metallopeptidase Domain 17</td>
</tr>
<tr>
<td>AICD</td>
<td>Activation-induced cell death</td>
</tr>
<tr>
<td>aKG</td>
<td>alpha-Ketoglutarate</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>APCs</td>
<td>Antigen presenting cells</td>
</tr>
<tr>
<td>APLs</td>
<td>Altered peptide ligands</td>
</tr>
<tr>
<td>aPD-1</td>
<td>anti-PD-1</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BFA</td>
<td>Brefeldin A</td>
</tr>
<tr>
<td>c-FLIP</td>
<td>FADD-like IL-1-beta-converting enzyme</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>CAFs</td>
<td>Cancer-associated fibroblasts</td>
</tr>
<tr>
<td>CAR-T cells</td>
<td>Chimeric antigen receptor T cells</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DCs</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>DN</td>
<td>Double negative</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FIH1</td>
<td>Factor inhibiting hypoxia-inducible factor 1 alpha</td>
</tr>
<tr>
<td>GMP</td>
<td>Guanosine monophosphate</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia-inducible factor</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule-1</td>
</tr>
<tr>
<td>IDO</td>
<td>Indoleamine-pyrrole 2,3-dioxygenase</td>
</tr>
<tr>
<td>IFN-g</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol trisphosphate</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Jak</td>
<td>Janus kinases</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MDSCs</td>
<td>Myeloid-derived suppressor cells</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MMP</td>
<td>Mitochondria membrane potential</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T-cells</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-Kappa-B</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>O₂</td>
<td>Molecular oxygen</td>
</tr>
<tr>
<td>OXPHOS</td>
<td>Oxidative phosphorylation</td>
</tr>
<tr>
<td>PHD proteins</td>
<td>Prolyl hydroxylase domain (PHD) proteins</td>
</tr>
<tr>
<td>PIP₃</td>
<td>Phosphatidylinositol (3,4,5)-trisphosphate</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>pMHC</td>
<td>Peptide-MHC complex</td>
</tr>
<tr>
<td>PPP</td>
<td>Pentose phosphate pathway</td>
</tr>
<tr>
<td>pVHL</td>
<td>Von Hippel–Lindau protein</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>SP</td>
<td>Single positive</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription proteins</td>
</tr>
<tr>
<td>TAA</td>
<td>Tumor-associated antigen</td>
</tr>
<tr>
<td>TAD</td>
<td>Transactivation domain</td>
</tr>
<tr>
<td>TAMs</td>
<td>Tumor-associated macrophages</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid cycle</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Tumor growth factor-beta</td>
</tr>
<tr>
<td>TILs</td>
<td>Tumor-infiltrating lymphocytes</td>
</tr>
<tr>
<td>TME</td>
<td>Tumor microenvironment</td>
</tr>
<tr>
<td>TN</td>
<td>Triple negative</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>Tregs</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>UDP</td>
<td>Uridine diphosphate</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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</tbody>
</table>
CHAPTER I:
REVIEW OF LITERATURE

Introduction

CD8 T cells are of particular importance in killing tumor cells and controlling tumor growth\textsuperscript{1–5}. Over the past two decades, scientists have taken advantage of the capacity of cytotoxic T cells to recognize and kill altered cells to generate anti-tumor T cell-based immunotherapies. These therapies have proven effective in many patients, yet a significant number fail to develop clinical responses against cancer for unknown reasons.

Many tumor-associated factors are known to inhibit effector T cell function; however, one crucial aspect of the tumor microenvironment that remains understudied in the context of the immune response is hypoxia in solid tumors\textsuperscript{6}. Unlike acute infections, during which transient hypoxia is generated mainly due to high cell density in an inflammatory environment\textsuperscript{7}, tumor-induced angiogenesis leads to pathological hypoxia\textsuperscript{8}. Poor organization of the tumor vasculature and leaky blood vessels give rise to oxygen-deprived regions in the tumor, consisting of higher O\textsubscript{2} concentration in the peripheral area that decreases toward the tumor core. While this hypoxic environment promotes cancer cell survival and proliferation, tumor-infiltrating T lymphocytes (TILs) may not be able to function properly in such conditions\textsuperscript{9}. In fact, T cell infiltration in most tumors is restricted to the periphery, suggesting a potential role of hypoxia on the depth of penetration.

Elucidation of inhibitory mechanisms and identification of key intervention points will provide therapeutic targets for the development of competent anti-tumor CD8 T cells. This study
focuses on determining the effects of tumor-associated hypoxia on CD8 T cells and the resulting limitations to anti-tumor T cell response.

**CD8 T Cell Maturation and Activation**

**Differentiation of T cells in the Thymus**

T cell differentiation initiates when fetal liver or bone marrow-derived precursor cells migrate to the thymus, where they undergo a series of changes\(^\text{10,11}\). The T cell receptor (TCR), the main characteristic that defines T cells, is a heterodimer constituted by either an \(\alpha\) and a \(\beta\) chain (\(\alpha\beta\) TCR) or a \(\gamma\) and a \(\delta\) chain (\(\gamma\delta\) TCR). The \(\alpha\beta\) TCR binds to a ligand (antigen) in the context of the major histocompatibility complex (MHC)\(^\text{12}\). While most T cells express \(\alpha\beta\) TCRs (65-70\%)\(^\text{13}\), up to 5\% of the total T cell population express a non-MHC restricted \(\gamma\delta\) TCR\(^\text{13}\). Similar to B cell antigen receptors, the set of genes that encode the TCR rearrange and give rise to a high diversity of TCRs in a healthy individual\(^\text{14}\), which is vital for an effective immune system. The rearrangement of these genes, known as V(D)J recombination, involves the recombination of the exons encoding the antigen-binding portions of the receptor. These are variability (V), diversity (D) and joining (J) gene segments. During this process, in the case of TCR \(\beta\) and \(\delta\) chains, a D segment and a J segment are first ligated together; then, a V segment is joined to the rearranged DJ segments in a second step. TCR \(\alpha\) and \(\gamma\) chains only contain V and J segments, and the recombination process occurs in one step\(^\text{15,16}\).

T cells express a TCR in association with the CD3 complex, which contains \(\gamma\), \(\delta\), \(\epsilon\) and \(\zeta\) invariable chains\(^\text{17,18}\). This complex binds to the TCR and allows for the transduction of TCR-stimulatory signals\(^\text{19-21}\), as discussed below. In addition, \(\alpha\beta\) T cells express a co-receptor that enhances binding to the peptide-MHC (pMHC) complex; this can be either CD4 or CD8, and each defines a different subset of T cells\(^\text{22,23}\). CD4 T cells have a variety of functions and can be
further sub-categorized into several lineages, including the classical classification as helper T cells (mainly Th1 and Th2) and regulatory T cells (Tregs), among others\textsuperscript{24}. CD8 T cells are known as cytotoxic T lymphocytes (CTLs) due to their capacity to kill target cells\textsuperscript{22,25}. All CD4 and CD8 T cells express αβ TCRs. CD4 binds to MHC class II while CD8 binds to MHC class I, enhancing and stabilizing the TCR-MHC interaction\textsuperscript{26–29}.

While in the thymus, T cells undergo multiple developmental stages that can be tracked by surface expression of the TCR-CD3 complex, CD4 and CD8 in combination with the expression of other markers, including CD117 (c-kit), CD25 (IL-2Rα) and CD44\textsuperscript{26,30,31}. CD4-CD8- double negative (DN) cells can thus be further subdivided into subsets according to their developmental stage. Initially, these cells are triple negative (TN) and do not express CD3, CD4 or CD8, and have not undergone TCR gene arrangements\textsuperscript{31}. There are four populations of DN cells: DN1 cells do not express CD3 and are CD44+CD25-. Dβ-Jβ rearrangement determines the transition into DN2 cells, which are CD44+CD25+\textsuperscript{32,33}. DN3 cells are CD44-CD25+ and have undergone TCR Vβ-DJβ rearrangement. This population expresses a pre-TCR, containing the pre-Tα chain and a rearranged β chain, along with low levels of CD3\textsuperscript{34}. Progression beyond this stage is limited by the expression of a functional (signaling) TCR complex. Finally, DN3 cells give rise to DN4 cells, which are CD44-CD25-\textsuperscript{35}. Rearrangement of the TCR α chain takes place subsequently and a TCR is expressed along with CD3 and both coreceptors (CD4+CD8+) in double positive cells (DP)\textsuperscript{12,17,18,36,37}.

Successful rearrangement and pairing of the TCR chains allows for a process known as positive selection, during which the cells are exposed to self-antigens in the context of self-MHC class I or class II, triggering development of single positive (SP) cells (CD4+CD8- or CD4-CD8+)\textsuperscript{14}. This positive selection process is critical for the survival of the cells; those T cells
unable to respond to TCR stimulus do not receive the adequate survival signals and die of neglect or anergy. On the other hand, cells that express a TCR that interacts with high avidity against self-antigen are either eliminated by clonal deletion\textsuperscript{40,41} or are diverted into a suppressive function, giving rise to Tregs, a process called clonal diversion\textsuperscript{42,43}. These mechanisms that occur in the thymus before T cells mature and enter circulation are referred to as central tolerance\textsuperscript{44} and allow for the production of self-tolerant yet effective T cell clones able to recognize antigens in the context of self-MHC\textsuperscript{39}. In addition, other mechanisms leading to anergy and deletion of self-reactive T cell clones can also take place in the periphery (peripheral tolerance)\textsuperscript{44}. Therefore, while T cells play an important role in the response against tumors, self-reactive T cells typically express a TCR with low affinity against ligand\textsuperscript{45–48}.

**Activation of Naïve T Cells**

Upon maturation, naïve T cells, defined as CD3\textsuperscript{hi}CD27+CD45RA+CD62L+CD69– in humans\textsuperscript{49} and as CD44\textsuperscript{lo}CD62L\textsuperscript{hi} in mice\textsuperscript{50}, leave the thymus and enter circulation. During the final stage of maturation, T cells also upregulate sphingosine-1-phosphate (S1P) receptor S1P\textsubscript{1}, which plays an important role in T cell migration. Compared to the concentration in tissues, S1P levels are higher in the lymph and the blood, creating a gradient that attracts T cells out of the thymus\textsuperscript{51,52}. Secondary lymphoid organs also have a high density of S1P, inducing T cell entrance to lymph nodes. Lymph nodes collect interstitial fluid from soft tissues, to which T cells are exposed. Lymphocytes enter the lymph node through the afferent vessel or through the high endothelial vessels, located in the paracortex. Naïve T cells upregulate CCR7, a receptor for chemokines CCL19 and CCL21, which directs them to the T cell zone within the paracortical region of the lymph node, where they become activated\textsuperscript{53–56}. The engagement of this receptor mediates T cell migration through the lymph node; cells lacking this receptor move more
quickly, while naïve T cells spend more time surveying the lymph node for antigen\textsuperscript{57,58}.

Eventually, lymphocytes travel through the medulla and exit through the efferent vessel\textsuperscript{59}.

As a T cell travels through peripheral lymphoid tissues, specialized antigen presenting cells (APCs), such as dendritic cells (DCs), may present an antigen to the T cell\textsuperscript{60}. Encounter with antigen initiates the T cell activation process, which consists of three essential steps: signal 1: TCR engagement; signal 2: co-stimulatory signals (such as CD28); and signal 3: inflammatory cytokines\textsuperscript{61}.

In addition to antigen presentation in the context of an MHC molecule (signal 1), APCs also provide T cells with further signals that induce full activation. These include ligands to T cell co-stimulatory receptors CD28, ICOS and CD40L\textsuperscript{62} (signal 2). These signals stimulate the autocrine production of interleukin-2 (IL-2) and other cytokines (signal 3), which further promote T cell activation and proliferation\textsuperscript{60}. T cells that only receive signals 1 and 2 become anergic and do not progress unless they receive signal 3, which allows the cells to undergo extensive proliferation and differentiation into effector T cells\textsuperscript{63}.

In addition to the stimulatory signals provided by APCs, helper CD4 T cells also contribute to the differentiation of naïve CD8 T cells into effector cells. CD40L-expressing CD4 T cells interact with both DCs and CD8 T cells positive for CD40, resulting in activation and enhanced antigen presenting function of APCs and activation of CD8 T cells\textsuperscript{64–67}. Activated DCs then produce IL-12 and IL-15, cytokines key in CD8 T cell activation\textsuperscript{68,69}, and upregulate 4-1BBL and B7, the agonistic ligands for 4-1BB and CD28, respectively\textsuperscript{70,71}. CD4 T cells also produce cytokines that are essential for the activation of CD8 T cells, including IL-2\textsuperscript{72} and IFN-\gamma\textsuperscript{73,74}. 
In order to control a rapidly proliferating pathogen during an infection, activated T cells must undergo clonal expansion to generate a pool of effector cells in a short period of time. These T cell clones recognize the same antigen and travel out of the priming site upon activation\textsuperscript{39,75,76}. Effector CTLs are equipped to clear the infection by killing target cells and producing pro-inflammatory cytokines, which further induce their own activation and proliferation and that of other immune cells\textsuperscript{77–79}. After the antigen has been cleared, effector T cells undergo clonal deletion and only a few antigen-reactive T cells remain. These are memory cells, which confer protection from recurring infections\textsuperscript{77,79}. In addition to the role of CD4 T cells in CD8 T cell priming, CD4 T cell help is required for formation of long-lasting CD8 memory T cells\textsuperscript{80}.

**T Cell Receptor Signaling Pathway**

Interaction of the TCR with the peptide-MHC (pMHC) complex, along with the appropriate co-stimulation, initiates a signaling cascade involving kinases that propagate signals inducing T cell activation and proliferation\textsuperscript{81}. Such interactions are determined by the affinity and the avidity. Binding affinity is defined as the strength of the interaction between a single TCR and a single pMHC epitope. It is inversely proportional to the dissociation equilibrium constant $K_D$: the lower the $K_D$ value, the greater the binding affinity of the receptor for its ligand\textsuperscript{82,83}. Structural avidity is determined by the binding strength of a cluster of TCRs to multiple pMHCs as well as co-receptors that aid this interaction\textsuperscript{84,85}. Functional avidity is defined by the biological response of a T cell to a given antigen at a specific concentration based on the quantification of effector functions, such as proliferation, cytokine production and cytotoxic activity\textsuperscript{84,86}.
Upon TCR engagement, activation of the Src kinases Lck and Fyn leads to phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) found on CD3 subunits. The ITAMs are defined by the motif YXXL/I-X6-8-YXXL/I (where X denotes any amino acid). In addition to enhancing the binding of the TCR to the pMHC complex, the co-receptor (CD8/CD4) highly contributes to triggering TCR signaling by recruiting Lck, which binds to the cytoplasmic tail of the co-receptor. Protein kinase ZAP-70 is recruited and activated upon phosphorylation by Lck. Activated Lck, Fyn, and ZAP-70 then phosphorylate multiple targets, including linker for activation of T cells (LAT). Recruitment of adaptor proteins to LAT eventually leads to downstream changes critical for T cell function, such as calcium (Ca\(^{2+}\)) flux, mitogen-activated protein kinase (MAPK, also known as Erk) activation, co-receptor upregulation and stimulation of cytokine release.

Conformation of the LAT signalosome induces subsequent activation of phospholipase C (PLC), leading to hydrolysis of phosphatidylinositol 4,5-bis phosphate (PIP\(_2\)) into diacylglycerol (DAG) and inositol-3-phosphate (IP\(_3\)). IP\(_3\) then induces Ca\(^{2+}\) signaling by acting on IP\(_3\) receptors in the ER and the plasma membrane. Ca\(^{2+}\) influx into the cytoplasm activates the Ca\(^{2+}\)-dependent calmodulin–calcineurin pathway, which induces the nuclear translocation of transcription factor nuclear factor of activated T-cells (NFAT). NFAT in turn induces gene transcription, resulting in T cell activation, proliferation and differentiation. The DAG signaling branch induces protein kinase C \(\theta\) (PKC-\(\theta\)), an important activator of transcription factor NF-\(\kappa\)B, and RasGRPs, which are critical activators of the MAPK pathway. Activation of c-Jun, downstream of both the Ca\(^{2+}\) pathway and PKC-\(\theta\), and c-Fos, by the MAPK pathway, results in the conformation activation of dimeric transcription factor AP-1.
Fig. 1. TCR Signaling Pathway. Activation of the TCR leads to a signaling cascade that induces T cell activation, survival and proliferation. Upon TCR engagement with an antigen in the context of the major histocompatibility complex (MHC) on the antigen presenting cell (APC), immunoreceptor tyrosine-based activation motifs (ITAMs) on the cytosolic tail of the CD3 complex are phosphorylated by the kinase Lck, brought near the complex by the TCR coreceptor (CD8 in this example). ZAP70 is then recruited to the TCR-CD3 complex and phosphorylated. Activation of ZAP70 induces a number of effects, including LAT activation. LAT acts as an adaptor protein, forming a signalosome that induces the activation of several pathways. Among these, Grb2 (Ras pathway activator), PI3K (which activates PDK1 and PKC-θ) and phospholipase C (PLC)-γ become activated. PLC-γ hydrolyzes PIP2 into second messengers DAG and IP3. While IP3 induces the Ca2+ pathway and subsequent activation of NFAT, DAG activates PKC-θ and the mitogen-activated protein kinase (MAPK) pathway, both of which lead to NF-kB activation. Both Ca2+ and PKC-θ result in the activation of c-Jun, while ERK induces c-Fos activation. Together, c-Jun and c-Fos (not depicted) compose the transcription factor AP-1. Engagement of CD28 with agonist B7.1/2 ligands further induces T cell activation by binding to PI3K and Grb2, leading to PDK1 and Ras activation, respectively.

Activation of the MAPK pathway is essential during T cell differentiation. Sustained ERK activation is required for CD69, IL-2 and IFN-γ expression, targets of AP-1. CD69 is an
early activation marker upregulated within hours after TCR activation, before even CD25 (IL-2Ra) expression (~1-2 days)\textsuperscript{100}. CD69 expression has been associated with T cell differentiation, tissue egress or retention and, more recently, metabolic regulation\textsuperscript{101}.

While TCR activation provides an initiating signal at the onset of T cell activation, in the absence of additional co-stimulation T cells become anergic and dysfunctional. Thus, co-stimulatory requirement allows for a mechanism for peripheral tolerance\textsuperscript{102}. CD28 is a co-stimulatory receptor that contains a YXXM signaling motif (specifically YMNM) and greatly enhances T cell activation in response to the target antigen. The extracellular domain of CD28 binds to B7 proteins, and is activated by phosphorylation of the YMNM motif in the cytoplasmic tail. This leads to interaction with the p85 subunit of PI3K and Grb2 in a competitive manner (Fig. 1). Eventually, the PI3K signaling pathway induces NFAT and NF-κB activation\textsuperscript{103}. Downstream effects of Grb2 binding lead to AP-1, NFAT and NF-κB activation. Thus, PI3K and Grb2 signaling pathways result in the regulation of cell cycle progression, apoptosis, cellular metabolism, and IL-2 transcription\textsuperscript{103,104}.

**Cytokine Production by Effector CD8 T Cells**

Cytokine production is a major function of effector T cells that is determined by their subset, differentiation state and quality of stimulation. In particular, CD8 T cells are known to produce tumor necrosis factor-alpha (TNF-α), interferon-γ (IFN-γ) and IL-2 when activated\textsuperscript{105,106}. Cytokines and chemokines attract and activate other immune cells, but may also have a deleterious effect on target cells\textsuperscript{107}.

One of the initial cytokines produced by CD8 T cells is IFN-γ, known for its important autocrine and paracrine effects. Autocrine production of IFN-γ induces CTL differentiation and activation by increasing T-bet upregulation and granzyme B production\textsuperscript{108}. Its presence is
necessary for optimal priming of T cells\textsuperscript{109–111}. CD8 T cell migration to site of injury and cytotoxic function\textsuperscript{112–115}. In addition, binding of IFN-γ to IFNGR induces upregulation of MHC I expression, improving TCR engagement and subsequent killing, and it directly induces cell death by ROS accumulation in target cells\textsuperscript{116,117}. IFN-γ has also been shown to induce cell cycle arrest, apoptosis and necroptosis in cancer cells\textsuperscript{117}. Therefore, IFN-γ is a key factor in the effector CD8 T cell response.

While TNF-α has important anti-inflammatory functions\textsuperscript{118,119}, I will focus here on its pro-inflammatory role regarding the effector functions of CD8 T cells. Two receptors have been described to recognize TNF-α: TNFR1, expressed in most cell types, and TNFR2, expressed mainly in immune cells\textsuperscript{118}. TNF-α promotes differentiation and survival of naïve CD8 T cells\textsuperscript{120}, both by acting directly on the cell and by boosting CD28-mediated production of IL-2\textsuperscript{121}. In effector CD8 T cells, TNF-α induces the production of serine protease granzyme B and pore-forming protein perforin, which act together to induce cell death in target cells. TNF-α is also necessary for the induction of FasL expression on CTLs; FasL mediates cell death by binding to Fas receptor on target cells. Thus, TNF-α is critical for T cell-mediated cytotoxicity\textsuperscript{118}.

As previously mentioned, T cell activation and differentiation into effector cells is highly dependent on CD28 signaling, which in turn drives IL-2 expression and production. Autocrine IL-2 production induces proliferation and survival during early activation of T cells. In fact, strong IL-2 production is mainly limited to naïve T cells and central memory T cells\textsuperscript{63,122,123}. Effector T cells only release low levels of this cytokine upon antigen recognition due to an IL-2-dependent negative feedback loop established after T cell activation\textsuperscript{63}.

Polyfunctionality, defined as the capacity of T cells to simultaneously produce multiple pro-inflammatory molecules, such as IFN-γ/TNF-α/IL-2, is a hallmark of protective
Different levels of TCR engagement and signaling are required to elicit the production of specific cytokines in effector T cells\textsuperscript{125,126}. Production of IFN-\(\gamma\) usually requires low TCR stimulation, closely followed by TNF-\(\alpha\)\textsuperscript{125}. However, increased TCR stimulation is necessary to elicit IL-2 production, only occurring in cells already producing IFN-\(\gamma\)\textsuperscript{126}. Therefore, IL-2-producing effector CD8 T cells often simultaneously produce IFN-\(\gamma\) and TNF-\(\alpha\) at larger amounts\textsuperscript{122} and are associated with increased cytotoxic activity\textsuperscript{124}.

**Antigen Clearance, Chronic Infections and T Cell Exhaustion**

During a primary response, once the levels of antigen have decreased, the majority of expanded effector T cell clones undergo apoptosis\textsuperscript{60}. This protects against further damage to the tissue induced by continuous release of inflammatory molecules\textsuperscript{127}. Apoptosis of effector T cells in this contraction phase is triggered by TCR signaling and T cell activation, known as activation-induced cell death or AICD. Fas-FasL interaction plays a central role in AICD, although other mechanisms have been described to also induce AICD\textsuperscript{128,129}, such as TNF-\(\alpha\) signaling\textsuperscript{118} and pro-apoptotic protein Bim activation\textsuperscript{130}. A small population of antigen-specific T cells remains, which subsequently develop into persisting memory T cells\textsuperscript{60,131}. These cells are functionally different from effector T cells in that they have a lower threshold of activation and respond more rapidly to antigen recognition. This population can therefore efficiently respond to and clear a recurring infection (secondary response)\textsuperscript{60}.

During chronic infection, memory T cell differentiation is altered by constant exposure to cognate antigen\textsuperscript{131}. In this context, the immune response fails and T cells become exhausted. Exhaustion is defined as a state of unresponsiveness in which activated T cells sequentially lose their effector functions and become suppressed\textsuperscript{132}. This phenotype is characterized by impaired cytokine production\textsuperscript{133}, impaired proliferation accompanied by the inability to become
quiescent\textsuperscript{134,135}, upregulation and sustained expression of co-inhibitory checkpoint receptors (such as PD-1\textsuperscript{136}, CTLA-4\textsuperscript{137}, LAG-3\textsuperscript{138,139} and TIM-3\textsuperscript{140}), and altered metabolism resulting in poor glucose use and mitochondrial dysfunction\textsuperscript{141}. Eventually, exhausted T cells undergo apoptosis, resulting in the elimination of the antigen-reactive T cell population before clearance of the target\textsuperscript{131}.

In autoimmune diseases driven by over-reactive T cells, targeting co-stimulatory molecules to manipulate T cell activity can help to slow down disease progress\textsuperscript{142}. Similarly, anti-tumor therapies can take advantage of co-inhibitory receptors expressed by activated T cells\textsuperscript{143}. Expression of both CTLA-4 and PD-1 is induced by activating signals in the T cells\textsuperscript{136,144} and is necessary for T cell tolerance maintenance\textsuperscript{145,146}. In particular, PD-1 is a critical regulator of T cell exhaustion\textsuperscript{132,146,147}. Its ligand, PD-L1 is often expressed by suppressive cells, such as Tregs, and tumor cells\textsuperscript{148–150}. PD-1/PD-L1 signaling strongly inhibits effector CD8 T cells in chronic infections and in the tumor, causing T cell dysfunction and inability to clear the antigen\textsuperscript{143,146}. Blocking the expression of either PD-1 or PD-L1 as well as CTLA-4 has been exploited as a strategy of anti-cancer immunotherapies\textsuperscript{151,152}.

**Effector T Cell Metabolism**

Energy status, nutrient availability and metabolism are associated with cell division. Cells must be in ideal conditions to enter cell cycle and proliferate. Resting T cells, such as memory and naïve T cells, use oxidative phosphorylation (OXPHOS) as the major source of ATP, a catabolic metabolism\textsuperscript{153}. On the other hand, while T cells undergo activation and expansion, they switch to glycolysis, an anabolic form of metabolism and a rapid source of ATP\textsuperscript{154}. This phenomenon occurs even when oxygen availability is not compromised (aerobic glycolysis) and despite the reduced ATP production efficiency of glycolysis, also known as the Warburg
A similar effect is observed in cancer cells and is pronounced in hypoxia: low oxygen tension forces the cell to use glycolysis as the main source of energy, promoted by hypoxia inducible factor (HIF) stabilization and transcriptional activity\textsuperscript{153,156}. In T cells, TCR signaling also activates HIF, which contributes to the metabolic switch observed in effector T cells\textsuperscript{157}.

Mitochondrial OXPHOS, or cellular respiration, is the main source of energy in eukaryotic cells. In this process, transfer of electrons between four enzymes occurs in a series of redox reactions, the electron transport chain (ETC). The transfer of electrons generates a proton gradient across the mitochondrial inner membrane that can then be used to drive the production of ATP\textsuperscript{158}. In brief, complex I, an NADH dehydrogenase and proton pump, transfers two electrons from NADH to ubiquinone, resulting in the transport of four protons out of the mitochondria. In an independent event, complex II oxidizes succinate and also transfers the electrons to ubiquinone. Cytochrome c oxidoreductase, or Complex III, is a proton pump that oxidizes ubiquinone, transferring electrons to cytochrome c and driving the translocation of four more protons across the membrane\textsuperscript{159}. The final step of the ETC is mediated by complex IV, cytochrome c oxidase. This enzyme accepts electrons from cytochrome c and transfers them to the final electron acceptor, an O\textsubscript{2} molecule that is reduced to water (H\textsubscript{2}O)\textsuperscript{160}. Finally, the proton gradient resulting from the ETC is utilized for ATP synthesis by a fifth OXPHOS complex, the F\textsubscript{0}/F\textsubscript{1} ATP synthase\textsuperscript{159} (Fig. 2).
Fig. 2. Oxidative Phosphorylation and the Electron Transport Chain. ATP synthesis by oxidative phosphorylation (OXPHOS) takes place in the mitochondria. For this process, glycolysis and the tricarboxylic acid (TCA) cycle contribute reduced cofactors NADH and FADH$_2$, which are used in the electron transport chain (ETC) as electron and proton donors. Electrons are transported through a series of carriers (complexes I-IV) to the final electron acceptor, molecular oxygen (O$_2$). O$_2$ accepts 2 electrons (e$^-$) and is combined with 2 protons (H$^+$) to produce water (H$_2$O). Protons are transferred out of the mitochondria by complexes I, III and IV, creating an electrochemical gradient across the mitochondrial inner membrane that makes the mitochondrial lumen negatively charged in relation to the cytosol. This gradient provides the driving force for the F$_0$F$_1$ ATP synthase, which synthesizes ATP from ADP.

Pyruvate, catabolized from glucose, may be converted into acetyl-CoA and enter the tricarboxylic acid (TCA) cycle, which generates the NADH necessary for ATP production by OXPHOS$^{161}$. During glycolysis, pyruvate is instead converted to lactate (Fig. 3). Lactate production regenerates the NAD$^+$ needed for the early steps of glycolysis, propagating the glycolytic cycle. While this pathway generates a lower yield of ATP in the cytoplasm than OXPHOS, it does so at a faster rate, fueling the rapid growth and proliferation of effector T cells. Moreover, it has been shown that glycolysis is necessary for cytokine production in effector T
cells. Additionally, glucose converted into glucose-6-phosphate enters the pentose phosphate pathway (PPP), critical for the biosynthesis of aromatic amino acids (Trp, Tyr and Phe), reduced NADPH and ribose-5-phosphate for the biosynthesis of nucleotides.

**Fig. 3. Glycolysis Pathway.** Glucose is degraded in the cytosol in a series of enzymatic steps that result in the production of pyruvate and its conversion to lactate. Pyruvate can also enter the mitochondria to be used in the tricarboxylic acid (TCA) cycle. Intermediates of the glycolytic pathway may be used as precursors in other metabolic pathways, such as glucose-6-phosphate (glucose-6-P), which can enter the pentose phosphate pathway (PPP). In addition, glycolysis generates NADH, which can be used as a cofactor for OXPHOS or in the degradation of pyruvate into lactate, generating NAD⁺. Fructose-6-P: fructose-6-phosphate; Fructose-1,6-biP: fructose-1,6-bisphosphate; GA3P: glyceraldehyde-3-phosphate; 1,3-BPG: 1,3-bisphosphoglycerate; 3PG: 3-phosphoglycerate; 2PG: 2-phosphoglycerate; PEP: phosphoenolpyruvate.
The TCA cycle is of particular importance for the production of macromolecules, as it provides the necessary intermediates that feed into other pathways. A higher biomass translates into faster cell division and growth. Glutamine is an important source of nucleotide precursors and nonessential amino acids\textsuperscript{165,166}, critical for T cell function and able to be incorporated into the TCA cycle as α-ketoglutarate (α-KG), driving the generation of macromolecules. Alternatively, glutamine may be consumed to produce pyruvate and then lactate, similar to glucose. Similar to PPP, this pathway also produces the NADPH required for lipid synthesis as well as glutathione production for redox homeostasis. Unlike glycolysis, however, this glutaminolysis can only result in ATP production by entering the TCA cycle and inducing OXPHOS\textsuperscript{154,167}.

Nucleotides, the activated precursors of nucleic acids, are necessary for replication of the genome. Thus, rapidly proliferating cells require an ample supply of nucleotides. Nucleotides are also involved in several essential processes separate from cell division: ATP and GTP participate as energy currencies in many biological processes, cyclic nucleotides (such as AMP and GMP) act as second messengers in signal transduction pathways, ATP hydrolysis is used for phosphorylation of molecules, and derivatives (such as UDP-glucose) are involved in metabolic functions\textsuperscript{168}. Most proliferating cells synthesize nucleotides and nucleic acids \textit{de novo}, from glucose, glutamine and CO\textsubscript{2}. Aspartate, derived mainly from glutamine, is a precursor of pyrimidine biosynthesis, while glycine and serine are used in the production of purines. ATP derived from glycolysis is needed for both pyrimidine and purine synthesis. Therefore, cell division does not only require nutrient acquisition, but is also energetically costly\textsuperscript{169}.
Mitochondrial Function in Effector T Cells

The mitochondrion plays an essential role in cell metabolism. Several biochemical processes occur in this organelle, the most relevant being cellular respiration and the TCA cycle\textsuperscript{170}. Metabolites and ATP generated in the mitochondria affect not only other metabolic pathways, but also the energetic fitness and function of the cell. In CD8 T cells, there is also a tight link between activation state of the cell and mitochondrial function\textsuperscript{167,171}.

Although effector T cells maintain OXPHOS after activation, cellular metabolism transitions mostly to glycolysis\textsuperscript{167,171}. As a result, they have reduced mitochondrial content in contrast to naïve or memory T cells, which rely on oxidative metabolism. Mitochondrial remodeling also occurs, characterized by expansion of cristae (folds of the mitochondrial inner membrane) as opposed to the tight morphology observed in OXPHOS-based metabolism, which provides an increase in surface area to accommodate the ETC\textsuperscript{167,172}. Mitochondrial function is also supported by active fusion and fission of mitochondria. Fusion involves the merging of both membranes of two or more mitochondria, further enhancing the ETC and OXPHOS\textsuperscript{173}. Elongated, hyperfused mitochondria are thus associated with higher ATP production. When activated T cells shift towards glycolytic metabolism, mitochondrial fission is favored, resulting in multiple fragmented mitochondria that make ETC less efficient. This seems to be a direct effect of TCR signaling\textsuperscript{161}. Therefore, mitochondrial fusion and fission regulate T cell metabolic function.

Mitochondria play an important role in Ca\textsuperscript{2+} signal regulation upon TCR activation. Free Ca\textsuperscript{2+} transport to the mitochondrial matrix is enabled by the proton gradient generated by the ETC. TCR-induced Ca\textsuperscript{2+} influx in the mitochondria induces ROS production, likely by increasing mitochondrial membrane potential (ΔΨm). ΔΨm is determined by the proton gradient
generated by complexes I, III and IV of the ETC and the electrical potential as a consequence of the charge separation across the inner membrane\textsuperscript{174}. Thus, $\Delta \Psi m$ is often used as a readout of mitochondrial response to T cell activation.

It has also been suggested that sustained Ca\textsuperscript{2+} levels after TCR engagement lead to nitric oxide (NO) production, which can compete with O\textsubscript{2} as a complex IV substrate. Alternatively, NO reacts with superoxide, giving rise to the radical peroxynitrite (ONOO\textsuperscript{–}), which inhibits several ETC enzymes. These two outcomes are associated with ATP depletion and mitochondrial hyperpolarization\textsuperscript{175}.

Mitochondrial reactive oxygen species (ROS) are critical for T cell activation and cytokine production. ROS are used as secondary messengers in signaling pathways, including TCR signaling. In fact, the looser conformation of mitochondrial cristae in effector T cells makes the ETC less efficient, possibly causing electrons to linger in the complexes and induce ROS production\textsuperscript{161}. While this might result in increased ROS-associated damage, it also provides signals necessary for T cell activation\textsuperscript{161,176}, including induction of NFAT and IL-2 production\textsuperscript{176,177}.

**Oxygen Distribution and Sensing**

Environmental cues determine the cellular metabolic and functional state. These include nutrients, cytokines, chemokines and other internal and external signals. Similarly, cells sense environmental O\textsubscript{2} levels and modulate their metabolic functions accordingly. O\textsubscript{2} is essential for several biochemical pathways in multicellular organisms\textsuperscript{178}. As the most significant O\textsubscript{2} consumers in the cell, mitochondria act as O\textsubscript{2} sensors and likely induce changes in cell function and metabolism by reducing OXPHOS and generating ROS. In addition, other systems that
allow the cell to sense and adjust to different O$_2$ levels play an important role in cell metabolism, function and fate, as will be discussed in this section.

O$_2$ is distributed by the blood from the lungs to the rest of the body through arteries, arterioles, metarterioles, and capillaries. Blood vessels are leaky, especially at the level of the capillaries$^{179,180}$. Capillaries spread through tissues in numerous branches. They are the smallest and most permeable blood vessels, with gaps between endothelial cells large enough to allow the passage of macromolecules and diffused gases (such as O$_2$ and CO$_2$), although leukocytes can actively migrate out of the blood vessel (extravasation) and travel through the interstitial matrix upon stimulation by environmental cues$^{181,182}$. Low O$_2$ blood is carried from the capillaries to the venules and veins back to the heart and the lungs. The fluid not recovered into the blood is collected by lymphatic vessels, which connect to a network of lymph nodes that refeeds into the blood. The lymph, constituted by plasma leaked from blood as well as interstitial fluid and immune cells (especially lymphocytes)$^{181,183}$, enters the lymph node through an afferent vessel and exits through an efferent vessel. In the node, the lymph is scavenged by cells of the immune system, serving as a filter for pathogens and foreign material$^{184,185}$. Since lymphatic vessels are very permeable at the tissue level, the interstitial fluid is equilibrated with the lymph, which presents similar concentrations of solutes and gases as those found in the tissue$^{186}$. However, once the interstitial fluid is collected after the gas and nutrient exchange has occurred, the concentration of nutrients and O$_2$ in the lymph is expected to be lower than that of oxygenated blood and the tissue$^{187}$. Therefore, immune cells may be subjected to a wide range of O$_2$ concentrations ([O$_2$]), as described below.

The highest [O$_2$] in the human body is found in the lungs, measured at about 14% O$_2$ in the alveoli, and it can reach levels as low as 1% in the skin$^{188}$. The lung is a unique anatomical
site with direct contact with the outside of the body and can therefore be a significant point of entrance of foreign matter and pathogens. In addition, it constitutes a common metastatic site due to the ease of access to the rest of body through the capillary network that serves the lung. The most prominent immune populations in the lung are macrophages, DCs and Tregs. While DCs and effector T cells play an important role in the control of viral infections and cancer, macrophages and Tregs establish immunological tolerance in the lung and can favor tumor colonization. As will be discussed later, high [O₂] plays a role in the regulation of anti-tumor response by T cells in the lung.

O₂ levels in lymphoid organs range between 1–4.5%. In the bone marrow, a highly vascularized tissue, [O₂] has been recorded at 1.5%. Mouse thymus O₂ levels have been reported to be under 1.3%, while the spleen can range from 3 to 4% O₂. Circulating T cells may be recruited from the well-oxygenated circulatory system to areas with lower O₂ tension; these may either be healthy tissues, such as lymphoid organs, or inflamed areas of injury or infection, referred to as sites of pathological hypoxia. Thus, the term “hypoxia” is inherently relative and is best defined as the level of O₂ at which normal cell functions are compromised.

The biological effects of hypoxia have been mostly studied in cancer cells in the context of solid tumors, which are often hypoxic. Despite enhanced angiogenesis by cancer cells, O₂ concentrations in solid tumors vary depending on cancer type between 0.3 and 4.2%, although most show a median [O₂] below 2%. Exposure to low levels of O₂ induces the expression of genes that allow cancer cells to survive in hypoxia, including vascular endothelial growth factor (VEGF) to induce angiogenesis and glycolysis-associated genes to support anaerobic glycolysis, among many others. Release of the VEGF family of growth factors also has an effect in lymphangiogenesis, which allows for increased lymph flow in the tumor but also
promotes tumor metastasis\textsuperscript{201–203}. Hypoxia also induces the expression of molecules that aid in the adhesion of tumor cells to endothelial cells, further enhancing tumor cell migration and metastasis\textsuperscript{204,205}.

Metabolic reprogramming in response to low [O\(_2\)] is mainly regulated by HIFs. These factors form heterodimers comprised of subunits \(\alpha\) and \(\beta\). HIF\(\beta\) is stably expressed, while the \(\alpha\) subunit is constantly degraded at high \([O_2]\). There are three isoforms of HIF\(\alpha\), of which HIF1\(\alpha\) is ubiquitously expressed and the best characterized. HIF\(\alpha\) is hydroxylated by O\(_2\)-dependent prolyl hydroxylase domain (PHD) proteins, members of the Fe(II) and 2-oxoglutarate-dependent oxygenase superfamily\textsuperscript{206,207}. Hydroxylated HIF\(\alpha\) is then recognized by E3 ubiquitin ligase von Hippel-Lindau protein (pVHL) and tagged for proteasomal degradation\textsuperscript{200,208}. Factor inhibiting HIF-1 (FIH1) is also a Fe(II)- and 2-oxoglutarate-dependent dioxygenase and its function depends on O\(_2\) availability in a similar way to PHD proteins\textsuperscript{207,209,210}. HIF\(\alpha\) contains two transcriptional activation domains (TAD): N-terminal or NTAD and C-terminal or CTAD\textsuperscript{211}. FIH1 protein regulates HIF\(\alpha\) function by hydroxylating an asparagine residue in the CTAD when O\(_2\) is available, suppressing HIF\(\alpha\) transcriptional activity by preventing binding of transcriptional coactivators\textsuperscript{210}. Since FIH1 remains active at lower O\(_2\) levels than PHD proteins\textsuperscript{209}, it may repress the function of HIF\(\alpha\) proteins that do not get degraded via proteasome. However, in vitro studies show that the optimal [O\(_2\)] for enzymatic activity of both PHDs and FIH1 function is much higher than the predicted intracellular [O\(_2\)]\textsuperscript{212–214}. Therefore, HIF\(\alpha\) hydroxylation acts as an O\(_2\) sensor, regulating gene expression directly in response to [O\(_2\)] changes\textsuperscript{215}.

HIF expression is stabilized in cells at \(\sim 5\%\) O\(_2\) or lower\textsuperscript{216}. PHD proteins use O\(_2\) as a substrate for hydroxylation, and therefore their activity is regulated by O\(_2\) availability. However, in HIF upregulation has been observed at higher O\(_2\) levels\textsuperscript{217}. PHD protein activity is regulated
by mitochondrial ROS: although the exact mechanism is yet to be determined, ROS generation by complex III of the ETC inhibits PHD proteins, resulting in HIFα stabilization\textsuperscript{217,218}. Therefore, HIF activity is not always associated with low [O$_2$].

While PHD proteins and HIFα are of particular interest in the hypoxic TME, their activities are also consequential in well-oxygenated areas. As mentioned, Tregs suppress effector T cell function in the lung. Here, PHD proteins regulate the relative proportion of Tregs and effector T cells: deletion of these O$_2$-dependent proteins in all T cell subsets results in enhanced anti-tumor effector T cell response due to a reduction of the Treg population, presumably via the increased stabilization of HIFα. In other words, the high oxygenation of the lung induces PHD protein activity in T cells, which preserves the Treg population in this tissue and in turn promote tumor invasion\textsuperscript{194}. Thus, different T cell subsets seem to have different responses to O$_2$ levels.

**T Cells in the Tumor Microenvironment**

Cancers originate from cells with acquired somatic mutations that result in aberrant cell division and behavior\textsuperscript{219}. Besides uncontrolled proliferation, tumor-specific mutations may render cancer cells either more visible to the immune system or allow them to escape it. While higher infiltration of cytotoxic CD8 T cells in the tumor is associated with positive prognosis\textsuperscript{220}, effective T cell response against tumors depends on multiple factors. Most tumor-reactive T cells express self-reactive TCRs with low affinity for their ligands and while high-affinity TCRs are often associated with off-target toxicities\textsuperscript{86}, low affinity TCRs may lead to poor anti-tumor responses\textsuperscript{83}. Certain mutations can lead to the increased expression of tumor-associated antigens (TAAs) from unmodified proteins; TAAs are self-antigens found at higher levels in cancer cells but expressed at low levels in healthy tissues or in sites with immune privilege (such as reproductive tissues)\textsuperscript{221–223}. However, immunogenicity of TAAs may be low, resulting in weak
anti-tumor T cell responses. Cancer mutations that result in the expression of aberrant proteins may give rise to new antigens exclusively expressed in the tumor, thus, these tumor neoantigens constitute an excellent target for tumor-reactive T cells. Unfortunately, neoantigen-reactive T cells are rare and predicted neoantigens in a given tumor do not always correspond with those actually expressed. Moreover, limitations such as low binding affinity of the antigen to the MHC or whether the antigen triggers a sufficient T cell response also hinder the use of neoantigens for immunotherapy.

While tumor antigen expression and recognition of the tumor by effector CTLs play a key role, effective T cell response against tumor depends on multiple factors. The tumor microenvironment (TME) is comprised of both cellular and non-cellular components that may contribute to tumor development. In solid tumors, these include cancer-associated immune and stromal cells, cytokines, chemokines and other factors released by cells or generated by the extracellular matrix. One aspect of the TME that has been understudied in the context of the immune response is hypoxia, characteristic of solid tumors. Tumor vasculature is structurally abnormal, with larger gaps between endothelial cells and higher branching of vessels. Moreover, solid tumors usually have very little to no lymphatic vessels. This poor organization of the tumor vasculature creates an oxygen gradient: higher O\textsubscript{2} concentrations are found in the perivascular areas, decreasing in areas of the tumor more distal of the blood vessel. Consequently, limited O\textsubscript{2} diffusion through the tumor generates hypoxic areas in which cancer cells are able to survive and proliferate while tumor-infiltrating T lymphocytes (TILs) may not be able to function properly. Tumor-associated hypoxia induces resistance to radiotherapy and chemotherapy, generates necrotic areas in the core of the tumor, and allows tumor cells to escape...
immune surveillance by controlling gene expression$^{227}$. Hypoxic tumors are therefore considered of poor prognosis and are often associated with metastasis$^{231}$.

Immune suppressive cells can play a critical role in T cell tolerance, but in the tumor this suppression results in the inhibition of CTL anti-tumor responses. Tumor associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs) produce suppressive factors that contribute to tumor persistence and inhibit CTL anti-tumor activity$^{127,232}$. Interestingly, hypoxia has been demonstrated to polarize these cells, favoring suppressive phenotypes$^{233}$. Cancer associated fibroblasts (CAFs) constitute a significant proportion of the tumor stroma and often secrete factors that favor tumor progression. Hypoxia reprograms CAFs to a more oncogenically-favorable state, mainly by inducing the production of cytokines such as TGF-β$^{180}$.

TGF-β, IL-6 and IL-10, among many others, are immunoinhibitory factors commonly found in the suppressive tumor$^{234}$. TGF-β inhibits effector CD8 T cell activation and function while it promotes Treg and MDSC recruitment and tumor growth$^{235,236}$. In cancer patients, intra-tumoral and systemic levels of TGF-β are usually high, generating an immune-suppressed environment$^{236}$. TGF-β blockade results in increased CD8 T cell tumor infiltration and killing$^{235,237}$. IL-10, produced by various types of immune cells, drives tumor progression and inhibits anti-tumor T cell response by directly suppressing APCs and T cells$^{238}$. This is further supported by the improved T cell function and tumor control observed with combined blockade of IL-10 and PD-1 treatment$^{234}$. Hypoxia promotes the production of suppressive cytokines in a variety of cells, including tumor cells, Tregs, TAMs and MDSCs$^{239–243}$.

Metabolic competition in the TME can also act as an immune checkpoint. MDSCs express high levels of arginase I, reducing the availability of arginine in the TME, which is
necessary for proper effector T cell function. Another enzyme that is overexpressed by suppressive cells in the TME is IDO (indoleamine-2,3-dioxygenase), which converts tryptophan into the immunosuppressive metabolite kynurenine. Glycolysis not only facilitates rapid tumor proliferation, it also results in high production of lactate, lowering the pH in the extracellular space and suppressing T cells.

Immune checkpoint receptors are upregulated by a variety of stimuli, including TCR activation. PD-1 and TIM-3 are checkpoint receptors that have been tightly associated with T cell exhaustion. PD-1, in particular, prevents TCR signaling. Upregulation of immune checkpoint ligands by tumor cells, such as PD-L1, constitutes another immune escape strategy. Hypoxia likely exacerbates this effect, as it has been demonstrated that upregulation of PD-L1 is induced in low O2 via HIFα on tumor cells, MDSCs, tumor-associated macrophages (TAMs) and DCs. In fact, PD-L1 blockade in hypoxia abrogates T cell suppression by MDSCs. Hypoxia also drives the differentiation of MDSCs into suppressive TAMs. Expression of inhibitory receptors CTLA-4 and OX40 on T cells is induced by HIFα, which is in turn stabilized in hypoxia. Thus, chronic exposure to hypoxia likely induces T cell exhaustion in the TME.

T cells are drawn to the tumor by cytokines and chemokines released at the tumor site. While expression of these receptors allows T cells to enter the tumor, it can prevent their exit. As in chronic infections, constant exposure to antigen renders TILs exhausted.

**B16 Melanoma Model**

The mouse melanoma B16 cell line was spontaneously generated in a B6 mouse. For decades, it has been widely used in the study of cancer, particularly in T cell anti-tumor response. Although B16 exhibits a large number of somatic mutations, similar to human
melanomas, only a small subset of these have been found to elicit an immune response, making it a poorly immunogenic tumor\textsuperscript{256}. It also presents immune cell-infiltration patterns similar to those of human melanomas\textsuperscript{256,257}. These patterns can be categorized in three immunotypes: having few immune cells (immunotype A), higher immune cell infiltration associated with intra-tumoral vasculature (immunotype B) and diffuse immune cell infiltration independent of the vasculature (immunotype C)\textsuperscript{258}.

Most melanoma tumors, including B16, are categorized as Immunotype B, with a high percentage of the infiltrating immune cells being T cells\textsuperscript{257,258}. While survival has been correlated with high CD8 T cell tumor infiltration, this is not the case for CD4 T cells. In fact, a significant proportion of CD4 TILs are Tregs and their presence is associated with poor prognosis in multiple cancer types, including melanoma\textsuperscript{258–261}. Moreover, B16 is characterized by its high secretion of TGF-β\textsuperscript{262,263} which supports the infiltration of Tregs, as explained above.

Tumor size correlates with intra-tumoral vessel size, with larger tumors associated with larger blood vessels\textsuperscript{264}. However, regardless of tumor stage (early, intermediate or established, large tumor), B16 has been found to constantly express angiogenic factors, such as VEGF, and HIFα, which also regulates vascular morphology and generation\textsuperscript{265,266}, throughout its progression\textsuperscript{264}. Interestingly, expression of TGF-β in B16 has been found at higher levels in the tumor vasculature compared to the tumor as a whole and to vessels irrigating healthy tissues, indicating an acquired suppressive phenotype of endothelial cells in the TME\textsuperscript{264}.

**T Cell-Based Cancer Immunotherapies**

T cell recognition and killing of target cells has been exploited for the development of anti-tumor therapies. These strategies have the main goal of boosting cytotoxic T cell activity by genetically manipulating target cell recognition, increasing the activating signals or removing
inhibitory signals that prevent normal effector cell function. The discovery of IL-2 as a T cell growth factor was a breakthrough in the field of immunology\textsuperscript{267}. Therefore, it is not surprising that one of the first drugs to be approved for cancer immunotherapy was IL-2, which was initially used to boost the immune system of metastatic patients\textsuperscript{268,269}. Since then, multiple approaches have been developed that can be divided into three categories: adoptive T cell therapies, immune checkpoint inhibitors and tumor vaccines\textsuperscript{270}. Here, I will briefly describe the first two.

Adoptive cell transfer (ACT) of tumor infiltrating lymphocytes (TILs) after ex vivo expansion allows for the isolation of tumor-specific T cells that can later be re-infused at larger numbers. However, the process of isolation of functionally effective, tumor-reactive cells is not always successful\textsuperscript{271}. ACT of TCR-engineered cells addresses this problem. This therapy consists of the introduction of a tumor-reactive TCR sequence in T cells in vitro for later re-infusion\textsuperscript{270,271}. While this therapy has demonstrated good results to some extent, certain limitations remain. Mainly, MHC (HLA) restriction limits the use of donor cells for different patients, while the effectiveness of anti-tumor response varies in tumor types and patients\textsuperscript{270,272}. The use of chimeric antigen receptor-modified T (CAR-T) cells has thus emerged as an alternative to TCR-engineered T cells. These cells are transduced with a synthetic receptor, typically composed of the antibody variable heavy (VH) and variable light (VL) chains, which are connected by a linker to form a single-chain variable fragment (scFv)\textsuperscript{273,274}. The scFv is specific against extracellular antigens, making the interaction between effector and target cells MHC-independent\textsuperscript{270,274}. The intracellular domain typically contains an activation domain, such as CD3-derived ITAMs, and one or more co-stimulatory domains, usually derived from CD28 or 4-1BB, although the variety and number of domains differs\textsuperscript{154}. Though treatment of blood
cancers with CAR-T cells has been very successful, this therapy remains ineffective in solid tumors for unknown reasons\textsuperscript{273}.

Therapies targeting co-inhibitory signals, such as checkpoint receptor inhibitors, have increased in popularity due to their durable results in responding patients\textsuperscript{249}. PD-1 and PD-L1 blockade might probably the most used checkpoint therapy in the last few years, proven to revert T cell exhaustion in TILs and improving T cell response against tumor as consequence. While the applicability of this therapy to wide variety of cancers makes it very attractive, it has been successful only in a subset of patients\textsuperscript{249} (estimated at 13\% of US patients by 2019)\textsuperscript{275}.

Overall, while the rapid progress of cancer immunotherapy has produced promising results, further research is required to fully elucidate the underlying factors causing the failure of T cell-based immunotherapies against solid tumors.

**Effects of Hypoxia on T Cells**

Early studies on the effects of O\textsubscript{2} levels on T cells focused on the comparison between atmospheric concentrations and physiologically-relevant O\textsubscript{2} tensions. Caldwell et al. (2001) compared the cytolytic function of mouse effector T cells at either 21\% or 2\% O\textsubscript{2}. This study found that while there were no differences in cytolytic function between the two conditions, T cells were less proliferative at 2\% O\textsubscript{2}\textsuperscript{198}. This was verified in human T cells, which showed a lower proliferation potential when activated at 5 or 10\% O\textsubscript{2} compared to 21\% O\textsubscript{2}\textsuperscript{276}. It was suggested that increased oxidative metabolism at atmospheric O\textsubscript{2} supports faster proliferation compared to T cells cultured at lower O\textsubscript{2}, which exhibit enhanced glycolysis\textsuperscript{277}. Another group proposed that reduced T cell proliferation upon activation at 1\% O\textsubscript{2} may be due to reduced expression of Kv1.3 K\textsuperscript{+} channels involved in Ca\textsuperscript{2+} signaling\textsuperscript{278}. 
While the reduction of T cell proliferative capacity at low O\textsubscript{2} is accepted\textsuperscript{198,276,278–280}, there is disagreement on the effects of hypoxia on T cell function. While one study showed increased apoptosis upon TCR activation at 2% O\textsubscript{2} compared to atmospheric conditions\textsuperscript{158}, a different report suggested instead that increased HIF expression prevents AICD at low O\textsubscript{2} (0-5\%)\textsuperscript{160} in response to TCR activation.

Cytokine production, a critical aspect of T cell function, has been shown to be altered in hypoxia (1% O\textsubscript{2} or lower). A study on mouse effector CD8 T cells\textsuperscript{281} demonstrated reduced pro-inflammatory cytokine production yet increased expression of suppressive cytokine IL-10. In contrast, oxygen levels seem to affect human T cell subtypes differently depending on the O\textsubscript{2} concentration: naïve and central memory T cells did not survive when cultured at low O\textsubscript{2} levels, while effector memory T cells have enhanced proliferation and lytic function in hypoxia\textsuperscript{282}. These discrepancies were attributed to the glycolytic phenotype observed in effector T cells.

In vivo, T cell activation levels were demonstrated to be higher in mouse cells localized to higher oxygenated regions\textsuperscript{283}. However, Xu et al. (2016) showed increased expression of proliferation marker Ki-67 in neuroblastoma-specific CAR-T cells that penetrated hypoxic areas of human tumor xenografts compared to cells located to non-hypoxic areas of the tumor\textsuperscript{284}.

Overall, research indicates that the effects of O\textsubscript{2} concentration on T cell proliferation and function may depend on culture conditions, the stimulus used for activation, and the T cell subtype (as well as species). This lack of consensus in the literature demonstrates the requirement to further characterize the effects of hypoxia on T cell function.
CHAPTER II:
MATERIALS AND METHODS

Culture Media and Reagents

Unless otherwise noted, cells were cultured in complete media supplemented with 10% FBS (Seradigm), 2 mM L-glutamine (Corning 25005CI) and 10 IU/mL penicillin and 10 µg/mL streptomycin (Corning 30001CI). RPMI 1640 with L-Glutamine (Corning 10040CV) and DMEM [+] 4.5 g/L glucose, L-glutamine, sodium pyruvate (Corning 10040CV) were used as indicated. For glucose uptake assays, glucose-free RPMI 1640 with L-Glutamine (Gibco 11879020) was used. Enzymatic dissociation of adherent cells was performed with trypsin at 0.05% with 0.53mM EDTA in HBSS w/o calcium and magnesium (Corning 25051CI). Buffers used: phosphate-buffered saline (PBS) (Dulbecco's Phosphate-Buffered Salt Solution; Corning 21031CV); Hanks' Balanced Salt Solution (HBSS) w/o calcium and magnesium (Corning 21021CV).

Mice

Animal work was done in accordance with the Institutional Animal Care and Use Committee of Loyola University Chicago. All mice were housed in specific pathogen free conditions prior to use. C57/BL6 and OT-I mice (expressing a TCR for OVA257-264 in the context of H2Kb) were obtained from The Jackson Laboratory. All measures were taken to minimize pain and suffering. Female and male mice of 6–12 weeks of age were used, with individual experiments using mice of a single sex.
B16 Melanoma Tumor Implantation and Processing

B16 melanoma cell line was obtained from Dr. Mary Jo Turk (Dartmouth). Cryopreserved B16 cells derived from low passage numbers (3-4) were thawed and cultured in complete DMEM in 150cm² flasks at 300,000 cells per flask for three days before tumor implantation. Cells were used when cultures reached about 60% confluency. Cells were then trypsinized and washed twice with PBS before re-suspending in the appropriate volume of PBS for a final concentration of 2 million cells/mL. Cells were kept on ice until injection into mice. Mice were shaved in the right flank in anticipation of tumor implantation. 100 µL (200,000 cells) of tumor cell suspension were intradermally injected per mouse.

When tumors reached 10 mm in diameter, mice were injected retro-orbitally with pimonidazole (Hypoxyprobe, HP6-100Kit) at 120 mg/kg in 100 µL. One mouse per group was injected with only PBS as negative control. After 90 to 120 min, mice were euthanized and tumor, thymus and spleen tissues were extracted and kept on ice. Spleens and thymi were dissociated and passed through a strainer to generate single cell suspensions. Tumors were first dissociated in a gentleMACS Dissociator (Miltenyi Biotec 130-093-235) and then digested by incubation in a solution of Liberase TM (Roche 05401127001) and DNase I (Roche 4536282001) for 30 min at 37 °C. Digestions reaction was neutralized by addition of FBS at a final concentration of 1%. Cells were then washed by centrifugation and re-suspended in PBS and filtered by passing suspensions though a strainer. Cell suspensions were then used for flow cytometry staining.

Culture of Effector T Cells

Spleens from were harvested and a single-cell suspension was prepared in complete RPMI. For each experiment, at least three different samples (individual spleens from separate
mice) were used. B6 splenocytes were cultured in 24-well plates with 0.1 µg/mL anti-mouse CD3 (aCD3) (Bio X Cell, clone 2C11), 0.1 µg/mL anti-mouse CD28 (BioLegend), 10 IU/mL rhIL-2 (NCI) and 50 ng/mL recombinant murine IL-15 (PeproTech) for 5 days, splitting wells as necessary by adding RPMI with IL-2 and IL-15 at the same concentrations. During the first 5 days, cells were cultured in a conventional incubator kept at 37 °C with atmospheric O₂ and 5% CO₂.

OT-I splenocytes were cultured with 0.1 µg/mL of peptide (New England Peptide), 10 IU/mL rhIL-2 and 50 ng/mL recombinant murine IL-15. Cells were stimulated with one of four different peptides: WT OVA peptide or N4, or altered peptide ligands (APLs) Q4, T4 or V4. APLs bind equally to H2Kᵇ, but differ in their binding to the OT-I TCR. Each peptide has a lower affinity than the previous one in the order listed, with V4 being 700-fold less potent than N4²⁸⁵.

1383I TCR-transduced human PBMCs²⁸⁶ were obtained from Dr. Michael Nishimura (LUC) following their laboratory’s SOP. Briefly, PBMCs from healthy donors were cultured for three days in AIM-V medium (Life Technologies, Carlsbad, CA) supplemented with 5% heat-inactivated human AB serum (hAB; Valley Biomedical, Inc., Winchester, VA), aCD3 (clone OKT3, Miltenyi Biotec), 300 IU/mL recombinant human IL-2 (rhIL-2; Novartis Pharmaceuticals Corporation, East Hanover, NJ) and 100 ng/mL recombinant human IL-15 (rhIL-15; NIH). Cells were transduced by spinoculation with supernatants prepared from a stable virus-producing PG13 cell line expressing 1383I TCR in the SAMEN vector. CD34 was used as transduction marker and to purify transduced cells. Cells were stored in liquid nitrogen by the Nishimura laboratory. After thawing in the laboratory, cells were rested in complete RPMI with 300 IU/mL rhIL-2 (NCI) and 50 ng/mL rhIL-15 (PeproTech) for 3 days. Cells were then counted and
separated for culture in normoxia (21%) or hypoxia (0.5%) at equal cell densities. Two days later, cells were plated at 1:1 ratio with T2 target cells loaded with cognate peptide (Tyro; New England Peptide) or in the presence of PMA/Ionomycin for >8 hours at the respective O$_2$ concentration. As negative controls, transduced effector T cells were also plated in the presence of T2 cells loaded with irrelevant peptide (MART-1) or no stimulus.

**Oxygen Levels of In Vitro T Cell Culture**

After priming, T cells were counted and plated at 500,000 cells/mL/well in 24-well plates either at atmospheric (21%), physioxic (5%) or hypoxic (0.5%) O$_2$ concentration and CO$_2$ at 5%.

In this document, I use the term “normoxia” in reference to culture conditions at atmospheric O$_2$ pressure in a conventional incubator, “physioxia” to refer to 5% O$_2$, corresponding to the [O$_2$] measured in healthy lymphoid organs in a physiological condition, and “hypoxia” for cultures at 0.5% O$_2$, representing a [O$_2$] lower than physioxia in the tissue.

Hypoxia was generated by culturing cells either in a hypoxia glove box (Coy Laboratory Products, Inc.) set at 0.5%, 5% CO$_2$ and 37 °C. To reach the correct [O$_2$], this machine flushes the culture workstation with N$_2$ gas. Alternatively, I used a modular incubator chamber (Billups-Rothenberg, Inc.; MIC-101). The latter was also used for culturing cells at 5% O$_2$. Desired [O$_2$] was achieved by flushing the chamber with a pre-made gas mix for 8 min at a flow rate of 20 L/min$^{287}$.

Media used for cultures at 5% or 0.5% O$_2$ were equilibrated to the corresponding concentration for at least 3 hours before use.

**In Vivo Activation of Lymph Node-Derived Effector T Cells**

B6 mice were injected with OVA peptide at 10 ug/mouse mixed at 1:1 ratio with adjuvant (TiterMax USA, Inc.; TiterMax Classic) in one hind paw. Five days later, mice were
euthanized and inguinal and popliteal lymph nodes were harvested and dissociated. Cells were counted and plated at the same number and concentration at 21%, 5% or 0.5% O₂. Two days later, effector cells were co-cultured with peptide-loaded target RMAS cells and supernatant was collected for IFN-γ ELISA.

**Re-stimulation of Effector T Cells**

After initial activation, cell cultures were maintained with IL-2 and IL-15 for 48 h in normoxia or hypoxia before re-stimulation. For pERK detection, cells were re-stimulated with beads (MACS) coated with 5 ug aCD3 for 30 to 120 min. To evaluate CD69 and IRF4 expression, cells were stimulated with plate-bound aCD3 at 0, 0.1 or 1 μg/mL for >4 h. Re-stimulation of cells for cytokine production was conducted overnight with plate-bound aCD3. Cytokine production was evaluated by incubating cells with protein ER-Golgi transport inhibitor brefeldin A (BFA) for subsequent intracellular cytokine staining (ICS) or without BFA for supernatant collection. Supernatants were stored at -20 °C until their use, avoiding repeated freeze-thaw cycles.

Tissue culture-treated 96-well plates were coated overnight at 4 °C with aCD3 at 5, 1, or 0.1 μg/mL diluted in PBS. As control, Armenian hamster IgG antibody (Bio X Cell BE0091) was used at the highest concentration of aCD3 used in the experiment. Plates were washed three times with PBS and cells were seeded for stimulation.

**T Cell Co-culture with Target Cells**

TAP-deficient T2 and RMAS cells were used as targets for human and mouse T cells, respectively, and were cultured in complete RPMI at atmospheric O₂ until time of assay.

For re-stimulation of antigen-specific mouse T cells, I used RMAS cells loaded with peptide. RMAS cells were counted and the number of cells needed was re-suspended in the
appropriate volume of medium for a final density of 10 million cells/mL in 15-mL conical tube. Peptide was loaded by adding the necessary amount of peptide to reach a concentration of 0.1 µg/mL (unless otherwise noted) and incubating cells at 37 °C for 2 hours protected from light, with occasional agitation by tube inversion. After incubation, cells were wash three times with PBS and re-suspended in RPMI for plating. In parallel, effector T cells were counted and the appropriate amount was collected and washed three times with PBS to remove cytokines from the medium. In all cases, target cells were incubated with effector cells at a 1:1 ratio. When a 24-well plate was used, I seeded 500,000 cells of each type per well. When a 96-well plate was used, I plated 100,000 cells of each type per well. As control, T cells were co-cultured with RMAS cells loaded with irrelevant peptide or no peptide. For ICS, BFA (2X) was added to the RMAS cell suspension before mixing with cells.

OT-I T cells were re-stimulated with the same peptide used for initial activation (N4, Q4, T4 or V4). In all cases an irrelevant peptide was used as control.

For antigen-specific human T cells, a similar procedure was performed. In this case, I loaded T2 cells with peptide at a cell density of 1 million/mL. Peptides were added at either 10 or 1 µg/mL final concentration.

**Stimulation with PMA and Ionomycin**

Effector T cells were re-stimulated in normoxia or hypoxia with PMA/Ionomycin in order to measure cytokine production. For this, PMA (Sigma-Aldrich P1585) was used at a final concentration of 20 ng/mL and Ionomycin (Sigma-Aldrich I0634) was used at 1.5 µM. A 2X solution of PMA/Ionomycin was added to the cell culture in a volume equal to the cell suspension. For ICS, BFA (2X) was added to the PMA/Ionomycin solution before mixing with cells.
T Cell Proliferation Curve

After 5 days of activation, I plated effector T cells at 500,000 cells/mL/well in 5 wells/spleen. For the following 5 days, one well/sample was collected each day. Cells were transferred to flow tubes, centrifuged and re-suspended in 1 mL PBS for subsequent counting by Trypan blue exclusion. Cells were centrifuged again and stained for Annexin V detection.

PD-1 Blockade of In Vitro-Generated Effector T Cells

After 48 h of separation into normoxia and hypoxia, T cells were incubated with anti-PD-1 antibody or IgG control for 2 hours before re-stimulation with aCD3. Alternatively, cells were blocked with anti-PD-1 for the duration of their exposure to hypoxia (48 h) before re-stimulation. Cells were then washed to remove supernatant and tested for cytokine production.

IFN-γ Enzyme-Linked Immunosorbent Assay (ELISA) and Multiplex Bead-Based Assay

Detection of IFN-γ in supernatants collected from activated T cells was evaluated by ELISA in specified experiments. For this, BioLegend ELISA kits for mouse (430801) or human (430101) IFN-γ were performed as per manufacturer instructions. Briefly, ELISA plates were coated overnight with capture antibody at the recommended concentration according to manufacturer’s protocol. The following day, wells were washed with PBS and Tween-20 at 0.05% (PBST) and blocked with either 5% FBS in PBS or 1% BSA in PBS for 1 h at RT.

Thawed supernatants were then added at a 1:100 dilution (in vitro-generated effector mouse T cells) or 1:10 dilution (in vivo-generated OVA-specific mouse effector T cells or 1383I TCR-transduced human T cells) and incubated for 2 h at RT. Plates were rewashed and biotinylated secondary antibody was added at recommended concentration. After washing, avidin-Horseradish Peroxidase (Av-HRP) was added and incubated for 30min at RT. Subsequently, wells were washed again and incubated with Tetramethylbenzidine (TMB), used as substrate.
Reaction was stopped after 20 min with TMB Stop Solution. Finally, absorbance was read in a microplate reader set to 450 nm.

For detection of an array of pro-inflammatory cytokines and chemokines, mouse T cell supernatants were tested by a multiplex bead-based assay at Eve Technologies (Mouse Cytokine Array / Chemokine Array 31-Plex; MD31). Factors studied in this array are listed in Table I.

**Table 1. Array of Mouse Pro-Inflammatory Cytokines and Chemokines**

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<td>IL-12 (p70)</td>
<td>MCP-1</td>
<td>VEGF</td>
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**Staining for Flow Cytometry Analysis**

**General Protocol**

Samples were collected in polystyrene round-bottom tubes or in 96-well plates and washed with PBS. Cell pellets were then re-suspended in surface antibody mix, prepared in autoMACS Running Buffer (Miltenyi Biotec 130-091-221). Samples were incubated with surface antibodies for 20 min at 4 °C in the dark and washed again with PBS. When possible or necessary, pellets were fixed with BioLegend Fixation Buffer (420801) and stored at 4 °C. For intracellular staining, following wash with PBS cell pellet was instead re-suspended in BD Cytofix/Cytoperm Fixation and Permeabilization Solution (BD Biosciences 554722) and incubated for 20 min at 4 °C. Cells were then washed with Intracellular Staining Permeabilization Wash Buffer (referred to as “Perm Wash buffer” from here on) (BioLegend 421002). Pellets were re-suspended with intracellular antibody mix (diluted in Perm Wash
buffer) and incubated for 1 h at RT. Samples were washed once more with Perm Wash buffer, fixed with Fixation buffer and stored at 4 °C. For detection of intranuclear or transcription factors (FOXP3, IRF4), eBioscience Fix/Perm FOXP3 kit (Cat. 00-5523-00) was used following similar steps according to manufacturer’s protocol.

Antibodies and dyes used are listed in Table 2 and Table 3, respectively. Data were acquired on BD LSR Fortessa (BD Bioscience) and CANTO II (BD Bioscience) flow cytometers using BD FACSDiva software (BD Bioscience). Results were analyzed with FlowJo software for Windows.

Table 2. Flow Cytometry Antibodies for Mouse Cells

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<th>Reference</th>
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Table 3. Dyes Used for Flow Cytometry Analysis.

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ERK Activation

For phospho-ERK (pERK) staining, cells were activated for the specified times and immediately fixed with Fixation buffer. Samples were incubated for 30 min at RT, transferred to 5-mL flow tubes and washed with PBS. Cells were then permeabilized with ice-cold methanol (90%) while agitating tubes on vortex and incubated at 4 °C for 30 min. Samples were then stored at -20 °C for later staining or washed right away with PBS. After wash, cells were stained with anti-pERK diluted in autoMACS buffer for 1 h at RT. After washing with PBS, cells were fixed with Fixation buffer and stored at 4 °C until analysis.

Apoptosis

For apoptosis analysis, staining of Annexin V was performed following surface antibody and viability dye (BioLegend Zombie Aqua) staining. Cell pellets were washed with PBS and then washed again with Annexin-V-Binding Buffer (BioLegend 422201). Anti-Annexin V antibody diluted in Annexin-V-Binding Buffer was added to samples, which were incubated for 15-min at RT. Samples were washed again with the same buffer and cell pellets were re-suspended in Fixation buffer.

Detection of ROS Content and ΔΨm

For detection of ROS, I used the indicator 2',7'-dichlorodihydrofluorescein diacetate (H$_2$DCFDA), which is oxidized in the presence of ROS and converted to the fluorescent form 2',7'-dichlorofluorescein (DCF)$^{288}$. H$_2$DCFDA was reconstituted in DMSO and stored at -20 °C.
at 1 M. Similarly, tetramethylrhodamine ethyl ester (TMRE), used to measure $\Delta \Psi_m$, was stored at -20 °C at a stock concentration of 1 mM. The accumulation of this dye in the mitochondrial matrix is inversely proportional to the $\Delta \Psi_m^{289}$. For labeling, cells cultured in normoxia or hypoxia were collected, centrifuged and re-suspended either in HBSS buffer with H$_2$DCFDA at a concentration of 1 μM or in a solution of TMRE in HBSS at 100 nM, separately. After a 30 min incubation at 37 °C, at atmospheric O$_2$, samples were washed twice with HBSS and re-suspended in HBSS for immediate analysis by flow cytometry.

**Cytosolic Ca$^{2+}$ Levels**

Cytosolic calcium levels were measured with Fluo-4 AM. For this, a solution of 25 mg of Pluronic F127 in 75 μL DMSO was prepared and incubated at 37 °C for at least one hour to dissolve. Cells cultured in either normoxia or hypoxia were washed and re-suspended at 10 million cells/mL in HBSS with 5% FBS. To this cell suspension, 20 μL/mL of freshly reconstituted Fluo-4 AM at 1.5 mM in DMSO as well as 5 μL/mL of Pluronic 127 solution were added. Cells were then incubated at RT on gentle rotation for 1 h at atmospheric O$_2$. After 3 washes with HBSS, cells were re-suspended in 5% FBS HBSS at 2 million cells/mL and analyzed by flow cytometry. Ca$^{2+}$ release from intracellular compartments was evaluated by adding ionomycin diluted in HBSS at a final concentration of 1.5 μM.

**Transduction Levels of Human T Cells**

Vector expression in transduced human T cells was analyzed by staining with PE-conjugated anti-human CD34 antibody (BioLegend 343606).

**Glucose Uptake Assay**

Cell cultures in 96-well plate were washed to remove medium. Pellets were then re-suspended with glucose-free RPMI supplemented with 5% FBS and antibiotics and incubated for
2 h. Fluorescent glucose analog 2-NBDG (Thermo Fisher Scientific N13195) was added at a final concentration of 100 µg/mL and cells were incubated for an additional 20 min. Cells were then collected and washed with PBS before analysis by flow cytometry.

**Transmission Electron Microscopy (TEM) Imaging**

After incubation for 48 h in hypoxia, cells were collected, washed with PBS and fixed by myself with a special fixative for TEM imaging. Samples were then processed by Dr. David Rademacher (LUC Imaging Facility). Images were taken by myself in a Philips CM120 120kV TEM. Mitochondrial size measurements were analyzed by myself with Image J by manually tracing the mitochondria. Only clearly discernible mitochondria were included in the analysis. Parameters used to compare mitochondrial morphology from cells cultured in normoxia and hypoxia were described by Picard et al. 2013: surface area or mitochondrial size (nm²); perimeter (nm); aspect ratio, calculated as [(major axis)/(minor axis)], reflects the “length-to-width ratio”; form factor (FF) [(perimeter²)/(4π·surface area)] reflects the complexity and branching aspect of mitochondria; circularity [4π·(surface area/perimeter²)] and roundness [4·(surface area)/(π·major axis²)] are two-dimensional indexes of sphericity (values of 1 indicate perfect spheroids).

**Mitochondrial DNA Quantification**

After 48 h of exposure to hypoxia, cells were collected and total DNA was purified using Qiagen DNeasy Blood & Tissue Kit (69504). DNA concentration was measured on Nanodrop Lite (Thermo Fisher Scientific) and adjusted to 3 ng/µL. For quantification of mitochondrial DNA (mtDNA), 5 ng of purified DNA were used per reaction in SYBR Green-based qPCR. Primers were used at final concentration of 0.4 µM. Primers targeting LPL: forward: GGA TGG ACG GTA AGA GTG ATT C; reverse: ATC CAA GGG TAG CAG ACA GGT (225 bp fragment).
To quantify mitochondrial DNA, I used ND1 (NADH dehydrogenase subunit 1) primers: forward: CCC ATT CGC GTT ATT CTT; reverse: AAG TTG ATC GTA ACG GAA GC (202 bp fragment). Amplification protocol was as follows: 1 cycle of 50 °C for 2 min, 95 °C for 10 in; 40 cycles of 95 °C for 15 sec, 60 °C for 1 min; 1 cycle of 95 °C for 15 sec, 60 °C for 1 min, 95 °C for 15 sec.

**RNA Sequencing**

Effector T cells from three different samples were pulled together after 48 h of exposure to normoxia or hypoxia. Cells were centrifuged and total RNA was isolated with miRvana miRNA isolation kit (Thermo Fisher Scientific AM1561). RNA was stored at -20 °C (for less than 1 week) until use. Samples were then sequenced (single-end 75 base pairs sequencing; output of 100 million reads per sample) on NextSeq (Illumina) and analyzed at Northwestern University Sequencing Core Facility. Differential expression of genes was determined using DESeq2. The cutoff for determining significantly differentially expressed genes was an FDR (fold discovery rate)-adjusted P value less than 0.05. The pathway analysis was done using Metascape. Gene Ontology (GO) analysis results were used to show differential expression of gene pathways significantly changed in hypoxic cells as compared to normoxic cells.

**Seahorse Analysis of Mitochondrial Stress**

Effector mouse T cells cultured at 21% or 0.5% O₂ were plated on poly-D-lysine-coated Seahorse culture plates at 200,000 cells/well in non-buffered RPMI (Agilent 103576-100) supplemented with 25 mM glucose and 2 mM glutamine. Cells were allowed to equilibrate to atmospheric air while kept at 37 °C, as per Seahorse protocol, for one hour and then analyzed using the MitoStress assay for Seahorse XFe96 (Agilent 103015-100). This assay was used to measure the O₂ consumption rate (OCR), mainly from mitochondrial activity, and the
extracellular acidification rate (ECAR), a surrogate for lactate production from glycolysis.

During the assay, cells were treated either with anti-IgG, aCD3 (5 ug-coated beads), PMA/Ionomycin or medium, followed by 2 mM oligomycin (ETC Complex V inhibitor; shows OCR due to ATP production), 0.5 mM FCCP (a proton ionophore that induces maximal OCR by allowing protons to flow into the mitochondria), and 100 mM rotenone/antimycin A (ETC Complex I and III inhibitors, respectively, and show non-mitochondrial OCR by inhibiting the ETC), following the protocol suggested by van der Windt et al. (2016). Seahorse Wave Desktop Software was used for analysis of results.

**Lysate Generation and Western Blot Analysis**

Cells were washed with PBS and cell pellet was re-suspended in RIPA buffer mixed with protease and phosphatase inhibitors (Thermo Fisher) to generate cell lysates. Protein concentration in lysates was calculated with a BSA-based assay and lysates were re-suspended in the same buffer to bring to a final concentration of 30 µg/20 µL. Electrophoresis of lysates and Western blot analysis was performed using the Bio-Rad system, including reagents and equipment, as per manufacturer instructions. Briefly, lysates were mixed with sample reducing agent (NuPAGE, Invitrogen NP0009) and LDS Sample Buffer (NuPAGE, Invitrogen NP0007) and boiled for 5 min (denaturing conditions). Gels (10% acrylamide; Bis Solution, 40% Acrylamide, Bio-Rad 1610146) were loaded with 25 µL sample and 7 µL protein ladder (Precision Plus Protein Dual Color Standards, Bio-Rad 1610374). Proteins were separated in 10x Tris/Glycine/SDS buffer (Bio-Rad 1610732) at 65-80 V. Transfer of proteins onto 0.2 µm PVDF membrane (Immun-Blot PVDF Membrane, Bio-Rad 1620177) was performed in a Bio-Rad Trans-Blot Turbo Transfer System with Trans-Blot Turbo Transfer kit (Bio-Rad 1704272). Membranes were then blocked with 5% non-fat milk (Cell Signaling Technology, 9999S) in Tris
buffer saline (TBS) for 1 hour at room temperature (RT). Antibodies were diluted in 3% BSA (Fisher Scientific BP1600-1) diluted in TBS. Membranes were incubated with primary antibody overnight at 4 °C, followed by three 10-min washes with 0.1% Tween 20 (Fisher Scientific BP337-50) in TBS (TBST). Incubation with secondary antibody diluted in 3% BSA-TBS was performed at RT for 1 h. Washes with TBST were repeated followed by a final wash with TBS. For signal detection, membranes were incubated with a 1:1 mix of luminol solution and peroxide solution (ECL™ Prime Western Blotting System, GE Healthcare RPN2232) and developed using Bio-Rad ChemiDoc MP imaging system (Cat. #1708280). Protein signal was then quantified using ImageJ 1.x software.

**Metabolomics Analysis**

Cells cultured in normoxia or hypoxia were collected, pelleted by centrifugation and washed with PBS. Supernatant was fully removed and cell pellets were snap-frozen in liquid nitrogen. Samples were stored at -80 °C until shipment on dry ice. Metabolomics analysis was performed by the Metabolomics Core Facility at Robert H. Lurie Comprehensive Cancer Center of Northwestern University. Protocol for analysis is described in Weinber et al. (2019). Samples were analyzed by High-Performance Liquid Chromatography and High-Resolution Mass Spectrometry and Tandem Mass Spectrometry (HPLC-MS/MS). The system consisted of a Thermo Q-Exactive in line with an electrospray source and an Ultimate3000 (Thermo) series HPLC. Data acquisition and analysis were carried out by Xcalibur 4.0 software and Tracefinder 2.1 software, respectively (both from Thermo Fisher Scientific). Statistical analysis was performed with Metaboanalyst R script.
Statistical Analysis

Statistical significance was determined by paired, two-tailed Student’s T-test using Graph Pad Prism version 6.0 in all experiments. Differences of P < 0.05 were considered significant. For curve comparisons, Graph Pad Prism was used to first generate linear regressions of each curve, then calculate the slope of the regression lines. To compare the lines, the program determines whether the slopes are significantly different; if the P value is lower than 0.05, the slopes are considered significantly different. The following symbols are used: ns (P> 0.05); * (P≤ 0.05); ** (P≤ 0.01); *** (P≤ 0.001); **** (P≤ 0.0001). For RNA-seq data, only gene expression changes with P< 0.05 were considered significant.
CHAPTER III:

RESULTS

Exposure of T Cells to Tumor Hypoxia

O₂ is not only necessary for respiratory metabolism, it is also a critical regulator of cell fate and function. Induction of angiogenesis by cancer cells result in the rapid formation of structurally abnormal vasculature around the tumor, resulting in uneven distribution of O₂ and nutrients throughout the tumor. Many reports have shown the effects of O₂ concentration on tumor cells and it is well known that hypoxia contributes to tumor growth and immune escape\textsuperscript{230,231,297–299}. However, very few studies focus on evaluating the effects of hypoxia on immune cells. I therefore first sought to determine how T cell distribution in the tumor is affected by hypoxia.

Previous reports indicate that tumor-reactive CD8 T cells mainly locate to the periphery of the tumor\textsuperscript{300,301}. To study the exposure of TILs to tumor-associated hypoxia in vivo, I utilized the melanoma mouse model B16 in combination with the hypoxia probe pimonidazole (PIMO). Cells exposed to O₂ levels of ~1.3% or lower in the tissue show as positive for PIMO, allowing to distinguish cells localized in hypoxic regions of the tumor\textsuperscript{302–304}. B16 was implanted in the flank of mice; once tumors reached 10 mm in diameter, mice were injected with PIMO (Fig. 4). One mouse was injected with PBS for staining control. After 1.5-2 h of injection, spleens and tumors where harvested and processed for flow cytometry analysis.
Fig. 4. Determination of Hypoxic T Cells in B16 Tumors. a. Experimental Design. Mice bearing >10 mm diameter B16 tumors were injected with pimonidazole (PIMO) 1.5-2 h before harvesting thymus, tumor and spleen. Tissues were dissociated and tumors were digested with collagenase and DNase before staining for flow cytometry analysis. b. Expected results. Cells positive for PIMO are exposed to hypoxia in the tumor, while PIMO-negative cells are located in non-hypoxic areas.

The gating strategy used to analyze these flow cytometry results is shown in Fig. 5a. For gating on PIMO⁺ lymphocytes in the tumor, I used spleen samples for size-based gating on CD3⁺ lymphocytes (open gate in SSC vs. FSC plot) and as a negative control for PIMO (Fig. 5, Fig. A.1). Thymus samples were used as a PIMO-positive control (Fig. 5), as this tissue has been shown to exhibit low [O₂]₁⁹⁷.
Fig. 5. Gating of Hypoxic T Cells in B16 Tumors. a. Representative gating strategy of a tumor sample. First, size-based gating was done in SSC vs. FSC plot, from which live CD3+ cells were selected. Live CD3+ cells were further divided into CD4 or CD8 T cells, as well as in PIMO+ (hypoxic) and PIMO− (non-hypoxic). b. PIMO expression in CD3+ cells from spleen, thymus and tumor. Spleen (oxygenated tissue) was used as negative PIMO control and thymus (low O2 concentration) was used a positive PIMO control. Fully stained samples from negative control mouse injected with only PBS (“No PIMO”) are compared to fully stained samples from mouse injected with PIMO. FMO (Fluorescence Minus One) control represents thymus sample from mouse injected with PIMO and stained with all antibodies except for anti-PIMO antibody.

I found that the proportion of T cells that were exposed to hypoxia (PIMO+) of the tumor was relatively low (Fig. 6), averaging less than 10% of total CD3+ cells (mean= 8.9%). Within this PIMO-positive T cell population (Fig. 6a), the proportion of CD8 T cells (mean= 9.5%) was lower than their CD4 counterparts (mean= 21.6%; P= 0.0008). However, I found no differences in the proportion of total CD8 (mean= 29.30) and CD4 T cells (mean= 23.11; P= 0.1866) (Fig. 6b) or differences in the proportion of non-hypoxic (PIMO-negative) CD8 (mean= 24.0%) and CD4 (mean= 32.3%; P= 0.0822) T cells (Fig. 6c). These results indicate that either a very low
proportion of CD8 T cells penetrate the hypoxic areas of the tumor or that hypoxia causes cell death in CD8 T cells that reach these areas.

**Fig. 6. Hypoxic T Cells in The Tumor.** a. Overlaid B16 tumor samples gated on live CD3+ CD8 T cells (top) and CD4 T cells (bottom). Gate denotes hypoxic (PIMO+) cells. b. Left: Proportion of total PIMO+ CD3+ T cells (PIMO+ gated on live CD3+ cells) in tumors. Right: Percentage of total CD4 and CD8 T cells (gated on live CD3+ cells). c. Left: Proportion of hypoxic CD4 and CD8 T cells (gated on PIMO+ live CD3+ cells). Right: non-hypoxic CD4 and CD8 T cells (gated on PIMO− live CD3+ cells). Results represent summary of 23 samples. Paired t-test analysis, P< 0.05.

**Tumor-Infiltrating Hypoxic T Cells Show an Effector-Like Phenotype**

T cells play major role in tumor control and their presence in the tumor is associated with good prognosis. Therefore, to test how hypoxia may affect the activation state of T cells, I measured the expression of activation markers CD62L, CD44, PD-1 and Tim-3 in hypoxic and non-hypoxic TILs in B16 tumors. I found that most TILs were antigen experienced (CD44-positive), and the expression of CD44 was unchanged when comparing hypoxic CD4 T cells or hypoxic CD8 T cells with their non-hypoxic counterparts (Fig. 7a-b).
Fig. 7. CD44+CD62Llo T Cells are Enriched in Hypoxic Areas of the Tumor. a. Gating on CD44 and CD62L in hypoxic (PIMO+, blue) or non-hypoxic (PIMO−, red) in CD4 and CD8 T cells. These cells were gated on CD3+PIMO+ or CD3+PIMO− cells. b. Proportion of total CD44+CD4 (left) and CD8 T cells (right). c. Proportion of PIMO+ or PIMO− CD44+CD62L− CD8 (left) and CD4 (right) T cells. Results represent summary of 23 samples. Paired t-test analysis, P<0.05.

However, there was a higher percentage of effector (CD44+CD62L−) CD8 T cells in hypoxia (82.7% of PIMO+ vs. 71.9% of PIMO− CD8 T cells; P=0.0360). Most CD4 T cells were found to be CD44+CD62L− regardless of their PIMO accumulation (90% in both PIMO+ and PIMO− CD4 T cells; P=0.8618) (Fig. 7c). These results suggest that while all CD8 TILs expresses CD44, effector CD8 T cells (with low CD62L expression) are enriched in hypoxic regions of the tumor.

Co-inhibitory receptors PD-1 and Tim-3 were also increased in hypoxic TILs (59.6% PD-1+ and 34.2% TIM-3+ of PIMO+CD3+ cells vs. 38.7% PD-1+ and 23% TIM-3+ of PIMO−CD3+ cells; P<0.0001 in both cases) (Fig. 8). These receptors are induced after TCR stimulation and indicate T cell activation; however, their expression and inhibitory activity are associated with T cell exhaustion. These results suggest that hypoxic tumor-infiltrating T cells are more likely to become exhausted than their non-hypoxic counterparts.
**Fig. 8. High Expression of Checkpoint Receptors in Hypoxic TILs.**  
**a.** Gating on TIM-3 and PD-1 in hypoxic (PIMO⁺) or non-hypoxic (PIMO⁻). Plot gated on live CD3⁺ cells in a B16 tumor sample.  
**b.** Proportion of PIMO⁺ or PIMO⁻ CD3⁺ T cells that are PD-1⁺ (left) and TIM-3⁺ (right). Results represent summary of 23 samples. Paired t-test analysis, P <0.05.

**In Vitro-Induced Hypoxia Inhibits CD8 T Cell Proliferation**

Our observations of melanoma TILs indicate that there are fewer CD8 T cells in the hypoxic areas than in the non-hypoxic areas of the tumor. Given the importance of oxygen in cell survival, I hypothesized that hypoxia induces CD8 T cell death. I thus sought to test CD8 T cell viability and proliferation under controlled oxygen conditions. I activated B6 mouse T cells in vitro by culturing whole splenocytes with aCD3/aCD28 agonist mAbs, IL-2 and IL-15 at atmospheric O₂ concentration (Fig. 9). Cells were split and media supplemented with cytokines was added as needed. After five days, cells were collected and replenished with fresh media and cytokines. I counted and seeded these activated T cells in new 24-well plates at 500,000 cells/mL/well and concentrations for further culture under normoxic and hypoxic conditions (21% and 0.5% O₂, respectively). To induce hypoxia, I used a glove box set at 37 °C, 5% CO₂ and 0.5% O₂. During the following five days, I took daily samples from cultures in normoxia or hypoxia to count live and dead cells by Trypan blue exclusion (Fig. 9). To study apoptosis and confirm viability, I stained the cells with anti-Annexin V Ab and with the Zombie aqua fixable viability dye.
Fig. 9. Generation of Effector T Cells In Vitro and Culture in Hypoxia. Experimental design followed for T cell culture in in vitro-induced hypoxia. a. Whole splenocytes were cultured in presence of stimulating aCD3 and aCD28 mAbs and cytokines IL-2 and IL-15. Cells were kept in culture for five days and then collected, counted and plated at the same cell density per well in 24-well plates. Fresh IL-2 and IL-15 were added and cultures were either maintained at normoxia (21% O2; in a conventional incubator) or transferred to hypoxia (0.5% O2; in a glove box). b. Cells cultured either in normoxia or hypoxia were counted and stained each day for the following 5 days (days 6-10 of culture) for dead and apoptotic cell content determination. For this, one well of the 24-well plates under each condition was taken per day.

After 5 days of culture of activated T cells under normoxia or hypoxia (day 10 of culture), my results show no difference in the proportion of total CD8 T cells (mean= 78.3% in normoxia, vs. mean= 77.2% in hypoxia, P= 0.554), indicating that this T cell subset was not selectively reduced by in vitro-induced hypoxia (Fig. 10, Fig. A.2).

Fig. 10. The Proportion of CD8 T Cells is Unchanged in Hypoxia Effector T cells generated in vitro from whole splenocytes were cultured for five days at either 21% or 0.5% O2. Left: Gating of CD8 and CD4 T cells in flow cytometry plot on Day 10 of culture. Plots shown were gated on total single cells. Right: Summary of results comparing the proportion of CD8 T cells in normoxia (21%) or hypoxia (0.5%) on Day 10 of culture. Results represent summary of 3 combined experiments with 3 independent samples each. Paired t-test analysis, P< 0.05.

While not reaching statistical significance in all measurements, the proportion of dead CD8 T cells (Zombie+) was slightly higher in hypoxia (16±11.8% in normoxia vs. 18.9±10.3% in hypoxia; P= 0.6), as shown by flow cytometry analysis (Fig. 11). However, I found no increase
in the proportion of apoptotic (Annexin V+) CD8 T cells in hypoxia (7.0±4.0% in normoxia vs. 9.6±4.2% in hypoxia on Day 10; P= 0.2) (Fig. 11). Moreover, I found a much larger number of live cells in normoxic cell cultures by cell counting with Trypan blue, while the total dead cell number was similar in both cultures (Fig. 11). Evaluation of the proliferation curves shows a higher slope in normoxic cultures (P= 0.00089), while hypoxic T cells seem to remain at similar numbers over time (Fig. 11). Contrary to my expectations, there was no difference in the number of dead cells between the two conditions (P= 0.3622). From these results, I conclude that CD8 T cell proliferation was reduced in hypoxia, while there were no indications of increased cell death, rejecting my initial hypothesis.

**Fig. 11. Reduced T Cell Proliferation in Hypoxia.** Effector T cells generated in vitro from whole B6 splenocytes were cultured for five days at either 21% or 0.5% O2. a. Plots gated on total CD8 T cells show live/dead cells and annexin V expression at 21% or 0.5% O2 on days 8 (left) and 10 (right) of culture. Plots shown were gated on total single cells. b. Summary of results comparing the proportion of live (left) and annexin V+ (right) CD8 T cells in normoxia (21%) or hypoxia (0.5%) over time. c. Proliferation (left) and death (right) curves of total cultures assessed by Trypan blue exclusion (slope of curve test, P< 0.05) in normoxia or hypoxia. Results represent summary of 3 combined experiments with 3 independent samples each. Paired t-test analysis, P< 0.05.
Hypoxia is known to be a global regulator of cell function and gene expression. To evaluate potential gene expression changes induced by hypoxia, an RNA-seq analysis was conducted on cultures exposed to normoxia or hypoxia for 48 h. While T cells were not purified for this analysis, approx. 80% of the culture corresponds to CD8 T cells (Fig. 10). Gene Ontology (GO) analysis results indicate a downregulation of gene pathways associated with cell cycle and mitotic cell division (Fig. 12). A list of significantly changed genes in hypoxia is shown in Table 4.

Fig. 12. Reduced Expression of Genes in Hypoxia. Effector T cells generated in vitro from whole B6 splenocytes were cultured for two days at either 21% or 0.5% O2. RNA was then purified from whole cultures kept under each condition and prepared for RNA-seq analysis. List shows the most significantly downregulated gene pathways in hypoxic T cells. The cutoff for determining significantly differentially expressed genes was an FDR-adjusted P value less than 0.05. Metascape was used for the pathway analysis. Results shown are a comparison between 3 independent samples cultured under each condition.

Notably, apoptosis-associated genes were also downregulated in hypoxia (Fig. 12, Table 4). I further confirmed this by evaluating hypoxic and normoxic CD8 T cells for their expression of pro-survival protein Bcl-2 (Fig 13; Fig. A.3). Interestingly, I found a higher content of Bcl-2 in hypoxic CD8 T cells (P= 0.0063), concurring with my viability results.
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### Table 4. Genes Changed in Hypoxic T Cells (Cont.)

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**Fig. 13. Increased Bcl-2 Expression in Hypoxic CD8 T Cells.** Effector T cells generated in vitro from whole B6 splenocytes were cultured for two days at either 21% or 0.5% O₂. 

(a) Histograms show Bcl-2 expression of cells cultured at 21% or 0.5% O₂ and an isotype control. Plots shown were gated on live CD8 T cells. 

(b) Summary results of Bcl-2 expression based on the MFI. Results of one representative experiment of 2, with 3 independent samples. Paired t-test analysis, P < 0.05.

From these data, I conclude that hypoxia reduces the effector T cell proliferation rate and does not cause apoptosis.

**Lower Expression of T Cell Homing Factor CD62L in Hypoxic CD8 T cells**

T cells upregulate the expression of CD44 after antigen encounter, which distinguishes effector T cells from naïve cells\(^{306}\). Similarly, TCR activation induces the downregulation of the homing molecule L-selectin (CD62L)\(^{307,308}\). Together, these two molecules are associated with cell migration and commonly used as T cell activation markers\(^{309,310}\). To test the activation status of hypoxic CD8 T cells, in vitro-activated B6 mouse T cells were further cultured under normoxic and hypoxic conditions. Five days later, I analyzed their expression of CD44 and CD62L by flow cytometry analysis. While there were no differences in the proportion of CD44+ cells (89.9% in normoxia vs. 88.5% in hypoxia; P = 0.689), I found a higher proportion of CD44+CD62L- effector CD8 T cells in hypoxia (66.7%) compared to cells in normoxia (34.8%; P = 0.058) (Fig. 14). These results reflect those seen in hypoxic CD8 TILs (Fig. 7).
Enhanced Expression of T Cell Inhibitory Co-Receptors in Hypoxic CD8 T Cells

TCR signaling is also followed by upregulation of inhibitory receptors, such as TIM-3 and PD-1\textsuperscript{135}, which can be used as markers of T cell activation. To test whether the upregulation of PD-1 and TIM-3 seen in vivo hypoxic TILs was mirrored in vitro, I measured the expression of these receptors on effector CD8 T cells after 48 h of exposure to hypoxia. As shown in Fig. 15 (Fig. A.4), I found that hypoxic effector CD8 T cells expressed both inhibitory receptors at higher levels (PD-1 MFI= 191 in normoxia vs. 226 in hypoxia of PD-1\textsuperscript{+} cells, P= 0.0016, and TIM-3 MFI= 464.5 in normoxia vs. 646.3 in hypoxia of TIM-3\textsuperscript{+} cells, P= 0.0013). While there was a higher proportion of PD-1\textsuperscript{+} CD8 T cells in hypoxia (18.2% in normoxia vs. 29.3% in hypoxia of PD-1\textsuperscript{+} cells, P= 0.0029), the percentage of TIM-3\textsuperscript{+} CD8 T cells was not significantly
different between the two conditions (21.5% in normoxia vs. 28.6% in hypoxia of TIM-3+ cells; P= 0.07). These results are similar to those found in hypoxic TILs.

**Fig. 15. Hypoxic CD8 T cells have increase PD-1 and Tim-3 upregulation.** Effector T cells generated in vitro from whole splenocytes were cultured for two days at either 21% or 0.5% O2. 

a. PD-1 and Tim-3 flow cytometry plot gated on CD44+CD62L- CD8 T cells at either 21% or 0.5% O2. b. Summary results of frequency of PD-1+ and Tim-3+ CD8 T cells at either 21% or 0.5% O2. 

Results of one representative experiment of 4 shown, with 4 independent samples. Paired t-test analysis, P< 0.05.

**Early TCR Signaling Is Impaired in Hypoxic CD8 T Cells**

Proper activation of T cells is necessary for their normal function. However, how hypoxia affects CD8 TCR activation is unknown. To determine how TCR signaling is affected by hypoxia, I generated in vitro-activated effector T cells from B6 mice. Among the early markers of T cell activation are CD69, a cell surface receptor, and IRF4, an important T cell transcription factor, both of which can be detected at the protein level after 2 hours of TCR activation\textsuperscript{101,311}. 

Following the previously described T cell activation protocol and after 48 h of exposure to
hypoxia (Fig. 16), I re-stimulated the cultures with aCD3 agonist mAb at 0.1 and 1 µg/mL. After 4 h of activation, I evaluated the expression of CD69 and IRF4 on CD8 T cells.

Fig. 16. Re-stimulation of Activated T Cells in Normoxia and Hypoxia. Experimental design. T cells from whole splenocytes were activated at atmospheric O₂ for 5 days. Cells were then cultured either in normoxia (21% O₂) or hypoxia (0.5% O₂) with cytokines for two additional days. On day 7, cells were re-stimulated with aCD3 agonist mAb for measurement of effector T cell function.

A larger proportion of CD8 T cells increased CD69 expression in normoxia (79.5%) than in hypoxia (61%; P= 0.01) at the highest aCD3 concentration ([aCD3]) used (Figs. 17-18).
Fig. 17. Reduced Expression of CD69 and IRF-4 in Hypoxic CD8 T Cells. In vitro-activated T cells from whole splenocytes were cultured for two days at either 21% or 0.5% O$_2$. Cells were stimulated with the indicated [aCD3] for 4 hours and tested for CD69 and IRF4 expression. Example of CD69 and IRF-4 gating in samples cultured at either 21% (top) or 5% O$_2$ (bottom). Plots shown were gated on live CD44+CD62L$^-$ CD8 T cells.

Among CD69-positive cells, CD69 expression per cell basis, as measured by the MFI, was higher in normoxic cells (MFI= 4247 vs. MFI= 2487 in hypoxia; $P= 0.0133$ at highest [aCD3]) (Fig. 18, Fig. A.5-A.6). While CD8 T cells under either condition were positive for IRF4, normoxic CD8 T cells increased the expression per cell basis of this marker to a higher extent than hypoxic T cells (MFI= 9378 in normoxia vs. MFI= 8325 in hypoxia; $P= 0.0133$ at highest [aCD3]) (Fig. 18). This response was dependent on TCR stimulation strength: higher [aCD3] resulted in higher CD69 and IRF4 expression, although percentage of CD69$^+$ cells reached a plateau at the lowest [aCD3] in normoxia. These data suggest early TCR activation is reduced in hypoxia.
Given the reduction in CD69 upregulation upon TCR stimulation in hypoxia, I hypothesized that early TCR signaling is inhibited in hypoxic T cells. To directly assess the effects of hypoxia in TCR signaling, I analyzed the activation status of a critical transcriptional component of the TCR downstream signaling, ERK. In vitro-generated effector T cells were exposed to normoxia and hypoxia as described before. After 2 days, cells were reactivated with aCD3 mAb in a time-dependent manner (0, 30, 60 and 120 min). Cells were kept at the indicated [O_2] throughout the experiment and collected at each time point and the levels of phospho-ERK were analyzed by flow cytometry.
Fig. 18. Hypoxic CD8 T Cells Have Reduced CD69 and IRF4 Upregulation Upon TCR Restimulation. Summary results of CD69 and IRF4 expression in live effector-like CD8 T cells. Effector T cells generated in vitro from whole splenocytes were cultured for two days at either 21% or 0.5% O2 and re-stimulated with aCD3 or IgG Ab control for 4 h. Top panel shows the proportion of CD69+ and IRF4+ cells and bottom panel shows the MFI levels of each protein within the respective positive population. Results of one representative experiment of 4 shown, with 4 independent samples. Paired t-test analysis, P<0.05.

While no phosphorylation of ERK was observed in control cells (IgG-treated), TCR stimulation induced ERK activation (Fig. 19). The highest activation level was found after 60 min of stimulation in normoxic T cells (60.3% of pERK+ CD8 T cells). In contrast, I found a lower proportion of pERK-positive cells T cells (peaked at 41.6% of CD8 T cells at 60 min; P=0.001) at all tested times under hypoxia.
Fig. 19. Reduced ERK Activation in Hypoxic CD8 T Cells. Effector T cells generated in vitro from whole splenocytes were cultured for two days at either 21% or 0.5% O₂ and re-stimulated with aCD3 for the specified times. a. ERK phosphorylation of CD8 T cells 21% (top) or 0.5% O₂ (bottom). Flow plot example of one sample gated on live CD8 T cells. b. Summarized results of proportion of pERK+ CD8 T cells (top) and pERK MFI in pERK+ CD8 T cells (bottom). Results of one representative experiment of 3 shown, with 3 independent samples. Paired t-test analysis, P< 0.05.

These results were further demonstrated by Western blot analysis of T cells re-stimulated for 1 h with and without aCD3 mAb. Fig. 20 (Fig. A.7) shows a higher band intensity of pERK in TCR-stimulated T cells from normoxic cultures compared to hypoxic cells (P= 0.0311), while the total content of ERK was not changed between conditions (P= 0.2624).
Fig. 20. Reduced ERK Activation in Hypoxic CD8 T Cells. Effector T cells generated in vitro from whole splenocytes were cultured for two days at either 21% or 0.5% O2. Cells were then re-stimulated with aCD3 for 1 h at 21% or 0.5%. Lysates were generated and ERK phosphorylation was measured by Western blot. a. Western blot analysis of one experiment. 30 µg of proteins were loaded per well. b. Summary results show the band area of phosphorylated (left) and total (right) ERK of the same experiment. Bands from samples in normoxia were compared to the corresponding sample in hypoxia visualized on the same membrane. Results of one representative experiment of 3 shown, with 3 independent samples. Paired t-test analysis, P< 0.05.

In addition, transcriptome analysis of effector T cells after 48 h of culture in normoxia and hypoxia by RNA-seq (Fig. 12) shows a significant reduction of genes associated with ERK/MAPK targets and MAPK signaling pathway.

**Hypoxia Prevents Activation-Induced T Cell Death**

TCR signaling strength correlates with AICD312,313. To further investigate the activation of the TCR signaling pathway in hypoxia, I treated in vitro-activated B6 T cells with 0.1 µg/mL aCD3 for 48 h at 21% or 0.5% O2 and evaluated their viability. This experimental strategy also mimics the encounter of T cells with cognate peptide in the tumor microenvironment from the moment they enter the hypoxic region. A higher proportion of dead CD8 T cells was found in
normoxia (77.1% vs. 41.4% in hypoxia; P< 0.0001) while the control cultures, treated with IgG isotype control antibody, did not show any difference between conditions (P= 0.543) (Fig. 21, Fig. A.8). These results further demonstrate the weaker TCR downstream signaling observed in hypoxic CD8 T cells.

**Fig. 21. Reduced AICD in Hypoxic CD8 T Cells.** Effector T cells generated in vitro from whole splenocytes were cultured in the presence of activating aCD3 mAb or IgG control for 48 h at either 21% or 0.5% O2. Proportion of dead cells was determined. **a.** Gating of dead cells in flow cytometry plots of one sample cultured with IgG control Ab (top) or aCD3 (bottom). Cells were gated on total CD8 T cells. **b.** Summary results of dead cell proportion in cultures stimulated with IgG control (top) or aCD3 (bottom). Results of one representative experiment of 3 shown, with 4 independent samples. Paired t-test analysis, P< 0.05.
CD8 T Cell Cytokine Production Is Reduced in Hypoxia

The secretion of pro-inflammatory cytokines is a key property tightly associated with the cytolytic function of CD8 T cells. Therefore, I went on to assess B6 mouse effector CD8 T cell production of pro-inflammatory cytokines IFN-γ, TNF-α and IL-2 in response to TCR activation with 0.1 µg/mL of aCD3 agonistic Ab after 48 h of culture in hypoxia (Fig. 16). As shown by flow analysis (Fig. 22), I found a severe reduction in cytokine-producing effector (CD44+CD62L-) CD8 T cells in hypoxia (mean = 11.2%) compared to normoxia (mean = 44.5%; P< 0.0001) (Fig. 23, Fig. A.9).

![Flow cytometry analysis of cytokine production (IFN-γ, TNF-α and IL-2) in CD44+CD62L- CD8 T cells re-stimulated with IgG control or aCD3 mAb either at 21% (left) or 0.5% O2 (right).](image)

**Fig. 22. Cytokine Production is Reduced in In Vitro-Induced Hypoxia.** Effector T cells generated in vitro from whole splenocytes were cultured for two days at either 21% or 0.5% O2 and re-stimulated overnight with IgG control or aCD3 mAb in the presence of BFA. **a.** Flow cytometry analysis of cytokine production (IFN-γ, TNF-α and IL-2) in CD44+CD62L- CD8 T cells re-stimulated with IgG control or aCD3 mAb either at 21% (left) or 0.5% O2 (right). **b.** Summary of total cytokine-producing effector CD8 T cells in each condition. Results of one representative experiment of 3 shown, with 3 independent samples. Paired t-test analysis, P< 0.05.

Specifically, a higher proportion of normoxic cells produced IFN-γ (41.8% vs. 6.3%; P= 0.0001), TNF-α (30.5% vs. 8.9%; P= 0.0026) and IL-2 (11.6% vs. 2.6%; P= 0.0173), in
comparison to hypoxic cells (Fig. 23). Interestingly, IL-2 production per cell basis, as measured by the median fluorescence intensity (MFI), was higher at low O₂ (P= 0.0109).

![Graphs showing cytokine production](image)

**Fig. 23. Lower Proportion of Cytokine-Producing Effector CD8 T Cells in In Vitro-Induced Hypoxia.** Effector T cells generated in vitro from whole splenocytes were cultured for two days at either 21% or 0.5% O₂ and re-stimulated overnight with IgG control or aCD3 mAb. Top panel shows the proportion of total IFN-γ-, TNF-α- and IL-2-producing effector CD8 T cells. Bottom panel shows the MFI of each cytokine within the population of cells positive for the cytokine. Results of one representative experiment of 3 shown, with 3 independent samples. Paired t-test analysis, P< 0.05.

To determine if the production of other inflammatory cytokines and chemokines is affected by hypoxia, I used a bead-based multiplex assay to test the culture supernatants of re-stimulated cells. Raw cytokine concentrations and their normalized values are shown in Fig. 24 (Fig. A.10) demonstrate a severe reduction in all tested parameters and that cytokine production inhibition in hypoxic cells is not limited to IFN-γ, TNF-α and IL-2. In contrast to previous reports, I did not find an increase in the production of IL-10 or other suppressive cytokines in hypoxic CD8 T cells.
Fig. 24. Lower Cytokine Production in Hypoxic T Cells. Effector T cells generated in vitro from whole splenocytes were cultured for two days at either 21% or 0.5% O_2 and re-stimulated overnight with aCD3 mAb. Supernatant was collected and analyzed. a. Concentration of cytokines/chemokines produced by B6 cells, assessed by bead-based multiplexing assay. Cytokines represented in graphs based on low, medium or high range of concentration. b. Representation of results in a, normalized to 100% (IL-10 was excluded). Results of one representative experiment of 2 shown, with 3 independent samples. Paired t-test analysis, P < 0.05.
These data indicate that hypoxia causes severe impairments in the ability of CD8 T cells to produce cytokines in response to TCR-mediated activation.

Higher expression of PD-1 and TIM-3 in hypoxic CD8 T cells (Fig. 15) suggested a potential mechanism of inhibition. In order to determine whether the lack of cytokine production was due to PD-1-mediated inhibition, I next incubated normoxic and hypoxic CD8 T cells with PD-1 blocking antibody (or with IgG as negative control) either for two hours before re-stimulation or for the duration of their exposure to hypoxia. Unfortunately, I did not observe rescue of IFN-γ production with PD-1 blockade (Fig. 25, A.11). I found that 57% of effector cells produced IFN-γ in normoxia with no increase upon PD-1 blockade (P= 0.265), while 23% of hypoxic cells were positive for IFN-γ production in control cultures and this percentage did not increase with anti-PD-1 treatment (P= 0.674). These results suggest that reduced cytokine production by CD8 T cells in hypoxia is not due to PD-1-mediated inhibition.
Fig. 25. PD-1 Blockade Does Not Improve Cytokine Production in Hypoxic T cells In Vitro.
Effector T cells generated in vitro from whole splenocytes were cultured for two days at either 21% or 0.5% O₂ with a blocking anti-PD-1 antibody (aPD-1) or IgG control (Ctrl.). Cells were then re-stimulated overnight with IgG control or aCD3 mAb in the presence of BFA. a. Flow cytometry analysis of cytokine production (IFN-γ, TNF-α and IL-2) in live CD44+CD62L- CD8 T cells treated with aPD-1 (top) or Ctrl. (bottom). b. Summary of results comparing cells cultured at 21% or 0.5% O₂ (top) and cells cultured with aPD-1 antibody or control at either O₂ condition (bottom). Results of one representative experiment of 2 shown, with 3 independent samples. Paired t-test analysis, P< 0.05.

Stronger TCR Engagement Partially Rescues Cytokine Production in Hypoxic Effector CD8 T Cells

TCR activation can be positively modulated by several factors, including the strength of the interaction between the TCR and its ligand. This effect can be mimicked by incubating B6 CD8 T cells with increasing concentrations of agonist aCD3 mAb. I hypothesized that a stronger TCR engagement would lead to improved T cell response as measured by cytokine production. To test this, I cultured CD8 T cells with 0 (IgG control), 0.1, 1 and 5 µg/mL of aCD3 in the presence of BFA for subsequent ICS of IFN-γ, TNF-α and IL-2. I found a dose-dependent response in both normoxic and hypoxic CD8 T cells, with higher concentrations inducing a higher proportion of cytokine-producing cells (Fig. 26, Table 5, Fig. A12). I compared the MFI
of each cytokine by subtracting the MFI obtained with no stimulation (IgG control) since T cells in hypoxia had a higher fluorescence. Similar to a previously described experiment (Fig. 23), higher IFN-γ MFI was found in normoxia while hypoxic T cells had a higher IL-2 MFI. In both conditions, the highest dose used (5 µg/mL) did not induce a further increase in the proportion of cytokine-producing cells, suggesting saturation of the TCR at 1 µg/mL. However, hypoxic CD8 T cells did not reach the same level of cytokine production as normoxic T cells even at the highest concentration used.
Fig. 26. Increasing TCR Stimulation Induces Higher Cytokine Production in Hypoxic T Cells. Effector T cells generated in vitro from whole splenocytes were cultured for two days at either 21% or 0.5% O$_2$ and re-stimulated overnight with IgG control or increasing concentrations of aCD3. 

**a.** Flow cytometry analysis of cytokine production (gating example of IFN-γ and TNF-α shown) of cells cultured either at 21% (top) or 0.5% O$_2$ (bottom). Cells were gated on live CD44$^+$CD62L$^-$CD8 T cells.

**b.** Summary of proportion of cytokine-positive cells (top) and MFI of each cytokine evaluated (bottom) comparing cells in normoxia (21% O$_2$; red) to hypoxia (0.5% O$_2$; blue).

Results of one representative experiment of 2 shown, with 3 independent samples. Paired t-test analysis, $P<0.05.$
Table 5. Dose-dependent cytokine production by effector CD8 T cells in normoxia or hypoxia. Cells were re-stimulated with the indicated concentrations of aCD3 mAb. P values determined by Student’s t-test.

<table>
<thead>
<tr>
<th>[aCD3] (μg/mL)</th>
<th>IFN-γ</th>
<th>TNF-α</th>
<th>IL-2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>21%</td>
<td>0.50%</td>
<td>P value</td>
</tr>
<tr>
<td>0</td>
<td>1.6</td>
<td>1.9</td>
<td>0.637</td>
</tr>
<tr>
<td>0.1</td>
<td>29.3</td>
<td>5.6</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>1</td>
<td>73.1</td>
<td>31.5</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>5</td>
<td>76.1</td>
<td>29.3</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

PMA/Ionomycin-Mediated T Cell Activation Partially Rescues T Cell Cytokine Production

I demonstrated that the strength of TCR engagement directly impacts T cell activation and cytokine production in hypoxia (Fig. 26). The reduced response found in hypoxic T cells, even with high affinity TCR-pMHC interactions, suggests a defect in the proximal TCR signaling pathway. I hypothesized that circumventing TCR-mediated activation would rescue effector CD8 T cell cytokine production in hypoxia. To rule out if this defect was solely dependent on reduced TCR signaling, I re-stimulated effector CD8 T cells with PMA/Ionomycin and compared the effects to aCD3 treatment. TCR-independent T cell activation with PMA (phorbol 12-myristate 13-acetate) induces PKC-θ activation, leading to MAPK pathway activation\(^{314}\). Ionomycin is a Ca\(^{2+}\) ionophore that allows for the influx of Ca\(^{2+}\)\(^{315}\). The combination of PMA and Ionomycin therefore induces cytokine production while bypassing early TCR signaling activation.

Activation with PMA/Ionomycin induced a greater proportion of total cytokine-producing CD8 T cells compared to stimulation with 1 μg/mL aCD3 (Fig. 27, A.13), both in normoxia (increase of 18%; P= 0.001) and hypoxia (increase of 13%; P= 0.004). However, only 50% of CD8 T cells produced IFN-γ in hypoxia compared to 90% in normoxia (P< 0.0001). Similar deficiencies were observed in TNF-α and IL-2 production. These data indicate that
besides interference with TCR signaling there are additional defects that preclude proper T cell function under hypoxia.

**Fig. 27. Re-stimulation with PMA/Ionomycin Induces Higher Cytokine Production in Hypoxic T Cells Than TCR-Dependent Stimulation.** Effector T cells generated in vitro from whole splenocytes were cultured for two days at either 21% or 0.5% O₂ and re-stimulated overnight with either aCD3 antibody or PMA/Ionomycin (PMA/Iono) in normoxia (21%) or hypoxia (0.5%). a. Flow cytometry analysis of cytokine production (gating example of IFN-γ and TNF-α shown) of cells cultured either at 21% (left) or 0.5% O₂ (right) comparing aCD3 and PMA/Iono stimulation. Cells were gated on live CD44+CD62L- CD8 T cells. b. Top: Summary of proportion of cytokine-positive cells (left) and MFI of each cytokine evaluated (right) comparing cells in normoxia (21% O₂; red) to hypoxia (0.5% O₂; blue). Bottom: Summary of results comparing cells stimulated with PMA/Iono to cells stimulated with aCD3. Results of one representative experiment of 2 shown, with 3 independent samples. Paired t-test analysis, P< 0.05.
Low TCR-pMHC Affinity Exacerbates Poor Effector T Cell Response in Hypoxia

The majority of endogenous tumor-reactive T cells have TCRs with low affinity for their targets, resulting in suboptimal effector CD8 T cell activity\textsuperscript{316}. If TCR-pMHC affinity is a factor that contributes to the defects that affect CD8 T cells under hypoxia is unknown. I used the TCR transgenic mouse model OT-I\textsuperscript{317}, specific for the H-2K\textsuperscript{b}-restricted ovalbumin (OVA) peptide\textsuperscript{318}. TCR peptide affinity was modulated by using the OVA peptide (N4) and the low affinity altered peptide ligands (APLs) Q4, T4 and V4. While these APLs differ in their potency to activate OT-I T cells, they have equal MHC-I binding\textsuperscript{285}. OT-I splenocyte suspensions were plated in the presence of IL-2 and IL-15 and one of the peptides listed above for 5 days. Cells were then either kept at 21\% or moved to 0.5\% O\textsubscript{2}. Forty-eight hours later, T cells were exposed to RMAS target cells loaded with the same peptide that was used during priming (Fig. 28).

![Diagram](https://via.placeholder.com/150)

**Fig. 28. Modulation of TCR Signaling Strength in OT-I T Cells Cultured in Normoxia and Hypoxia.** OT-I splenocytes were activated with IL-2, IL-15 and either the OVA peptide or an APL at atmospheric O\textsubscript{2}. After 5 days, cultures were split into normoxia (21\%) or hypoxia (0.5\%) and maintained with cytokines. Two days later, cells were re-stimulated with the same peptide used for priming.

The production of the pro-inflammatory cytokines IFN-\(\gamma\), TNF-\(\alpha\) and IL-2 was assessed by flow cytometry (Fig. 29). While the proportion of cytokine-producing effector CD8 T cells was dependent on TCR affinity in both conditions (Table 6), I found that the response to all
peptides was lower in hypoxia, particularly in cultures with the lowest affinity peptide, V4 (Fig. 29, Table 6).

Fig. 29. Hypoxic OT-I Effector CD8 T Cells Have Higher Cytokine Production in Hypoxic T Cells Re-stimulated with High Affinity Peptides. Effector T cells generated in vitro from whole OT-I splenocytes were cultured for two days at either 21% or 0.5% O$_2$ and re-stimulated overnight with one of four OVA variants with different affinities (N4, Q4, T4, V4) in normoxia (21% O$_2$) or hypoxia (0.5% O$_2$). a. Flow cytometry analysis of cytokine production (gating
example of IFN-γ, TNF-α and IL-2 shown) of cells cultured either at 21% or 0.5% O₂ with each peptide. Cells were gated on live CD44+CD62L- CD8 T cells. b. Summary results: proportion of total- and triple-cytokine-producing effector CD8 T cells; proportion of total effector CD8 T cells positive for each cytokine tested; and cytokine production per cell basis (MFI) within cytokine-positive cells. Results of one representative experiment of 3 shown, with 3 independent samples. Paired t-test analysis, P< 0.05.

Table 6. TCR Affinity-Dependent Cytokine Production by Effector OT-I CD8 T Cells in Normoxia or Hypoxia. Cells were re-stimulated with the indicated APLs. P values determined by Student’s t-test comparing normoxia (21% O₂) to hypoxia (0.5% O₂).

<table>
<thead>
<tr>
<th>APL</th>
<th>IFN-γ+</th>
<th>TNF-α+</th>
<th>IL-2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>21%</td>
<td>0.50%</td>
<td>P value</td>
</tr>
<tr>
<td>N4</td>
<td>89.10</td>
<td>51.50</td>
<td>0.0086</td>
</tr>
<tr>
<td>Q4</td>
<td>93.13</td>
<td>35.17</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>T4</td>
<td>88.43</td>
<td>33.50</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>V4</td>
<td>61.73</td>
<td>7.62</td>
<td>0.0004</td>
</tr>
</tbody>
</table>

Additionally, the proportion of double- and triple-cytokine-producing cells was also severely reduced in hypoxia (Fig. 30, Fig. A.14). This is better illustrated by the non-cytokine producing portion of V4-activated T cells, which increased from 33.4% in normoxia to 87.3% in hypoxia (P= 0.0013). These data confirm that hypoxia reduces CD8 T cell pro-inflammatory cytokine-producing capacity and that TCR activation with very low affinity peptides exacerbates this phenotype.
Fig. 30. Reduced Proportion of Multifunctional CD8 T Cells in Hypoxia. Effector T cells generated in vitro from whole OT-I splenocytes were cultured for two days at either 21% or 0.5% O₂ and re-stimulated overnight with one of four OVA variants with different affinities (N4, Q4, T4, V4) in normoxia (21% O₂) or hypoxia (0.5% O₂). Pie charts show the average proportion of cytokine-producing cells in each combination of the cytokines tested (a) or the proportion of 0-, 1-, 2- and 3-cytokine-producing cells (b). Gated on live cytokine-producing CD44+CD62L-CD8 T cells.

Results of one representative experiment of 3 shown, with 3 independent samples.
**In Vivo-Generated Effector CD8 T Cells Fail to Respond in Hypoxia**

While our in vitro model is conducive to study effector CD8 T cells, in order to test physiologically relevant conditions, I next sought to assess the function of in vivo-generated effector CD8 T cells when exposed to hypoxia. To do this, I immunized the footpad of B6 mice with OVA peptide in adjuvant (Fig. 31).

![Flowchart: In Vivo-Generated Effector CD8 T Cells in Normoxia, Physioxia and Hypoxia](image)

**Fig. 31. Evaluation of In Vivo-Generated T cells in Normoxia, Physioxia and Hypoxia.** B6 mice were vaccinated with OVA and adjuvant in the right foot pad. Five days later, inguinal and popliteal lymph nodes were extracted and cell suspensions were made. Cells were then cultured for 2 days at 21%, 5% or 0.5% O₂ before re-stimulation with OVA-loaded RMAS target cells at the same [O₂].

After five days, mice were euthanized and inguinal and popliteal draining lymph nodes harvested. Lymphocytes were isolated and cultured at atmospheric O₂ (21%), physioxia (5% O₂) or hypoxia (0.5% O₂). After two days, I collected the cells, counted them and plated 100,000 cells/well from each culture in 96-well plates that were kept at the same [O₂] as the original cultures. I re-stimulated the cells with RMAS cells loaded with OVA peptide or with no peptide (100,000 cells/well for a 1:1 ratio); as controls, I stimulated the cells with PMA/Ionomycin or with no treatment. In this case, I collected the culture supernatant after overnight incubation and tested the production of IFN-γ by ELISA.

Cells reactivated at 21% O₂ had a higher production of IFN-γ (1510 pg/mL) than those cultured at 5% O₂ (674 pg/mL; P= 0.0377) (Fig. 32, Fig. A.15). However, I found that very low O₂ levels (0.5%) prevented this response (448.7 pg/mL; P= 00005 compared to 5%). These results suggest that effector CD8 T cells generated under physiological conditions, similar to
those generated in vitro, are impaired to respond to antigen in hypoxia. Importantly, this also indicates that [O\textsubscript{2}] plays a critical role in T cell response.

**Fig. 32. In Vivo-Generated Effector T Cells Fail to Produce IFN-γ Under Hypoxic Conditions.** Effector T cells generated in vivo from whole OVA-immunized B6 mice were cultured for two days at either 21% or 0.5% O\textsubscript{2} and re-stimulated overnight with the OVA peptide (N4; left) or PMA/Ionomycin (PMA/Iono; right) in normoxia (21% O\textsubscript{2}), physioxia (5% O\textsubscript{2}) or hypoxia (0.5% O\textsubscript{2}). Graphs show ELISA results of IFN-γ supernatant concentration. Results of one representative experiment of 2 shown, with 3 independent samples. Paired t-test analysis, P< 0.05.

**TCR-Transduced Human T Cells Fail to Respond to Antigen in Hypoxia**

Given the current translational effort to treat cancer with T cell-based immunotherapies, I tested if TCR-transduced T cells are also affected by hypoxia. For this, IFN-γ production was measured in human T cells transduced with tyrosinase (Tyro peptide)-specific TCR 1383i\textsuperscript{286}. For this, activated T cells were transduced and purified based on their expression of the transduction marker CD34 and cryo-preserved until their use. After thawing, cells were rested for three days with cytokines. I cultured them for an additional 48 h at 21% or 0.5% O\textsubscript{2} and stimulated with T2 target cells loaded with Tyro peptide or an irrelevant peptide. For control, I also incubated the cells with PMA/Ionomycin or with no treatment. I found that 1383i-transduced T cells in hypoxia produce nearly no IFN-γ upon activation (246.8 pg/mL vs. 6015 pg/mL in normoxia; P= 0.0024) (Fig. 33a). To determine if this reduction was the result of reduced transgene expression, I stained for the expression of CD34 in T cells after normoxia and hypoxia. Surprisingly, hypoxic T cells expressed higher levels of CD34 than normoxic T cells construct (5.65% in
normoxia vs. 29.9% in hypoxia; P= 0.0002) (Fig. 33b). Stimulation with PMA/Ionomycin induced a higher response both in normoxia (64644 pg/mL) and hypoxia (21278 pg/mL; P=0.0001); however, hypoxic cells did not reach the same level of activation as that of normoxic T cells. These data further demonstrate the inhibitory effects of hypoxia on T cells, and that these are not restricted to mouse T cells.

**Fig. 33. Hypoxia Impairs IFN-γ Production of TCR-Transduced Human T Cells.** 1383I TCR-transduced human T cells were cultured for two days at either 21% or 0.5% O₂ a. Cells re-stimulated with tyro peptide (top) or PMA/Ionomycin (bottom). Graphs show ELISA results of IFN-γ supernatant concentration. Results represent summary of 4 different donors. b. Top: Flow cytometry plots of transduction marker CD34 in 1383I TCR-transduced T cells. Gated on live cells. Bottom: Summary results of 3 different donors. Graphs show the percentage of CD34+ live CD8 T cells (left) and the MFI of CD34 in positive cells (right). Paired t-test analysis, P< 0.05.

**Increased Mitochondrial ROS Production in Hypoxia Is Independent of TCR Signaling**

Mitochondria are key to T cell metabolism and function, not only for energy production, but also for their role in cell signaling. In fact, mitochondrial reactive oxygen species (ROS) serve as mediators of Ca²⁺ signaling upon TCR activation to drive cytokine production.⁴¹⁹

Interestingly, mitochondria-derived ROS are responsible for HIF1α stabilization and hypoxia is known to increase ROS generation.⁴²⁰ Given that both TCR signaling and hypoxia are predicted
to increase ROS production, I sought to determine whether ROS content would increase in hypoxic T cells upon TCR stimulation. For this, I re-stimulated in vitro-generated B6 effector CD8 T cells with either aCD3 or IgG control Ab after 48 h of exposure to hypoxia and measured total ROS content by using the fluorescent indicator H2DCFDA by flow cytometry (Fig. 3, Fig. A.16). As predicted, a higher basal ROS content was found in hypoxic T cells (average MFI= 598.0 in normoxia vs. MFI= 2131 in hypoxia; P= 0.0011). However, TCR engagement with aCD3 induced ROS in B6 cells in normoxia, while failing to further increase ROS content in hypoxia (average MFI= 1069 in normoxia vs. MFI= 1361 in hypoxia; P= 0.4414). This result is consistent with the lower response to TCR signaling I have observed in hypoxic T cells in terms of cytokine production and ERK activation.
Fig. 34. Hypoxia Drives High ROS Production in T Cells. Effector T cells generated in vitro from whole B6 splenocytes were cultured for two days at either 21% or 0.5% O2 and treated overnight with either aCD3 antibody or IgG control in normoxia (21%) or hypoxia (0.5%). a. Flow cytometry analysis of ROS with indicator H2DCFDA. Cells were gated on live lymphocytes based on size. b. Top: Summary of H2DCFDA MFI comparing cells in normoxia (21% O2; red) to hypoxia (0.5% O2; blue). Bottom: Summary of H2DCFDA MFI comparing cells treated with aCD3 or IgG control at 21% O2 (left) or 0.5% O2 (right). Results of one representative experiment of 3 shown, with 3 independent samples. Paired t-test analysis, P< 0.05.

Increased Mitochondrial Membrane Potential in Hypoxia Is Independent of TCR Signaling

Another effect of TCR signaling is the polarization of the mitochondrial inner membrane. Mitochondria use the proton gradient across their inner membrane to drive ATP production in the electron transport chain (ETC)\textsuperscript{174}. TCR stimulation further induces OXPHOS-dependent ATP production, resulting in transient mitochondrial hyperpolarization in response to Ca\textsuperscript{2+} and ROS signaling\textsuperscript{175,321}. In order to determine the effects of hypoxia on the mitochondrial membrane potential (∆ψm) of effector CD8 T cells upon TCR stimulation, I re-stimulated activated B6 T cells after 48 h of culture in normoxia or hypoxia with aCD3 mAb or control mAb. To measure ∆ψm, I used the probe TMRE, which accumulates in the negatively charged mitochondrial
matrix and can be detected by flow cytometry. Hypoxic CD8 T cells had a higher basal Δψm (MFI= 116.5 in normoxia vs. MFI= 440.0 in hypoxia; P= 0.0012) and TCR activation with aCD3 mAb only induced a significant increase in Δψm in normoxic T cells (MFI= 318.0 in normoxia vs. MFI= 307.7 in hypoxia; P= 0.8755) (Fig. 35, Fig. A.17).

**Fig. 35. Hypoxia Induces High ΔΨm in T Cells.** Effector T cells generated in vitro from whole B6 splenocytes were cultured for two days at either 21% or 0.5% O2 and treated overnight with either aCD3 antibody or IgG control in normoxia (21%) or hypoxia (0.5%). a. Flow cytometry analysis of ΔΨm with indicator TMRE. Cells were gated on live lymphocytes based on size. b. Top: Summary of TMRE MFI comparing cells in normoxia (21% O2; red) to hypoxia (0.5% O2; blue). Bottom: Summary of TMRE MFI comparing cells treated with aCD3 or IgG control at 21% O2 (left) or 0.5% O2 (right). Results of one representative experiment of 3 shown, with 3 independent samples. Paired t-test analysis, P< 0.05.

Mitochondrial polarization is in part attributed to Ca2+ concentration in the cytosol and inside the mitochondria. Therefore, I sought to determine the relative cytosolic Ca2+ content in normoxic and hypoxic B6 spleen-derived T cells. For this, I loaded the cells with Ca2+ indicator Fluo-4 AM, which passively diffuses into live cells and binds to cytosolic Ca2+322. There was a higher baseline concentration of intracellular Ca2+ in hypoxic T cells (MFI= 1199 in normoxia...
vs. MFI=1689 in hypoxia; P=0.0185) (Fig. 36, Fig. A.18). Stimulation with ionomycin induced the release of Ca$^{2+}$ from intracellular stores in both conditions (MFI=3863 in normoxia vs. MFI=6120 in hypoxia; P=0.0239), with a higher release in hypoxia. These results demonstrate that cytosolic [Ca$^{2+}$] is higher in hypoxic T cells.

**Fig. 36. High Cytosolic Ca$^{2+}$ in Hypoxic T Cells.** Effector T cells generated in vitro from whole B6 splenocytes were cultured for two days at either 21% or 0.5% O$_2$. Then, cells were loaded with Ca$^{2+}$ probe Fluo-4 AM and treated with ionomycin (Iono) or PBS (not treated). a. Flow cytometry analysis of Ca$^{2+}$ content comparing baseline (PBS; purple) and release from intracellular stores with ionomycin (Iono; yellow) either in normoxia (21% O$_2$; left) or hypoxia (0.5% O$_2$; right). b. Flow cytometry analysis of Ca$^{2+}$ content comparing cells cultured in normoxia (21% O$_2$; red) and hypoxia (0.5% O$_2$; blue) treated with PBS (left) or ionomycin (right). c. First two graphs: Summary of Fluo-4 AM MFI comparing cells treated with aCD3 or IgG control at 21% O$_2$ or 0.5% O$_2$. Last two graphs: Summary of Fluo-4 AM MFI in cells treated with PBS (baseline) or Ionomycin in normoxia (21% O$_2$) or hypoxia (0.5% O$_2$). Results of one representative experiment of 2 shown, with 3 independent samples. Paired t-test analysis, P<0.05.
Mitochondrial Structure and Content Are Not Affected by Hypoxia in T Cells

Activated T cells undergo a metabolic switch associated with changes in mitochondrial content and structure that support energy production mainly through glycolysis\textsuperscript{167}. Due to potentially detrimental effects of mitochondrial-derived ROS and the important role of O\textsubscript{2} for cellular respiration\textsuperscript{323}, I predicted that hypoxic T cells would have decreased mitochondrial mass in order to reduce ETC use and ROS production. I thus assessed the mitochondrial mass of spleen-derived activated T cells after 48 h of culture in hypoxia or normoxia. by measuring their mitochondrial DNA (mtDNA) content relative to nuclear DNA by quantitative real-time PCR (qRT-PCR). Contrary to my predictions, mtDNA quantification showed no significant differences in mitochondrial content between normoxic and hypoxic cells (p= 0.2928) (Fig. 37).

![Fig. 37. Mitochondrial T cell Content is Not Changed in Hypoxia](image)

Effect T cells generated in vitro from whole B6 splenocytes were cultured for two days at either 21% or 0.5% O\textsubscript{2}. Whole cultures were collected and mitochondrial DNA (mtDNA) was quantified in T cells cultured in normoxia (21%) or hypoxia (0.5%). Graph shows relative content of mtDNA to nuclear DNA based on qRT-PCR analysis. Results represent summary of 3 combined experiments. Paired t-test analysis, P< 0.05.

In addition, I evaluated mitochondrial morphology in activated T cells by use of transmission electron microscopy (TEM). Following two days of hypoxia, activated T cells demonstrated no significant differences in mitochondrial number and morphology compared to normoxic controls (Fig. 38). Moreover, the prevalence of discrete and round mitochondria rather than elongated mitochondrial networks in both cultures suggests that activated T cells maintain a
glycolytic phenotype consistent with effector-like T cells\textsuperscript{171} both in normoxia and hypoxia (Fig. 38).

**Fig. 38. Mitochondrial Morphology is Not Changed in Hypoxic Effector T Cells.** Effector T cells generated in vitro from whole B6 splenocytes were cultured for two days at either 21\% or 0.5\% O\textsubscript{2}. Whole cultures were collected and prepared for TEM analysis to evaluate mitochondrial morphology. **a.** TEM images of normoxic (21\%; left) or hypoxic (0.5\%; right) cells focused on mitochondria. **b.** Summary of results. Each graph represents a parameter used to evaluate mitochondrial shape, number and size. Results represent summary of 3 combined experiments. Paired t-test analysis, P< 0.05.

**Use of Glycolysis Is Enhanced in Hypoxic CD8 T Cells**

As previously mentioned, T cells use glycolysis as their main source of energy during the effector phase while keeping low levels of mitochondrial OXPHOS. Since hypoxia also induces
a higher use of glycolysis, I hypothesized that hypoxic effector CD8 T cells would have increased glycolysis in comparison to their normoxic counterparts.

To test this hypothesis, I first measured glucose uptake in normoxic and hypoxic effector T cells from B6 mice after 48 h of culture under either condition. For this, I incubated T cells with the fluorescent glucose analog 2-NBDG following 2 hours of glucose starvation. After washing off the medium, the cells were analyzed by FACS. As expected, I found a higher glucose uptake by CD8 T cells in hypoxia compared to normoxia (Fig. 39, Fig. A.19), indicating that there might be a higher usage of glycolysis by T cells in hypoxia.

![Graph of glucose uptake](image)

**Fig. 39. Increased Glucose Uptake in Hypoxic Effector T Cells.** Effector T cells generated in vitro from whole B6 splenocytes were cultured for two days at either 21% or 0.5% O2. Cells were then glucose-starved and incubated with glucose analog 2-NBDG to measure glucose uptake. Left: Flow cytometry analysis gated on live CD8 T cells. Right: Summary of 2-NBDG MFI results in cells cultured under each condition. Results of one representative experiment of 3 shown, with 4 independent samples. Paired t-test analysis, P< 0.05.

To further demonstrate the higher use of glycolysis in hypoxic effector T cells, I went on to evaluate glycolysis in hypoxic T cells using the Mitochondrial Respiration Agilent Seahorse XF Cell Mito Stress Test (Agilent). For this, splenocytes were cultured in conditions favoring T cell activation for 5 days in normoxia and then maintained for two more days in either normoxia or hypoxia with cytokines. This assay uses different inhibitors to target each component of the ETC to study the respiratory capacity of the cells. In addition, it continually measures the pH of the culture medium (extracellular acidification rate or ECAR) as a readout of glycolysis-derived
lactate. As an additional stimulus, I used aCD3-coated beads, empty beads, PMA/Ionomycin or no treatment (medium) (Fig. 40a). In accordance with higher glucose uptake in hypoxia, I found that ECAR was augmented in hypoxic T cells (Fig. 40b) under all treatments. Stimulation with PMA/Ionomycin did not increase ECAR in either normoxic or hypoxic cells (P= 0.3938 in normoxia; P= 0.1621 in hypoxia), yet both normoxic and hypoxic cells were responsive to TCR stimulation, increasing the glycolytic rate upon aCD3 treatment (P< 0.0001 in normoxia; P= 0.0009 in hypoxia) (Fig. 40c). Other compounds used in this experiment will be described below, as their effects are more relevant to modulate mitochondrial oxygen consumption and ETC function.
**Fig. 40. Hypoxia Induces Higher Glycolysis in Effector T Cells.** Effector T cells generated in vitro from whole B6 splenocytes were cultured for two days at either 21% or 0.5% O₂. Whole cultures under either condition were then prepared for Seahorse analysis. **a.** Glycolytic rate of cells cultured in normoxia (21%; red) or hypoxia (0.5%; blue). ECAR curves of each treatment shown (Empty: empty beads; aCD3: aCD3-coated beads; Media; P/I: PMA/Ionomycin). **b-c.** Proton efflux rate (PER) was used to measure acidification rate of media. Comparison between normoxic or hypoxic T cells after each stimulus (**b**) and between resting (basal) and stimulated (PMA/Iono or aCD3) cells cultured under each condition (**c**). Results of one representative experiment of 2 shown, with 5 independent samples. Paired t-test analysis, P< 0.05.

**Mitochondrial Respiration Is Inhibited in Hypoxic T Cells**

The low capacity of hypoxic T cells to further generate ROS and increase mitochondrial polarization upon TCR engagement, in combination with their increased glycolytic activity, lead us to hypothesize that mitochondrial function is reduced in hypoxia. To test this, OXPHOS profiles of T cells in hypoxia or normoxia were also determined using the Seahorse Mito stress assay. This analysis allows for the direct measurement of mitochondrial oxygen consumption rate (OCR) as well as the proton-coupled ATP generation and respiration capacity of the cell. As
previously described, I stimulated the cells with either aCD3-coated beads, empty beads, PMA/Ionomycin or no treatment. Curves of the overall OCR results with each stimulus are shown in Fig. 41 (Fig. A.20).

**Fig. 41. Reduced Mitochondrial O2 Consumption in Hypoxic Effector T Cells.** Effector T cells generated in vitro from whole B6 splenocytes were cultured for two days at either 21% or 0.5% O2. Whole cultures under either condition were then prepared for Seahorse analysis. Graphs show OCR curves of each treatment (Empty: empty beads; aCD3: aCD3-coated beads; Media; P/I: PMA/Ionomycin) from Seahorse analysis of T cells cultured in normoxia (red) or hypoxia (blue). Results of one representative experiment of 2 shown, with 5 independent samples. Paired t-test analysis, P< 0.05.

Among the parameters measured, basal respiration and maximal respiratory capacity were reduced in hypoxia, indicating lower use of mitochondria in hypoxic T cells both at resting conditions and when stress or maximal work is induced (Fig. 42). This was further demonstrated by the spare respiratory capacity (SRC) in TCR-activated cells. While treatment with either empty or aCD3-coated beads induced a higher SRC in normoxic cells, the ratio of SRC in aCD3-activated cells to SCR in cells treated with empty beads was higher in normoxia (P= 0.0352) (Fig. 44a). Interestingly, higher non-mitochondrial respiration was found in resting hypoxic cells, indicating that more O2 is used for cellular processes other than OXPHOS. This further demonstrates reduced mitochondrial activity in hypoxia.
Mitochondrial Respiration Is Reduced in Hypoxic Effector T Cells (Part I).

Effector T cells generated in vitro from whole B6 splenocytes were cultured for two days at either 21% or 0.5% O₂. Whole cultures under either condition were then prepared for Seahorse analysis. Graphs show parameters measured by Seahorse analysis of T cells cultured in normoxia (red) or hypoxia (blue) based on O₂ consumption. Displayed from top to bottom: ATP production, spare respiratory capacity, coupling efficiency, acute response. Treatments used: Empty: empty beads; aCD3: aCD3-coated beads; Media; P/I: PMA/Ionomycin.

Results of one representative experiment of 2 shown, with 5 independent samples. Paired t-test analysis, P<0.05.

Both proton leak and coupling efficiency of proton efflux to ATP production were not changed in hypoxia at resting conditions (Fig. 43). Coupling efficiency (CE) appears to be reduced in hypoxia with the use of beads (both control and aCD3-coated); however, the ratio of CE in TCR-stimulated (aCD3) to CE in non-stimulated (empty) cells was significantly higher in normoxic cells compared to hypoxic cells (P= 0.0352) (Fig. 44b).
Effector T cells generated in vitro from whole B6 splenocytes were cultured for two days at either 21% or 0.5% O₂. Whole cultures under either condition were then prepared for Seahorse analysis. Graphs show parameters measured by Seahorse analysis of T cells cultured in normoxia (red) or hypoxia (blue) based on O₂ consumption. Displayed from top to bottom: non-mitochondrial O₂ consumption, basal respiration, maximal respiration, proton leak. Treatments used: Empty: empty beads; aCD3: aCD3-coated beads; Media; P/I: PMA/Ionomycin.

Results of one representative experiment of 2 shown, with 5 independent samples. Paired t-test analysis, P< 0.05.

I also found that hypoxic cultures had reduced ATP production across all treatments and that stimulation with aCD3 (acute response) induced higher OCR in normoxic T cells (Fig. 43). Overall, these results demonstrate a lower mitochondrial function and respiratory function in hypoxic T cells.
Fig. 44. Higher mitochondrial spare respiratory capacity and coupling efficiency in normoxic effector T cells. Effector T cells generated in vitro from whole B6 splenocytes were cultured for two days at either 21% or 0.5% O₂. Whole cultures under either condition were then prepared for Seahorse analysis. Graphs show summary results of spare respiratory capacity ratio (a) and coupling efficiency ratio (b) between cells treated with aCD3 and empty beads from normoxic (21%) or hypoxic (0.5%) cultures. Results of one representative experiment of 2 shown, with 5 independent samples. Paired t-test analysis, P< 0.05.

Metabolic Profile Suggests Inhibited Effector T Cell Function in Hypoxia

Functional dysregulation, mitochondrial inhibition and increased glycolysis seen in hypoxic T cells suggest a global metabolic change. To further study the metabolic characteristics of T cells under hypoxic conditions, I analyzed the metabolome of normoxic and hypoxic B6 T cells (Fig. 45). I found a wide range of amino acids to be reduced in hypoxia, including arginine, a critical modulator of the immune response\textsuperscript{324}, and tryptophan, an essential amino acid that drives T cell proliferation\textsuperscript{325}. Important intermediates of phospholipid biosynthesis, such as cytidine triphosphate (CTP), cytidine diphosphate (CDP)-choline and choline\textsuperscript{326}, were also reduced in hypoxia.
Fig. 45. Metabolic profile of T cells cultured in normoxia and hypoxia. Effector T cells generated in vitro from whole B6 splenocytes were cultured for two days at either 21% or 0.5% \( \text{O}_2 \). Whole cultures under either condition were then prepared for metabolomics analysis. Graph shows a heat map of the top 35 metabolites (ranked by t-test P value) significantly changed between T cells cultured at 21% (green, right side) and at 0.5% \( \text{O}_2 \) (red, left side). Results shown are a comparison between 3 independent samples cultured under each condition.

Several metabolites found to be reduced in hypoxic T cells are involved important metabolic pathways predicted to be inhibited in hypoxia, such as the tricarboxylic acid cycle (TCA) cycle and the pentose phosphate pathway (PPP) (Fig. 46). Increased glycolysis induces pyruvate degradation into lactate\(^{327}\), preventing the synthesis of alanine (Ala) and valine (Val).
from pyruvate, as well as the formation of erythroose-4-phosphate (E4P) from the glycolysis intermediate 3-phosphoglycerate (3PG)\textsuperscript{159}. 3PG is a precursor of phenylalanine (Phe), Trp and tyrosine (Tyr), all three of which were found to be reduced in hypoxia. Glutamate (Glu), also reduced in hypoxia, can feed into the TCA cycle to synthesize α-ketoglutarate (α-KG).

Interestingly, α-Hydroxyglutarate (2-HG), formed from α-KG, was found to be enriched in hypoxic cells, in accordance to previous reports showing HIF1-dependent 2-HG accumulation in T cells\textsuperscript{328}.

![Fig. 46. Metabolic Pathways Affected by Hypoxia and Their Interactions. Schematic shows intermediates found changed in spleen-derived activated B6 T cells cultured for two days at either 21% or 0.5% O\textsubscript{2}, as per metabolomics analysis results. Intermediates found to be increased in hypoxia are shown in red and those found to be reduced are shown in blue.](image)

In addition to a plethora of amino acids, I also found the concentration of pyrimidine biosynthesis intermediates to be altered in hypoxia. Among these, dihydroorotate, a precursor of the pyrimidine synthesis pathway, was accumulated in hypoxic T cells, while uridine diphosphate (UDP), thymidine and the high energy molecules ATP and CTP, were reduced (Fig.
RNA-seq results (Fig. 12) also indicate a reduction of deoxyribonucleotide metabolic process. These results indicate a shutdown of metabolic pathways that are important for the synthesis of macromolecules and energy production.

**Media Supplementation with Uridine Increases Proliferation of Hypoxic T Cells**

It has been shown before that uridine availability is critical for cell proliferation\textsuperscript{329,330}, which is largely inhibited by hypoxia in effector T cells. Hypoxic T cell metabolomics (Fig. 45) and gene expression (Fig. 12) results indicate an inhibition of nucleotide synthesis pathway. Given the particular importance of uridine as a precursor for nucleotides and consequently DNA synthesis and replication, I tested its effects on hypoxic T cells. For this, I cultured activated T cells for 72 h either in hypoxia with increasing concentrations of uridine with the purpose of rescuing effector T cell function. These results show that while there was no change in cytokine production (not shown), cell proliferation was increased in T cells cultured with uridine (Fig. 47, Fig. A.21). Although the highest concentrations induce a higher number of dead cells, all concentrations induced a similar increase in live T cell number (489,000 cells in control cultures vs. 1.2 million cells at 400 μg/mL; \( P = 0.0001 \)). These results suggest that uridine induces T cell proliferation in hypoxia, possibly by rescuing DNA synthesis.
Fig. 47. Partial Rescue of Hypoxic T Cell Proliferation by Uridine Supplementation.
Effector T cells generated in vitro from whole B6 splenocytes were cultured for three days at 0.5% O2 with increasing concentrations of uridine. Total number of live (left) and dead (right) cells in cultures assessed by Trypan blue exclusion in hypoxia. Results of one representative experiment of 3 shown, with 3 independent samples. Paired t-test analysis, P< 0.05.
CHAPTER IV:
DISCUSSION

Low Infiltration of CD8 T Cells in Hypoxic Regions of the Tumor

Solid tumors are characterized by poor vasculature organization that results in areas with low oxygen concentration. In this study, I evaluated the infiltration of endogenous B6 T cells in B16 melanoma depending on O$_2$ availability. My results with the hypoxia probe PIMO show that most CD3$^+$ TILs were not exposed to hypoxia in the tumor, suggesting that they localize to non-hypoxic areas. Given the irregular distribution and permeability of intra-tumoral vessels, areas near the blood supply are expected to have enhanced oxygenation, which often coincide with the periphery of the tumor. Studies using PIMO demonstrate that binding of the probe in areas of the tumor distal from the blood vessel (distances greater than 100 $\mu$m from the vessel) . Thus, B16-infiltrating T cells, the majority of which were PIMO$^+$, mostly infiltrate tumor regions likely corresponding to areas near blood supply or the periphery of the tumor. This is supported by reports showing that most TILs localize to the periphery of the tumor.

Interestingly, it has been shown that T cells that penetrate deeper in the tumor (potentially hypoxic areas) are enriched for tumor-reactive cells and those T cells remaining at the periphery have low anti-tumor cytolytic activity. However, I did not test the function of TILs. Future investigation on how O$_2$ levels in the TME relate to antigen recognition by T cells would clarify if there is a relation between this and T cell infiltration.

A higher ratio of CD8/CD4 T cells has been correlated with positive outcomes. While there were similar proportions of total CD4 and CD8 T cells in the tumor, I found a lower
percentage of CD8 than CD4 T cells in hypoxic areas of the tumor. I demonstrated through in vitro experiments that hypoxia causes suppression of CD8 T cell proliferation but has no effect on cell survival, leading us to reject the original hypothesis that hypoxia induces CD8 T cell death. These results suggest that CD8 T cell proliferation may also be reduced in the suppressive hypoxic TME. While reduced proliferation was also found in hypoxic CD4 T cells in vitro, the culture conditions used here favor the expansion of effector-like cells, especially CD8 T cells. The potential suppressive nature of other cells in the tumor and the regulatory role of hypoxia on such cells were not represented in vitro cultures.

There are several potential explanations for the difference between CD4 and CD8 T cell proportions in the hypoxic regions of the tumor; one of them may be the suppressive action of tumor-associated endothelial cells on CD8 T cells. Increased tumor angiogenesis has been correlated with reduced CD8 T cell infiltration in the tumor. ICAM-1, a molecule critical for T cell extravasation and migration into the tumor, is inhibited in the intra-tumoral vasculature by VEGF\(^ {338-340}\), whose production is in turn enhanced by hypoxia. In fact, hypoxia promotes angiogenesis by inducing HIFα-mediated expression of VEGF in tumor cells\(^ {341}\). In addition to hindering CD8 T cell infiltration into the tumor in this way, endothelial cells exposed to hypoxia in vitro have increased expression of FasL, resulting in CD8 T cell death by Fas receptor engagement\(^ {342}\). Moreover, CD4\(^ +\) Treg cells express the anti-apoptotic molecule c-FLIP, which protects them from this Fas-mediated cell death, favoring the accumulation of this suppressive T cell subset in hypoxic regions of the tumor\(^ {342}\). While this would explain a general reduction of CD8 T cell tumor infiltration, it does not necessarily explain a reduction in the hypoxic areas of the tumor, since it would imply exposure of CD8 T cells and endothelial cells to hypoxia near the blood vessel, which is unlikely.
Another potential reason for this imbalance is the diversity of CD4 T cells that can differentially infiltrate the tumor. CD4 T cells are more plastic than CD8 T cells and may play multiple roles in the tumor. While a subset of CD4 T cells mediates anti-tumor effector functions, necessary for optimal tumor clearance\textsuperscript{343,344}, CD4 T cells can differentiate into several suppressive subsets which may be enhanced by hypoxia, including Treg and Th17 cells\textsuperscript{345}. CD4 T cells may differentiate into either Tregs or Th17 depending on environmental cues. TGF-β is a strong CD8 T cell suppressor\textsuperscript{236} and a Treg differentiation factor that is heavily secreted in the melanoma TME\textsuperscript{346}. In fact, TGF-β production has been associated with increased Treg tumor infiltration\textsuperscript{346}, which mediates immunosuppression by increasing TGF-β release as well as IL-10\textsuperscript{345}. Hypoxia further induces TGF-β production by both tumor and Treg cells through the action of HIF-α\textsuperscript{347,348}. On the other hand, Th17 CD4 T cells have also been associated with increased tumor growth\textsuperscript{349}. In the tumor microenvironment, presence of TGF-β induces Treg differentiation, but presence of both TGF-β and IL-6 can induce Th17 cell fate by inhibiting FoxP3 expression and increasing the Th17 transcription factor ROR-γ. There is debate on whether hypoxia favors the differentiation of one subset over the other: however, it induces a suppressive phenotype in both\textsuperscript{243,349,350}. In the case of Th17 cells, increased IL-10 production has been found in hypoxia\textsuperscript{349}. While I did not investigate the differentiation markers associated with CD4\textsuperscript{+} T cells in the tumor and, to my knowledge, there are no reports on their distribution in the tumor, both Th17 and Treg T cells are likely to be found and function in hypoxic areas of the tumor\textsuperscript{351}.

Other cell types typically found in the TME can exert a suppressive effect on CD8 T cells. Among these, MDSCs and TAMs produce reactive nitrogen species and have been shown to nitrate chemokines in the TEM, inhibiting T cell migration to the tumor core\textsuperscript{352}. While it has
not been shown in the hypoxic tumor, this activity seems to be enhanced by HIFα stabilization. Hypoxia induces recruitment and transformation of healthy fibroblasts into CAFs, which have been shown to remodel the extracellular matrix to support cancer progression and inhibit anti-tumor immune function. This hypoxia-associated suppressive environment may inhibit CD8 T cell infiltration.

While I demonstrated that hypoxia does not directly cause CD8 T cell apoptosis in vitro, prolonged tumor infiltration of effector CD8 T cells may result in exhaustion due to continuous exposure to antigen and suppressive factors of the TME, eventually leading to programmed cell death. T cell apoptosis in the TME has been associated with PD-L1, which is upregulated in hypoxic TAMs, MDSCs and tumor cells. Similar to in vitro-generated hypoxic B6 CD8 T cells, I found that TILs in hypoxia had increased expression of checkpoint receptors PD-1 and TIM-3 compared to non-hypoxic cells. Thus, it is plausible that the reduction of CD8 T cells in hypoxic areas of the tumor is the result of exhaustion-associated apoptosis as an indirect effect of hypoxia. This also suggests PD-1/PD-L1 signaling as a potential mechanism of tumor escape under hypoxia.

It may be suggested that, instead of a reduction in CD8 T cell proliferation or migration into the hypoxic TME, hypoxia induces downregulation of the CD8 coreceptor. While I found a CD3+ population that was double-negative for CD4 and CD8, these cells were mostly present in non-hypoxic areas of the tumor, similarly to CD4 and CD8 T cells. In addition, I did not observe a similar population nor a reduction of the proportion of either CD4 or CD8 T cells in in vitro hypoxic culture, indicating that hypoxia does not induce CD8 downregulation. Therefore, these results suggest a different CD3+ cell type. Although I did not investigate markers specific for these populations, some NKT cells and γδ T cells have been described to present a
CD3+CD4-CD8- phenotype. Alternatively, a defective CD4+CD8- T cell population has also been described in several types of tumors, including B16 melanoma.363

While other studies have shown that T cells mainly locate to the periphery of the tumor, to my knowledge, there are no reports associating this location with O2 availability. Many studies use the expression of HIFα and its targets as a surrogate for the presence of hypoxia; however, HIFα can be stabilize under non-hypoxic conditions. Here, I found a significant reduction of hypoxic CD8 T cells infiltrating tumor compared to CD4 T cells. Further study of tumor samples including markers for different T cell types would help explain the mechanisms that allow CD4 T cells to infiltrate hypoxic areas at a higher degree than CD8 T cells. In addition, visualization of tumor sections to determine the physical location of these populations in combination with endothelial cell markers and PIMO would contribute to understanding the relation between vasculature, O2 distribution in the tumor and T cell infiltration.

**Enhanced Activated Phenotype in the Hypoxic TME May Not Associate with Increased Function**

I found that the majority of TILs were antigen-experienced, as determined by CD44 expression, regardless of their distribution in hypoxic or non-hypoxic areas. While similar proportions of CD62L- CD4 T cells were found in hypoxic and non-hypoxic regions, I discovered a higher proportion of CD62L- CD8 T cells in the hypoxic population. CD62L is normally downregulated upon T cell activation, indicating that hypoxia induces CD8 T cell differentiation into an effector phenotype. However, CD62L downregulation may be a consequence of the inhibitory action of MDSCs. It has been well documented that T cells lose the expression of this homing molecule in the TME, likely due to the action of MDSCs. This suppressive cell type is frequently found in tumors, including B16 melanoma and is
known to become more suppressive in hypoxia\textsuperscript{366,367}. MDSCs enhance Treg recruitment and tumor growth\textsuperscript{365,368,369}, but also inhibit tumor-infiltrating CTLs through multiple mechanisms, including the induction of CD62L downregulation on T cells\textsuperscript{366}. MDSCs express the enzyme ADAM17 (a disintegrin and metalloproteinase domain 17), able to cleave the ectodomain of L-selectin and impair the ability of naïve T cells to migrate to activation sites\textsuperscript{364}. Alternatively, CD62L can be downregulated by increased HIF activity in hypoxic CD8 T cells\textsuperscript{328,370}. Consistent with this, in vitro cultures exposed to hypoxia also demonstrated downregulation of CD62L. Since TCR activation can lead to HIF\(\alpha\) stabilization, it is plausible that hypoxia further enhances this effect, resulting in CD62L downregulation and effector cell differentiation.

Loss of CD62L expression along with upregulation of PD-1 and TIM-3 in hypoxic CTLs suggests CD8 T cells acquire a more activated effector phenotype in hypoxia. In fact, PD-1 blockade in vitro resulted in no improvement of hypoxic CD8 T cell effector function, suggesting this checkpoint does not mediate the observed inhibition in proliferation and cytokine production, as will be discussed below. However, T cell expression of immune checkpoint receptors in the tumor, in combination with other suppressive factors and constant exposure to antigen, can result in enhanced inhibition under hypoxia. This can ultimately lead to fewer infiltrating CD8 T cells in the hypoxic TME.

**Reduced Cytokine Production in Hypoxic T Cells**

Inflammatory cytokine production is a hallmark of cytotoxic CD8 T cells\textsuperscript{371}. I found that CD8 T cells from B6 mice cultured in hypoxia failed to produce IFN-\(\gamma\), TNF-\(\alpha\) and IL-2 at the same level as cells in normoxia, suggesting that hypoxia impairs effector T cell function. In normoxia, I found that cytokines were produced following a hierarchical pattern, typical of the effector type 1 T cell response\textsuperscript{120,126,372,373}: IFN-\(\gamma\) and TNF-\(\alpha\) were produced at high frequency
and quantity, while IL-2 was produced at very low levels. The production of each cytokine has a different threshold; IFN-γ and TNF-α only require TCR signaling at low intensity and/or short length, while a strong and sustained signal is necessary to produce IL-2\textsuperscript{126,374}. While production of multiple cytokines does not necessarily happen simultaneously\textsuperscript{375}, it has been shown that IL-2 is usually produced by the same cells that produce IFN-γ and TNF-α and not by a different set of cells\textsuperscript{106}, similar to what I observed in both in B6 and OT-I CD8 T cells in normoxia. However, cytokine production was altered in hypoxia.

Hypoxic CD8 T cells showed a reduced capacity to produce pro-inflammatory cytokines and chemokines. I studied a set of 31 chemokines and cytokines including factors with pro- and anti-inflammatory properties. Hypoxic T cells demonstrated uniform reduction of all cytokines tested, suggesting there was no biased differentiation under this condition into a suppressive phenotype. Instead, this global inhibition of cytokine production in hypoxia suggests a defect in TCR signaling. Interestingly, neither normoxic nor hypoxic cultures produced anti-inflammatory cytokine IL-10\textsuperscript{131}. This is in contrast to a previous report that showed that CD8 T cells under hypoxia not only have increased IFN-γ, but also enhance IL-10 production\textsuperscript{279}. Another study on mouse CD4 T cells showed improved functions in hypoxia, with a higher production of IFN-γ at 1% O\textsubscript{2}\textsuperscript{376}. Unlike my experimental strategy, in this case, effector cells were generated under hypoxic conditions from isolated CD4 T cells. Nevertheless, CD4 and CD8 T cells have different growth requirements and functions, and hypoxia might exert different effects on CD4 T cells.

T cell functional avidity can be studied by controlling antigen dose\textsuperscript{377,378}. Here, I modulated OT-I CD8 T cell response with different affinity APLs, as TCR-antigen affinity also regulates cytokine production\textsuperscript{316}. Experiments with the OT-I model allowed me to study the influence of this parameter on hypoxic CD8 T cell responses. As expected, lower cytokine
production correlated with low antigen affinity. However, the response to low affinity peptide in hypoxia was drastically reduced compared to normoxia. The composition of cytokine-producing OT-I T cells also changed in hypoxia. The frequency of polyfunctional cells was reduced while 1-cytokine- and non-cytokine-producing cells increased in hypoxia, especially with low affinity antigen. Cells producing two or more cytokines, or polyfunctional T cells, provide a more effective response against antigen by generating multiple effector molecules at once. This is particularly important in the study of tumor-reactive T cells, whose affinity for self-antigens is usually low. High affinity TCRs are more sensitive to low quantities of antigen. The OVA peptide N4 has an affinity of ~5 μM and it is about 700 times more potent than V4, the lowest affinity OVA APL used in my experiments. Naturally occurring TCRs have affinities between 1-100 μM, with self-reactive TCRs within the lower range. Therefore, these results suggest that low affinity tumor-reactive T cells stimulated in hypoxia may be less efficient at antigen clearance.

Aside from affinity, co-stimulation and structural avidity also contribute to TCR engagement and T cell response. One explanation for reduced responses in hypoxia is the possible alteration of the expression and clustering of adhesion and co-stimulatory receptors that aid in TCR engagement at the immune synapse, known to affect TCR signaling and T cell sensitivity to antigen. In responses to weak stimuli, clustering can amplify the signal, for which actin-dependent cytoskeletal remodeling is required. Although this has not been explored in T cells under hypoxia, cytoskeleton regulation by hypoxia and HIFα has been reported for other cells and can potentially have an effect in TCR clustering and signal amplification with low affinity antigens.
However, I found similar results using the open TCR repertoire model B6 stimulated with aCD3, which is enough to induce and maintain TCR adhesion to target. Stimulation of normoxic or hypoxic B6 T cells with increasing [aCD3] generated a dose-dependent effect, though cytokine production in hypoxic CD8 T cells did not reach the same level as in normoxic controls. While there was a partial rescue in hypoxia at higher stimuli, a plateau of cytokine production was reached at 1 µg/mL of aCD3. This indicates that stimulation with aCD3 concentrations at TCR binding saturation could not further enhance the response and that other factors, such as downstream effector molecules, may be at play.

While I demonstrated that cytokine production in hypoxia can be improved to a certain extent by modulating TCR activation via CD3, my results suggest that failure to increase T cell response is possibly due to inhibited TCR signal-transducing molecules. I was able to confirm that there is a defect downstream of the proximal TCR signaling pathway by activating B6 T cells via TCR-independent stimulation. The combination of the ionophore ionomycin with the PKC activator PMA failed to rescue T cell response in hypoxia, indicating a defect downstream of PLCγ targets DAG and IP3, which in turn activate MAPK and Ca2+ signaling, respectively. This was further demonstrated by the low phospho-ERK and CD69 levels reached in hypoxia upon TCR activation with aCD3, indicating a defect in crucial steps of early TCR signaling. Here, the stimulation time used to test ERK phosphorylation was relatively long (30-120 min), given that ERK activation occurs within minutes of TCR engagement. However, the prolonged activation of ERK in normoxia indicates a potential maintenance of the signal by other stimuli, such as growth factors or cytokines. This suggests then that autocrine signaling in hypoxia is impaired. This can be due to lower production of cytokines in hypoxic cells, as has been observed in >5 h activation experiments, or to the reduced expression
of cytokine receptors. Future experiments to discern this would help better understand the TCR signaling defects in hypoxic T cells. Additionally, transcriptome analysis of effector T cells demonstrated a significant reduction of ERK/MAPK targets and MAPK signaling pathway, associated with metabolic control. Given the importance of these pathways on cell function, this suggests a global impact of hypoxia on T cells.

Our in vivo model further demonstrated the negative effects of hypoxia on T cell cytokine production. While after 5 days of activation in the LN T cells are expected to be terminally differentiated, those T cells cultured at either 21% or 5% were able to produce cytokines. In contrast, LN-derived T cells failed to produce cytokines in hypoxia. Importantly, these results further show that effector T cells activated in vivo, at physiological conditions, are impaired in hypoxia. It is thus expected that T cells that originate in the LN and travel to the hypoxic regions of the tumor would have a similar response upon encounter with antigen.

Although these results demonstrate that hypoxic CD8 T cells have a reduced capacity of producing and secreting pro-inflammatory cytokines, as shown by both flow cytometry of cells cultured with BFA and by ELISA, the level of regulation of this inhibition was not determined. This can be achieved by evaluating the expression of cytokines such as IFN-γ at the transcriptional level as well as the activity of potential transcription factors involved in their regulation. Given the reduction of ERK activation in hypoxia, it can be predicted that AP-1 activity is reduced in hypoxia as well. NFAT and NF-κB, which are critical for T cell activation and are downstream of TCR signaling\textsuperscript{397}, may also be affected. In addition, it would be interesting to learn the activation state of the Jak-STAT pathway in hypoxia, which is downstream of IL-2 and IL-15 signaling\textsuperscript{398}, the cytokines used in this study to stimulate CD8 T cells in culture.
In addition to altered cytokine production, reduced AICD following TCR activation further suggests that TCR signaling is compromised in hypoxia, as AICD is dependent on TCR signaling strength. Previous reports showed that anti-apoptotic proteins of the Bcl-2 family are increased by action of HIFα. In line with this, results show that Bcl-2 was upregulated in hypoxia while apoptosis-related genes were downregulated, providing an alternative explanation for reduced AICD in hypoxia. AICD is mediated by activation of the Fas pathway, leading to Bcl-2 cleavage by caspases. Moreover, Bcl-2 overexpression has been shown to partially prevent apoptosis mediated by antigen stimulation. Thus, reduction of AICD in hypoxia is likely due to inhibited TCR signaling enhanced by dysregulated apoptotic machinery.

However, another study in human T cells showed that AICD reduction at O$_2$ concentrations below 5% was associated with HIFα protein stabilization and an increase in the expression of adrenomedullin, which has been found in other instances to prevent AICD. In order to determine whether AICD is prevented solely by increased HIFα in hypoxic T cells, a similar experiment can be conducted at 21% O$_2$ in the presence of CoCl$_2$, or any other HIFα inducer. However, further analysis of hypoxic effector T cells upon TCR stimulation should be conducted to discern what other factors, including Bcl-2, transcription factors, caspases and even ROS production, may be involved in the control of apoptosis in hypoxia. One way this can be achieved is by conducting simultaneous transcriptomics and proteomics analyses of TCR-activated T cells in hypoxia and normoxia, which can now be performed at the single-cell level. Moreover, understanding if and how the factors that prevent AICD also inhibit TCR signaling activation and cytokine production would open the possibilities of modulating different pathways to trigger a stronger T cell response against antigen in hypoxia.
Mitochondrial Dysfunction and Metabolic Change in Hypoxia Lead to Poor Effector T Cell Proliferation and Function

Mitochondria play an essential role in T cell signaling, not only by generating ROS for fine-tuned TCR signal transduction, but also by regulating Ca\(^{2+}\) flux and participating in multiple metabolic pathways that determine energy production and usage\(^{406,407}\). In spite of the lower efficiency of ATP production, T cell activation induces a metabolic switch that favors glycolysis over OXPHOS\(^{408}\). Faster generation of ATP in the cytosol from glycolysis is thought to allow rapid growth and proliferation\(^{154}\). This phenomenon occurs upon TCR activation, which induces HIFα stabilization even when oxygen availability is not compromised\(^{409}\), promoting glycolysis. However, T cell proliferation decreases at lower O\(_2\) concentrations, especially at hypoxic levels, as demonstrated by my results. As will be discussed below, metabolic changes in hypoxia may lead to the poor proliferation observed in hypoxic cultures as compared to T cells in normoxia.

While HIFα expression is usually attributed to low O\(_2\) tensions, it was demonstrated that it occurs as a result of ROS production by the mitochondrial ETC\(^{295}\). In fact, TCR-induced ROS generation by mitochondrial complex III drives HIFα stabilization, which in turn induces glycolysis and cytokine production, among other effects, resulting in T cell activation\(^{176,406}\). ROS production also determines T cell fate after activation, and high levels result in cell death\(^{410,411}\). Interestingly, I found that TCR activation did not induce ROS increase in hypoxia, as it did in normoxia. This is perhaps a mechanism of the cell to avoid apoptosis.

Based on the common effects downstream of HIFα stabilization and TCR activation, one would expect enhanced effector T cell function in hypoxia. However, my results show that while chronic exposure to hypoxia induces higher glycolytic activity, it does not result in increased T cell function. High ΔΨ\(_m\) is often associated with higher ETC activity in the mitochondria and
thus, associated with OXPHOS-based metabolism. In hypoxia I found that despite increased
ΔΨm, T cells demonstrated both reduced OXPHOS-dependent ATP production and total cellular
ATP content. A similar phenotype has been observed in autoimmune diseases type I diabetes\textsuperscript{412}
and systemic lupus erythematosus (SLE)\textsuperscript{413}, where T cells show persistent mitochondrial
hyperpolarization: while normally the proton gradient created by the ETC across the inner
mitochondrial membrane is used to drive ATP formation, in this case the energy of ATP is used
by the F\textsubscript{0}F\textsubscript{1} ATP-synthase to transport protons outside the mitochondria against gradient, leading
to ATP depletion and ROS generation\textsuperscript{414}. Unlike what I observed in hypoxic T cells, high ROS
production in SLE induces T cell necrosis (causing inflammation in patients), while in type I
diabetes increased ROS leads to higher production of IFN-γ (and higher T cell reactivity)\textsuperscript{412,415}.

The level of accumulation of TMRE in the mitochondrion depends on the relative
difference in charges across the mitochondrial inner membrane\textsuperscript{416}. Thus, increased ΔΨm in
hypoxic T cells may be due to either a further decrease of the negative charge inside the
mitochondrion or an increase in the positive charge of the cytosol. The former may be induced
by higher proton transport out of the mitochondrion driven by the ETC, as described in SLE. The
latter could be caused by higher accumulation of positive ions in the cytosol. This is in line with
the increased baseline levels of cytosolic Ca\textsuperscript{2+} concentration I found in hypoxic mouse T cells. In
human T cells, a group showed that hypoxia induces a reduction in baseline cytosolic Ca\textsuperscript{2+}
concentrations due to inhibited expression of the potassium-activated Ca\textsuperscript{2+} channels Kv1.3,
resulting in depolarization of the plasma membrane\textsuperscript{417,418}. Although mouse T cells express
multiple members of the Kv1 channel family (suggesting a redundant function)\textsuperscript{419}, the RNA-seq
results revealed an increased expression of the gene encoding Kv1.3 channel (Kcna3). While
protein levels were not determined, higher presence of Kv1.3 channels would allow increased
Ca\(^{2+}\) entrance to the cell. Additional investigation is needed to understand the underlying mechanism of increased \(\Delta \Psi m\) in mouse T cells and the differences between mouse and human T cell behavior in hypoxia.

Mitochondrial hyperpolarization and elevated ROS content may suggest a better function of mouse T cells in hypoxia by improving TCR signaling and effector functions. Instead, I found a halt in T cell activity. TCR activation in hypoxic CD8 T cells resulted in a smaller relative increase of ROS content and \(\Delta \Psi m\) compared to normoxic controls, suggesting that baseline saturating levels of ROS and high \(\Delta \Psi m\) alter the threshold required for TCR signal transduction and impair it. This could then translate into lower cytokine production in hypoxic T cells.

While I found no differences in mitochondrial content or morphology between T cells cultured in normoxia and hypoxia, reduced OXPHOS in hypoxia suggests mitochondrial structural changes linked to reduced ETC use. Moreover, increased ROS generation in hypoxia has been shown to be mainly originated from the mitochondria\(^{217}\). Therefore, more loosely shaped mitochondrial cristae are expected in hypoxic T cells\(^{406}\), inducing a less efficient electron transport and ROS formation. Improved visualization of mitochondrial cristae structural detail in TEM images could help discern this. A previous report found that TILs in human head and neck squamous cell carcinoma have a significantly lower mitochondrial content compared to peripheral T cells\(^{420}\). While this study did not focus on hypoxia in the TME, TILs where found to have fewer mitochondria with a round shape, typical of glycolysis-based metabolism\(^{163}\). A similar approach including a hypoxia probe, such as PIMO, would further illustrate the regulation of mitochondria in tumor-infiltrating T cells entering hypoxia.

I found that in addition to low OXPHOS, hypoxic T cells had reduced content of important metabolic intermediates, suggesting inhibition of the TCA cycle, PPP and DNA
synthesis pathways. Uridine plays an important role in the pyrimidine de novo biosynthesis pathway and can promote DNA synthesis. Medium supplementation with this nucleotide in hypoxic T cell cultures resulted in partial rescue of cell proliferation. Additionally, I found that dihydroorotate, precursor to orotate, is increased in hypoxic T cells. The enzyme dihydroorotate dehydrogenase (DHODH) is responsible for this conversion, which occurs in the mitochondrial matrix and results in the generation of electrons that are taken up by ubiquinone and transported to complex III of the ETC. Orotate is then used to produce UMP, linking OXPHOS to the pyrimidine de novo biosynthesis pathway. Therefore, it would be no surprise for OXPHOS inhibition in hypoxia to be associated with reduced DHODH activity, resulting in the accumulation of dihydroorotate and thus, reduced pyrimidine synthesis. Supplementing the cell culture with uridine likely circumvents the production of UMP from orotate, promoting DNA replication and cell proliferation.

As previously described, effector T cells under normal physiological conditions have increased glycolysis to support T cell proliferation by providing rapid ATP production while keeping low levels of OXPHOS. However, the importance of mitochondrial function for the generation of macromolecules in proliferating T cells is more apparent in hypoxia, where T cells become more glycolytic but lack the necessary metabolic intermediates to sustain their effector function.

The overall model of the effects of hypoxia on effector CD8 T cells is shown in Fig. 48.
**Fig. 48. Multiple Metabolic and Signaling Pathways Are Altered in Hypoxic Effector CD8 T Cells.** Results in this study show a reduction in TCR signaling strength, while checkpoint inhibitors, such as PD-1, are upregulated. Altered OXPHOS and ETC regulation lead to high ROS production, while other metabolic pathways are inhibited. These include the TCA cycle, a major pathway in the production of macromolecules that promote cell division and function. As a result, reduced nucleic acid synthesis may inhibit T cell proliferation in hypoxia. In line with this, there is an increase in glycolysis, presumably due to HIFα activity. While glycolysis, ROS production and increased Ca²⁺ flux are associated with cytokine production and improved T cell effector function, the data indicate that high baseline levels do not result in improved function upon TCR activation. Red arrows show increase, blue arrows show decrease.

**Suffocated CD8 T Cell Phenotype Shares Characteristics of Exhausted T Cells**

I have found certain similarities between the phenotype of hypoxic T cells I describe here and that of exhausted T cells reported in the literature. Among these, poor proliferation, low production of pro-inflammatory cytokines, especially IFN-γ, TNF-α and IL-2, and upregulation of activation/exhaustion markers have been shown to characterize dysfunctional or exhausted T cells in chronic disease¹³¹,¹⁴¹,²²⁴. However, some critical differences are worth pointing out.
Exhaustion is often associated with increased production of suppressive cytokines, including IL-10\textsuperscript{131}. Interestingly, CD69 is highly expressed in exhausted T cells\textsuperscript{224} and seems to play an important role in regulating TIL exhaustion\textsuperscript{425}. Metabolic changes also influence this dysfunctional state. PD-1, a major regulator of exhaustion, has been shown to inhibit effector CD8 T cell function and regulate T cell metabolism\textsuperscript{141}. Exhausted T cells have reduced use of OXPHOS and mitochondrial depolarization\textsuperscript{141}. While Glut-1 expression is enhanced\textsuperscript{426}, glucose uptake and glycolysis is reduced in exhausted T cells\textsuperscript{141}. Surprisingly, mitochondrial depolarization was associated with increased ROS production and apoptosis. Partial reversal of the exhausted phenotype has been observed upon PD-1 blockade in vivo. Accordingly, higher presence of ROS in the tumor due to hypoxia inhibits T cell function and can induce T cell apoptosis\textsuperscript{131,427}.

Although ROS content was higher in hypoxic T cells, I did not see increased apoptosis in this study. As explained, HIFα has a protective action on hypoxic cells, inducing the expression of survival genes and promoting metabolic changes that allow cells to adapt to low O\textsubscript{2} environments\textsuperscript{399,409}. While hypoxic T cells were shown to have altered metabolic and mitochondrial function, mitochondrial hyperpolarization indicates conserved membrane integrity of the organelle. PD-1 can regulate T cell metabolism and it has been suggested that upregulation of this checkpoint receptor in the tumor can further drive T cell suppression by inducing OXPHOS and fatty acid oxidation\textsuperscript{428}. However, a study that used the B16 model to evaluate mitochondrial content in TILs found that mitochondrial content in the tumor is reduced, corresponding to a more glycolytic metabolism, and that PD-1 blockade treatment did not rescue this phenotype\textsuperscript{420}. 
Overall, while the phenotypes of exhausted and suffocated T cells do not overlap in all aspects, both T cell states are critically relevant in anti-tumor T cell responses. As described before, the suppressive characteristics of the TME are enhanced by poor oxygenation of the tissue. Therefore, it is likely that hypoxic TILs acquire a more severe exhausted phenotype than their non-hypoxic counterparts. It remains to be shown whether the suffocated phenotype is fully reversible. However, strategies directed at improving hypoxic T cell function may also decrease T cell exhaustion in this population. For example, modulation of the nucleotide synthesis pathway is likely to also enhance exhausted T cell proliferation. While PD-1 blockade did not seem to induce further activation of hypoxic T cells in my in vitro experiments nor change mitochondrial status in vivo\textsuperscript{420}, this therapeutic approach may induce further T cell infiltration in hypoxic areas of the tumor. On the other hand, while effector function of hypoxic T cells was enhanced by increasing TCR-pMHC affinity, T cells transduced with high affinity TCRs or CARs tend to become exhausted more quickly in the tumor\textsuperscript{429,430}.

**Culture Conditions and Inconsistent Results Across Studies on Effects of In Vitro-Induced Hypoxia on T Cells**

Previous reports on T cells subjected to hypoxia suggest a variety of conclusions that seem to be highly dependent on culture conditions. Typically, in vitro T cell expansion takes advantage of aCD3 and aCD28 agonist antibodies, which provide signals 1 and 2, respectively, for T cell activation. Culture supplementation with IL-2 has been shown to induce T cell proliferation and differentiation\textsuperscript{431,432}, while addition of IL-15 prolongs the life of T cells\textsuperscript{431,433,434}. However, the concentrations of each of these factors along with the choice of culture medium, supplementation with or without IL-15 or other cytokines, percentage of serum used and other potential additives can lead to different outcomes in terms of activation level,
composition of the T cell population and survival time in culture. In this study, I activated spleen-derived T cells with a set of conditions that result in the preferential expansion of CD8 T cells, including supplementation with both IL-2 and IL-15, in addition to aCD3 and aCD28 stimulatory antibodies. β-mercaptoethanol (2-ME) is a reducing agent commonly used in mouse T cell cultures to improve T cell activation and survival\textsuperscript{435}. A report showed no differences upon 2-ME addition when T cells were cultured at 7 or 21\% O\textsubscript{2}\textsuperscript{436}. However, in experiments studying the effects of hypoxia (1\% or lower), addition of a reducing agent may lead to altered results by preventing the formation of O\textsubscript{2} radicals\textsuperscript{437}, a characteristic of hypoxic environments.

A critical parameter that distinguishes the culture conditions used here from others is the concentration of aCD3, aCD28 and IL-2 used. Given the artificial nature of these growth factors, the concentration and combination used is widely arbitrary. With my strategy, I intended to mimic the in vivo expansion of T cells before the entrance of activated T cells to the tumor microenvironment. In fact, I confirmed the failure of T cells to produce cytokines with my in vivo model to generate cytotoxic T cells.

However, reports describing an improved function of T cells in hypoxia utilize much higher concentrations of these factors (up to 500 times greater), likely supplying signals and stimuli far outside physiological levels. An example of this is a study in which the authors attained enhanced proliferative, lytic and anti-tumor functions by priming naïve OT-I CD8 T cells in hypoxia\textsuperscript{438}. While this outcome might not be representative of physiological conditions, it provides a potential strategy to overcome the effects of hypoxia in the tumor.

**Therapeutic Aspects of T Cell Function in Hypoxia**

While anti-cancer immunotherapies have improved the overall T cell response against the tumor by manipulating TCR affinity, inducing co-stimulatory signals, taking advantage of
neoantigen-specific TCRs and blocking inhibitory receptors, most of the patients treated with ACT are not responsive\textsuperscript{439}. Given the low frequency of immunogenic tumor neoantigens, low affinity TCRs represent the majority of tumor-specific T cells in patients\textsuperscript{440}. In addition, an inherent factor of solid tumors that is often overlooked when studying anti-tumor T cell responses is hypoxia. Depending on the size and type, tumors have varying degrees of hypoxia, and negative clinical outcomes are correlated with levels of hypoxia in the tumor\textsuperscript{227}. Mouse models have shown that tumors as wide as 1 mm can be hypoxic (O$_2$ measured at ~0.7\%)\textsuperscript{441}. Here, I show that hypoxia inhibits T cell response against antigen, especially in low affinity TCR-pMHC interactions. Moreover, human T cells transduced with the Tyro-reactive TCR 1383I\textsuperscript{286} (a CD8-independent, high affinity TCR) failed to respond to antigen stimulation in hypoxia. While further investigation on CAR-T cells in hypoxia might shed some light on their inability to clear solid tumors, one published study showed that anti-CD19 CAR-T cells have impaired function in hypoxia\textsuperscript{442}. A different study using anti-GD1 neuroblastoma CAR-T cells showed improved function at low O$_2$ (2.5\%)\textsuperscript{284}, a physiologically relevant concentration. While there is a risk of higher immunotoxicity caused by the use of high affinity TCRs, this approach partially rescued suffocated CD8 T cells in hypoxia. Moreover, cytotoxicity of hypoxic T cells in the tumor and its relation to T cell response-modulating factors needs to be further investigated.

A comparison of tumor infiltration level based on O$_2$ availability between tumor-reactive T cells with different TCR-pMHC affinities would help further identify potential issues T cells encounter in the TME. In addition, further investigation is needed to compare the infiltration of T cells in the hypoxic regions of the tumor depending on the expression of TAAs, neoantigens or self-antigens by the tumor. This study focused on endogenous T cells that spontaneously enter the tumor. However, it would be interesting to learn how tumor-reactive vs. other T cells that are
attracted to the TME are distributed in non-hypoxic and hypoxic areas. Increased presence of CD8 T cells with low expression of CD62L and upregulation of PD-1 and TIM-3 were found in hypoxic areas; this population may potentially be tumor-reactive and relevant to the anti-tumor response in comparison to cells located in the non-hypoxic TME. Isolation and TCR sequencing of these T cells could be employed to determine their reactivity and the level of TCR affinity for tumor antigens.

My results on TCR-transduced T cells highlight hypoxia as a critical aspect of the TME. Including the evaluation of in vitro-expanded T cells under both physiological (5% O$_2$) and hypoxic (<1% O$_2$) conditions as part of pre-clinical ACT tests can be highly beneficial for therapeutic design. Similarly, measurement of tumor hypoxia levels in relation to T cell tumor infiltration can be useful for patient evaluation. The hypoxia probe pimonidazole is not toxic and has been used in humans before$^{227}$. It can be administered orally or intravenously in patients$^{230}$ prior to the collection of biopsies. O$_2$ levels in the tumor may constitute a prediction factor of the anti-tumor response. This would allow to determine not only the infiltration of T cells, but also other suppressive populations usually found in the TME, such as MDSCs, Tregs, CAFs and TAMs, among others.

Additionally, more pre-clinical data on such suppressive populations and their spatial distribution in the TME are needed. As explained before, most of the studies available that focus on the evaluation of hypoxic cells in solid tumors use HIFα as a surrogate to measure their exposure to hypoxia. Therefore, models using pimonidazole would overcome this issue and would provide a better understanding of the interactions between different cell types in the tumor as well as their physical localization respect to O$_2$ levels. This type of study would further our knowledge on potential targets in the TME that can be applied to the clinic in the future.
APPENDIX A:

SUPPLEMENTAL FIGURES
**Fig. A.1. Representative Gating Strategy of A Tumor Sample.** Size-based gating was done in SSC vs. FSC plot, from which live CD3$^+$ cells were selected. Live CD3$^+$ cells were further divided into CD4 or CD8 T cells, as well as in PIMO$^+$ (hypoxic) and PIMO$^-$ (non-hypoxic). A time gate is also shown for CD3$^+$ cells. Top: fully stained sample from mouse injected with PIMO. Bottom: fully stained sample from negative control mouse injected with only PBS.
Fig. A.2. Representative Gating Strategy of In Vitro-Activated Spleen-Derived T Cells.

Effector T cells generated in vitro from whole B6 splenocytes were cultured for five days at either 21% or 0.5% O₂. Flow cytometry plots shown from Day 10 of culture. Size-based gating was done in SSC vs. FSC plot, from which single cells were selected. Quantification of total live cells or total CD8 T cells was done within this population. Top: cells cultured in normoxia. Bottom: cells cultured in hypoxia.
**Fig. A.3. Increased Bcl-2 Expression in Hypoxic CD8 T Cells.** Effector T cells generated in vitro from whole B6 splenocytes were cultured for two days at either 21% or 0.5% O₂. Left: Histograms show Bcl-2 expression of cells cultured at 21% or 0.5% O₂ and an isotype control. Plots shown were gated on live CD8 T cells. Right: Summary results of Bcl-2 expression based on the MFI. Each exp. was performed with at least 3 independent samples. Paired t-test analysis, P< 0.05.

**Fig. A.4. Hypoxic CD8 T Cells Have Increase PD-1 and Tim-3 Upregulation.** Effector T cells generated in vitro from whole splenocytes were cultured for two days at either 21% or 0.5% O₂.

- **a.** PD-1 and Tim-3 flow cytometry plot gated on CD44+CD62L- CD8 T cells at either 21% or 0.5% O₂.
- **b.** Summary results of frequency of PD-1+ and Tim-3+ CD8 T cells (top) and PD-1 MFI of PD-1+ and Tim-3 MFI of Tim-3+ CD8 T cells (bottom).

Each exp. was performed with at least 3 independent samples. Paired t-test analysis, P< 0.05.
Fig. A.5. Hypoxic CD8 T cells have reduced CD69 and IRF4 Upregulation Upon TCR Re-stimulation (Repeat 2). Summary results of CD69 and IRF4 expression in live effector-like CD8 T cells. Effector T cells generated in vitro from whole splenocytes were cultured for two days at either 21% or 0.5% O2 and re-stimulated with aCD3 or IgG Ab control. Top panel shows the proportion of CD69+ and IRF4+ cells and bottom panel shows the MFI levels of each protein within the respective positive population. Each exp. was performed with at least 3 independent samples. Paired t-test analysis, P< 0.05.
Fig. A.6. Hypoxic CD8 T cells have reduced CD69 and IRF4 Upregulation Upon TCR Restimulation (Repeat 3). Summary results of CD69 and IRF4 expression in live effector-like CD8 T cells. Effector T cells generated in vitro from whole splenocytes were cultured for two days at either 21% or 0.5% O₂ and re-stimulated with aCD3 or IgG Ab control for 4 h. Top panel shows the proportion of CD69⁺ and IRF4⁺ cells and bottom panel shows the MFI levels of each protein within the respective positive population. Each exp. was performed with at least 3 independent samples. Paired t-test analysis, P < 0.05.
Fig. A.7. Reduced ERK Activation in Hypoxic CD8 T Cells. Effector T cells generated in vitro from whole splenocytes were cultured for two days at either 21% or 0.5% O2 and re-stimulated with aCD3. ERK phosphorylation was measured after 1 hour of re-stimulation with aCD3 activation during the specified times at 21% or 0.5%. a. Western blot analysis of one experiment. b. Summary results show the band area of phosphorylated (left) and total (right) ERK of the same experiment. Phospho-ERK (pERK) bands were normalized to total ERK protein bands.

Each exp. was performed with at least 3 independent samples. Paired t-test analysis, P< 0.05.
Repeat 2

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Fig. A.8. Reduced AICD in Hypoxic CD8 T Cells. Effector T cells generated in vitro from whole splenocytes were cultured in the presence of activating aCD3 mAb or IgG control for 48 h at either 21% or 0.5% O2. Proportion of dead cells was determined. a. Gating of dead cells in flow cytometry plots of one sample cultured with IgG control Ab (top) or aCD3 (bottom). Cells were gated on total CD8 T cells. b. Summary results of dead cell proportion in cultures stimulated with IgG control (top) or aCD3 (bottom).

Each exp. was performed with at least 3 independent samples. Paired t-test analysis, P< 0.05.
Fig. A.9. Lower Proportion of Cytokine-Producing Effector CD8 T Cells in In Vitro-Induced Hypoxia. Effector T cells generated in vitro from whole splenocytes were cultured for two days at either 21% or 0.5% O₂ and re-stimulated overnight with IgG control or aCD3 mAb. Graphs show the proportion of total IFN-γ-, TNF-α- and IL-2-producing effector CD8 T cells. Each exp. was performed with at least 3 independent samples. Paired t-test analysis, P< 0.05.
Fig. A.10. Increasing TCR Stimulation Induces Higher Cytokine Production in Hypoxic T Cells. Effector T cells generated in vitro from whole splenocytes were cultured for two days at either 21% or 0.5% O2 and re-stimulated overnight with IgG control or aCD3. Supernatant was collected and analyzed. Graphs show the concentration of cytokines/chemokines produced by B6 cells, assessed by bead-based multiplexing assay. Cytokines represented in graphs based on low, medium or high range of concentration.

Fig. A.11. PD-1 Blockade Does Not Improve Cytokine Production in Hypoxic T Cells In Vitro. Effector T cells generated in vitro from whole splenocytes were cultured for two days at either 21% or 0.5% O2 with a blocking anti-PD-1 antibody (aPD-1) or IgG control (Ctrl.). Cells were then re-stimulated overnight with IgG control or aCD3 mAb. Summary of results. Each exp. was performed with at least 3 independent samples. Paired t-test analysis, P< 0.05.
Fig. A.12. Increasing TCR Stimulation Induces Higher Cytokine Production in Hypoxic T Cells. Effector T cells generated in vitro from whole splenocytes were cultured for two days at either 21% or 0.5% O₂ and re-stimulated overnight with IgG control or increasing concentrations of αCD3. Graphs show the proportions of cytokine-positive cells comparing cells in normoxia (21% O₂; red) to hypoxia (0.5% O₂; blue). Cells were gated on live CD44+CD62L- CD8 T cells. Each exp. was performed with at least 3 independent samples. Paired t-test analysis, P< 0.05.
Fig. A.13. Re-stimulation with PMA/Ionomycin Induces Higher Cytokine Production in Hypoxic T Cells Than TCR-Dependent Stimulation. Effector T cells generated in vitro from whole splenocytes were cultured for two days at either 21% or 0.5% O₂ and re-stimulated overnight with either aCD3 antibody or PMA/Ionomycin (PMA/Iono) in normoxia (21%) or hypoxia (0.5%). Graphs show the proportions of cytokine-positive cells comparing cells in normoxia (21% O₂; red) to hypoxia (0.5% O₂; blue). Cells were gated on live CD44+CD62L-CD8 T cells.

Each exp. was performed with at least 3 independent samples. Paired t-test analysis, P< 0.05.
Fig. A.14. Reduced Proportion of Multifunctional CD8 T Cells in Hypoxia. Effector T cells generated in vitro from whole OT-I splenocytes were cultured for two days at either 21% or 0.5% O₂ and re-stimulated overnight with one of four OVA variants with different affinities (N4, Q4, T4, V4) in normoxia (21% O₂) or hypoxia (0.5% O₂). Pie charts show the average proportion of 0-, 1-, 2- and 3-cytokine-producing CD44⁺CD62L⁻ CD8 T cells. Each exp. was performed with at least 3 independent samples. Paired t-test analysis, P< 0.05.
**Fig. A.15. In Vivo-Generated Effector T Cells Fail to Produce IFN-γ Under Hypoxic Conditions.** Effector T cells generated in vivo from whole OVA-immunized B6 mice were cultured for two days at either 21% or 0.5% O₂ and re-stimulated overnight with the OVA peptide (N4; left) or PMA/Ionomycin (PMA/Iono; right) in normoxia (21% O₂), physioxia (5% O₂) or hypoxia (0.5% O₂). Graphs show ELISA results of IFN-γ supernatant concentration. Each exp. was performed with at least 3 independent samples. Paired t-test analysis, P< 0.05.

**Fig. A.16. Hypoxia Drives High ROS Production in T Cells.** Effector T cells generated in vitro from whole B6 splenocytes were cultured for two days at either 21% or 0.5% O₂ and treated overnight with either aCD3 antibody or IgG control in normoxia (21%) or hypoxia (0.5%). Graphs show summary of H₂DCFDA MFI comparing CD8 T cells in each condition. Each exp. was performed with at least 3 independent samples. Paired t-test analysis, P< 0.05.
**Fig. A.1.** Hypoxia Induces High ΔΨm in T Cells. Effector T cells generated in vitro from whole B6 splenocytes were cultured for two days at either 21% or 0.5% O₂ and treated overnight with either aCD3 antibody or IgG control in normoxia (21%) or hypoxia (0.5%). Graphs show summary of TMRE MFI comparing cells in each condition. Each exp. was performed with at least 3 independent samples. Paired t-test analysis, P< 0.05.

**Fig. A.18.** High Cytosolic Ca²⁺ in Hypoxic T Cells. Effector T cells generated in vitro from whole B6 splenocytes were cultured for two days at either 21% or 0.5% O₂. Then, cells were loaded with Ca²⁺ probe Fluo-4 AM and treated with ionomycin (Iono) or PBS (not treated). Flow cytometry analysis of Ca²⁺ content comparing cells cultured in normoxia (21% O₂; red) and hypoxia (0.5% O₂; blue) treated with Iono (left) or PBS (right). Summary graph shows Fluo-4 AM MFI comparing Iono-treated cells at 21% O₂ or 0.5% O₂. Each exp. was performed with at least 3 independent samples. Paired t-test analysis, P< 0.05.
Fig. A.19. Increased Glucose Uptake in Hypoxic Effector T Cells. Effector T cells generated in vitro from whole B6 splenocytes were cultured for two days at either 21% or 0.5% O₂. Cells were then glucose-starved and incubated with glucose analog 2-NBDG to measure glucose uptake. Left: Flow cytometry analysis gated on live CD8 T cells. Right: Summary of 2-NBDG MFI results in cells cultured under each condition. Each exp. was performed with at least 3 independent samples. Paired t-test analysis, P< 0.05.
Repeat 2

OCR IgG

ECAR IgG

OCR CD3

ECAR CD3

Fig. A.20. Reduced Mitochondrial O2 Consumption in Hypoxic Effector T Cells. Effector T cells generated in vitro from whole B6 splenocytes were cultured for two days at either 21% or 0.5% O2. Whole cultures under either condition were then prepared for Seahorse analysis. Graphs show OCR and ECAR curves of each treatment (IgG: IgG-coated beads; aCD3: aCD3-coated beads) from Seahorse analysis of T cells cultured in normoxia (red) or hypoxia (blue). Each exp. was performed with at least 3 independent samples. Paired t-test analysis, P< 0.05.
Fig. A.21. Partial Rescue of Hypoxic T Cell Proliferation by Uridine Supplementation. Effector T cells generated in vitro from whole B6 splenocytes were cultured for three days at 0.5% O₂ with increasing concentrations of uridine. Total number of live (left) and dead (right) cells in cultures assessed by Trypan blue exclusion in hypoxia. Each exp. was performed with at least 3 independent samples. Paired t-test analysis, P< 0.05.
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VITA

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In August 2013, Lourdes enrolled in the Biochemistry and Molecular Biology Master’s program at Loyola University Chicago in Dr. Jose Alejandro Guevara’s laboratory. In 2014, she switched to the PhD program within the same track. She completed her PhD in Dr. Guevara’s laboratory, where she studied the effects of tumor-associated hypoxia on effector CD8 T cells. After completion of her graduate studies, Lourdes will continue her postdoctoral research in the Guevara Laboratory, where she plans to further study the molecular mechanisms that cause T cell dysfunction in hypoxia.