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Mechanisms Behind Hypoxia-Driven Resistance to Immunotherapy in the Tumor Microenvironment

Alexandra Meyer[1] and Luke Riggan[#]

[1]Phillips Exeter Academy
[#]Advisor

1. ABSTRACT

Tumor formation requires rapid proliferation of malignant cells, which consume large amounts of oxygen from the microenvironment to meet metabolic demands. The resulting tumor microenvironment (TME) is usually low in oxygen compared to healthy tissue and left in a hypoxic state. Immune cells in the tissue rely on oxygen for energy production, therefore immune function is often inhibited in the TME. Novel immunotherapy treatments aim to reinvigorate the immune system, thus making hypoxia a concrete barrier against immunotherapeutics targeting solid tumors. Furthermore, oxygen levels are highly variable depending on the tissue, raising the question of the influence of physoxia on immune cell survival in hypoxic counterparts. This review aims to provide insight into the mechanisms that influence this question, using an in-silico approach, in order to understand how the field can improve immunotherapy treatments for patients.

2. Introduction to the Immune System

The TME consists of many components, including tissue-specific tumor cells and stromal cells such as fibroblasts, endothelial cells, and immune cells. Additionally, non-cellular components such as collagen, fibronectin, hyaluronan, and laminin are present in the TME, which stem from the extracellular matrix (Baghban et al., 2020). Innate immune cells such as dendritic cells, natural killer (NK) cells, macrophages, myeloid-derived suppressor cells (MDSCs), and neutrophils can all be found in the TME, while adaptive cells such as B and T cells are also present (Hinshaw & Shevde, 2019). Dendritic cells initiate adaptive immune responses by identifying and presenting antigens to T cells (Granucci et al., 2005), making them vital to antitumor immunity (Veglia & Gabrilovich, 2017). NK cells were identified as "natural killers" due to their ability to release perforins and granzymes to kill cancerous and stressed cells without any prior antigen sensitization (W. Hu et al., 2019; Yoon et al., 2015). Where NK and dendritic cells are known for their anticancer properties, MDSCs are specific to the TME and suppress T cell function to promote tumor growth. Derived from hematopoietic stem cells (HSCs), myeloid precursor cells transform into immature myeloid cells (IMCs), which generally differentiate into macrophages, DCs, and granulocytes. In the TME, IMCs have been found to differentiate into colonies of MDSCs instead (Gao et al., 2021). MDSCs are targeted to improve T cell function against cancer, and thus many T cell-focused immunotherapies (Gabrilovich & Nagaraj, 2009). Multiple groups have shown MDSCs to suppress activated intertumoral T cells by inhibiting IL-2 production (Almand et al., 2001; Diaz-Montero et al., 2009; Ostrand-Rosenberg & Sinha, 2009). Additional studies in mice showed that MDSCs might also block CD8+ and CD4+ T cell activation (Bronte et al., 1998; Gabrilovich et al., 2001; Mazzoni et al., 2002; Ostrand-Rosenberg & Sinha, 2009; Sinha et al., 2005). Macrophages are immune cells derived from Macrophage Dendritic Cell Precursors (MDPs) with two polarizing functions—M1 and M2. M1 macrophages increase the release of cytokines to elicit an inflammatory response and destroy pathogens. Conversely, M2 macrophages trigger cell proliferation and repair processes (Orecchioni et al., 2019). The two subtypes also have polarizing influences on the

onset of a tumor: M1 cells promote cytotoxicity in an antitumor response, whereas M2 cells aid tumor metastasis and inhibit anti-tumor T cell function (K. B. Long & Beatty, 2013; Pan et al., 2020).

Both B cells and T cells are present in the TME, the two key players of adaptive immunity that also interact with the innate immune system. T cells are derived from HSCs and travel to the thymus for development. All T cells possess T cell receptors (TCRs) complementary to a specific antigen. T cells are presented with their antigen fragment by antigen-presenting cells (APCs) through an MHC molecule, which holds the peptide antigen fragment and binds to the TCR to activate the T cell. When this occurs, the T cell, classified as naïve until it interacts with its specific antigen, proliferates and differentiates into an activated T cell which can then begin an immune attack on the pathogen. TCR - MHC binding requires the assistance of co-receptors, specifically CD4 or CD8 (Seder & Ahmed, 2003). CD8+ T cells differentiate into cytotoxic T cells; alternatively, CD4+ T cells develop into helper T cells. Cytotoxic CD8+ T cells recognize antigen fragments when presented by MHC-I complexes, triggering activation and the release of cytotoxic granules into their targets (Seder & Ahmed, 2003). The assistance of many signals from APCs and helper T cells may be needed to enlist CD8+ T cells for activation. In a $CD4^{-/-}$ knockout mouse model, which lacks helper T cells, a CD8+ response would still be present, although dampened (Caruso et al., 1999). Conversely, a knockout mouse model which lacks APCs will undergo next to zero activation (Velilla et al., 2006). Helper CD4+ T cells have many subtypes, although all are activated when presented with antigen fragments on MHC-II molecules by APCs (Seder & Ahmed, 2003). CD4+ T cells may release cytokines, activate immune cells, and aid in B cell antibody production to regulate the adaptive immune response. Regulatory T cells (Tregs) are another subcategory of T cells that may be either CD4+ or CD8+. Tregs inhibit T cell function to regulate the immune response and maintain homeostasis, thus inhibiting autoimmune development (Kondělková et al., 2010). Tregs may also inhibit the anti-tumor immune response through such mechanisms, and are often found near solid tumors due to their chemical attraction to chemokine gradients found in the TME (Ohue & Nishikawa, 2019). Recent literature has proposed that the following may be mechanisms of Treg suppression: regulation of co-stimulatory molecules such as CTLA-4/CD80/CD86 that may prevent T cell interaction with APCs (Grosso & Jure-Kunkel, 2013; Ovcinnikovs et al., 2019, p. 4), various MHC II complexes and receptors (Andrews et al., 2017, p. 3; Liang et al., 2008; Piechnik et al., 2013), granzymes/perforins (C.-H. Li et al., 2011), cytokine secretion of IL-10 (Mittal et al., 2015), TGF-ß (Wrzesinski et al., 2007), and IL-35 (Bettini et al., 2012; Pylayeva-Gupta, 2016), as well as IL-2 reception (Busse et al., 2010).

B cells are less versatile than T cells but still serve a crucial role by producing antibodies that bind to antigens and either neutralize or destroy invaders. Although not all B cell function is reliant on T cell signaling, T cells are capable of activating B cells to begin antibody production (Hoffman et al., 2016). Many studies have also pointed to B cell antitumor properties, although more research must be done before a definitive conclusion can be made regarding their role in the TME (Downs-Canner et al., 2022).

3. Immunotherapy

The goal of immunotherapy is to reinvigorate the antitumor immune response. The two main approaches include immune checkpoint blockade (ICB) which aims to reduce suppressive signals in the TME as well as chimeric antigen receptor T (CAR-T) cell treatments which add proinflammatory T cells specific to tumor antigens.

3.1 Immune Checkpoint Blockade

When a pathogen is detected, APCs, primarily dendritic cells and macrophages, present antigen fragments to complementary T cells (Hamilos, 1989). TCR engagement triggers signaling pathways that result in cytokine production (IL-

1, TNFs, Interferons, IL6, IL10, and TGF-ß) (DeLeo et al., 1996; C.-M. Hu et al., 2002; Lappin et al., 2001; Y.-J. Liu, 2007; Ozaktay et al., 2006), cell proliferation/differentiation, and cell survival (figure 1). TCR activation directly correlates to the expression of surface inhibitory receptors found on the T cell. Since the T cell is highly activated, it assumes that the detected pathogen is being successfully suppressed, thus causing the T cell to shut down through an inhibitory protein complex as a method to prevent any autoimmune activity (Simon & Labarriere, 2017, p. 1).

During tumor pathogenesis, immune cells in the TME are suppressed due to the engagement of surface inhibitory receptors on various immune cells (Korman et al., 2022). The ligands to these receptors can be found directly on tumor cells or other suppressive immune cell subsets (Muñoz-Fontela et al., 2016). Allison and colleagues found the first T-cell attenuating receptor in 1996, known as cytotoxic T lymphocyte antigen 4 (CTLA4) (Korman et al., 2022). CTLA4 has two ligands, CD80 and CD86 (Sansom, 2000, p. 28), although it is unclear why two ligands exist and the differences in their functions (Halliday et al., 2020, p. 86). Many other inhibitory receptors have since been defined on T cells, NK cells and macrophages (Zarrin & Monteiro, 2020). The PD-1/PDL-1 protein interaction is one such example that is well understood in the field. PD-1 is a transmembrane protein expressed on T and other immune-related cells. Under normal immune function, PD-1 binds PD-L1 to help control the body's immune response, preventing autoimmunity. Although commonly found on APCs, tumor cells may also express PD-L1 which inhibits the anti-tumor immune response and promotes tumor metastasis (figure 1).

Immune checkpoint blockade (ICB) immunotherapy targets inhibitory receptors such as CTLA4, PD-1, and PDL-1 to stop cancer cells from deterring immune activation. ICB therapy blocks inhibitory ligands on tumor cells from binding with their complementary surface receptors on T cells, thus stopping immune function from being blocked in the TME (figure 2). ICB has been an FDA-approved therapy since 2011 (Wei et al., 2018), beginning with anti-CTLA 4 before the innovation of six PD-1/PDL-1 targeted therapeutics. Standard approved treatment for PD-1/PDL-1 receptors using ICB includes monoclonal antibodies known as mAbs (Twomey & Zhang, 2021). Monoclonal antibodies consist of two sets of polypeptides, two light and two heavy. Disulfide linkages warp the protein into a Y-shape with a molecular weight of approximately 150kD (Rosenberg, 2015). Three anti-PD-1 and three anti-PDL-1 mAbs have been approved by the FDA. Pembrolizumab, nivolumab, and cemiplimab are antibodies used to combat PD-1 receptors, while atezolizumab, durvalumab, and avelumab are targeted against PDL-1 ligands (Twomey & Zhang, 2021). Novel research also seeks to combine ICB therapies with other immunotherapeutics for maximal results (Twomey & Zhang, 2021).

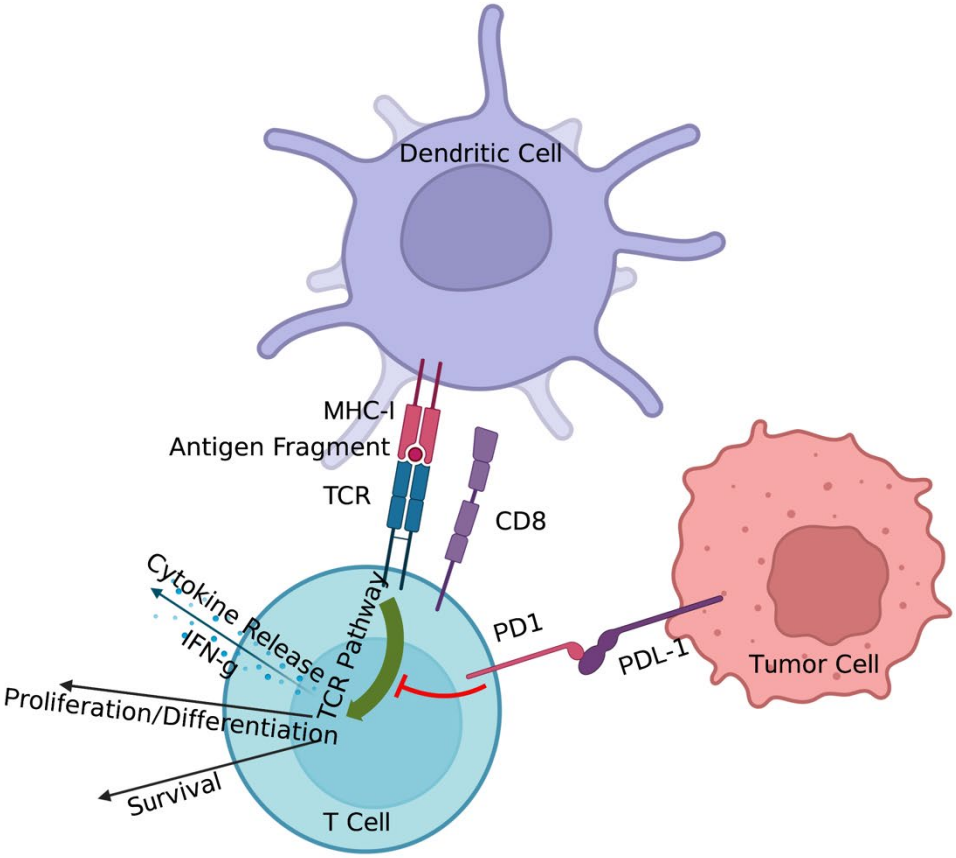

Figure 1: T Cell Interaction with APCs vs Tumor Cells

In this figure, the dendritic cell, acting as an APC, presents an antigen fragment to the T cell on the MHC-I complex. T cell receptors (TCRs) bind to the MHC molecule, triggering the release of cytokine IFN-γ along with cell proliferation/differentiation and cell survival (Twomey & Zhang, 2021). When the T cell identifies an antigen fragment, it recognizes a threat in its environment and thus triggers an immune response against it. Thus, the cell must promote its survival and proliferation to contribute to the immune attack and release cytokines that aid pathogen destruction. T cells express the inhibitory receptor PD-1 to modulate immune activity. When cytokine production increases, the T cell subsequentially expresses greater numbers of PD-1 molecules under the assumption that the number of pathogens has decreased. Thus, it increases the probability that PD-1 will bind to its complementary ligand PD-L1, inhibiting cell proliferation, activation, and cytokine production. PD-1 activation causes the cell to uptake phosphatase SHP-2 molecules near TCRs, causing dephosphorylation and attenuation in TCR pathways that inhibit their function (Ai et al., 2020, p. 1). Select tumor cells also express PD-L1, inhibiting T cell function in the TME and decreasing immune response against the tumor.

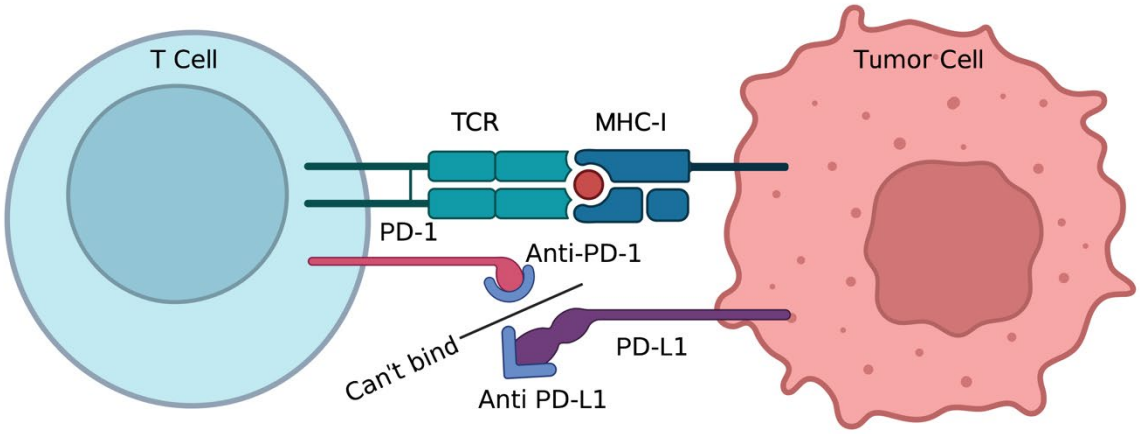

Figure 2: Immune Checkpoint Blockade as Combatant against PD-1/PD-L1 on Tumors

Without interference from the PD-1/PD-L1 protein complex, T cells register a tumor cell through its TCR binding to an MHC molecule, which triggers an immune response. A significant barrier in targeting immunotherapeutics against solid tumors is that many tumor cells possess PD-L1 ligands. When PD-1 proteins on T cells and other lymphocytes bind to PD-L1, it initiates an inhibitory pathway that terminates T cell activation and, thus, the antitumor response. Immune checkpoint blockade (ICB) is an immunotherapeutic that binds to PD-1 or PD-L1, respectively, blocking the protein from forming a complex with its complementary receptor or ligand. Anti-PD-1 binds directly to PD-1 on T and other immune cells to stop it from binding to PD-L1 on tumors. Conversely, Anti-PD-L1 binds to the ligand on tumor cells to inhibit binding to PD-1. Both serve as an effective form of immunotherapy for a subset of cancers.

3.2. CAR-T

Chimeric Antigen Receptor T cells (CAR-T) are a method of immunotherapy that genetically engineers a patient's T cells *in vitro* to express chimeric antigen receptors (CARs). CARs are designed using components of mAbs to identify specific tumor-related antigens, allowing T cells to quickly identify cancerous cells and initiate an immune response against them. CAR-T cells were first developed in 1993 by Zelig Eshhar who invented what is regarded as the 1st generation of CAR-T (Eshhar et al., 1993). The 1st generation's short longevity made it difficult to implement in clinical trials, resulting in the second generation in 2003. The second generation was the first time CD14 was the target of CAR-T cell receptors, leading to eventual clinical success in 2011 (Wang et al., 2018). CD14 antigens are commonly expressed in malignant cancers, resulting in more inflammation in the TME and increased cytotoxicity (Cheah et al., 2015, p. 14). Since the development of 4th generation CAR-T, the FDA has begun approving these treatments for clinical practice as of 2017, each design improving the efficacy of the immunotherapeutic (Wang et al., 2018).

CARs consist of extracellular, transmembrane, and intracellular domains. The extracellular single chain variable fragment (scFv) uses components of mAbs to recognize specific tumor antigens (Wang et al., 2018). Most variation of 4th generation treatment occurs in the scFv, with some models recognizing two or more antigens (Grada et al., 2013; Zah et al., 2016). The transmembrane domain most commonly features proteins derived from CD3ζ, CD4, CD8, or CD28, which connect the extracellular and intracellular domains (Bridgeman et al., 2014, p. 3; Wang et al., 2018). 4th generation CAR-T cells are comprised of both a co-stimulatory domain and a nuclear factor of activated T cells (NFAT) in their intracellular domains, although there is significant variation between different generations. Intracellular domains control CAR-T cell activation when target antigens bind to the scFv and are where each generation of CAR-T differs from the next. The 1st generation featured CD3ζ alone, the second a co-stimulatory domain along with CD3ζ, the third two co-stimulatory domains, before the recent derivation of the 4th generation (figure 3). The co-stimulatory domain improves CAR-T longevity *in vivo* while the NFAT helps control cytokine production, making 4th generation CAR-Ts among the most successful (Wang et al., 2018).

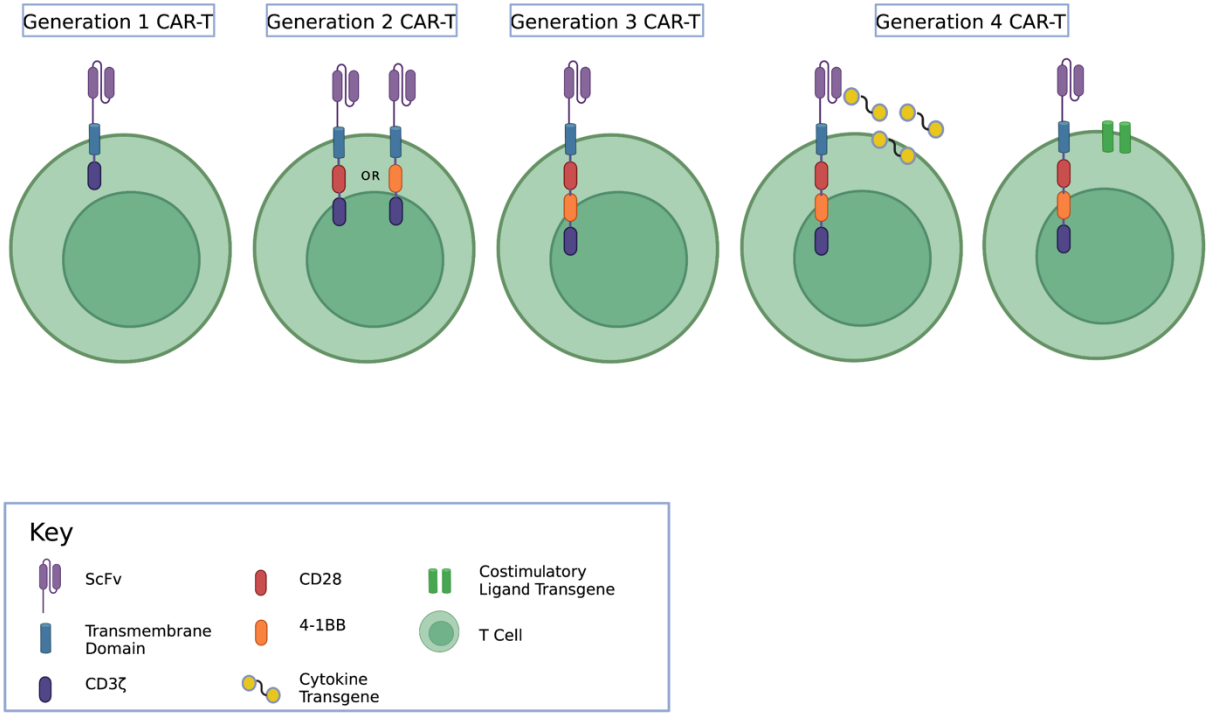

Figure 3: Generations of Chimeric Antigen Receptor Anatomy

The figure models the four generations of CAR-Ts, each including the ScFv extracellular domain, a transmembrane domain, and the intracellular protein CD3 ζ. The first generation only contains these features, while the 2nd generation also includes either a CD28 or 4-1BB intracellular protein, the third contains both CD28 and 4-1BB, and the fourth contains the same CAR protein from the third along with either a cytokine transgene or a costimulatory ligand transgene. Future CAR generations may have other features, although the graph above depicts the most successful CAR-T models (Subklewe et al., 2019).

When activated, CAR T therapeutics initiate a T cell response against target antigens, triggering the release of cytokines, granzymes, and perforins that aid in the destruction of target cells (Hartmann et al., 2017). CAR-T therapy utilizes the T cell's natural cytotoxicity and immune control to rally the most effective attack against invading cancer cells. To date, there are six FDA-approved CAR-T cell therapeutics. All approved therapeutics are targeted at blood-borne cancers such as leukemia and lymphoma, although trials are ongoing to innovate more successful solid tumor-based strategies (He et al., 2021). Many approved CAR-T therapeutics target CD19, an antigen expressed on cancerous and normal B cells alike. Axicabtagene ciloleucel was the first FDA-approved CAR-T cell therapy and highly successful treatment for non-Hodgkin lymphoma (NHL)(King & Orozco, 2019). Other CAR-T therapies use tisagenlecleucel, lisocabtagene maraleucel, Brexucabtagene autoleucel, and idecabtagene vicleucel, which target B-cell acute lymphoblastic leukemia (B-ALL), high-grade B-cell lymphoma (HGBCL), mantel cell leukemia (MCL), multiple myeloma (MM) respectively (table 1) (King & Orozco, 2019). With clinical trials and earlier research stages ongoing, there is hope that more CAR-T immunotherapeutics will soon be approved. Regardless, the metabolic conditions of the TME remain one of the biggest hurdles for optimizing CAR-T and other T cell-based therapeutics against solid tumors.

Table 1: CAR-T FDA-Approved Immunotherapeutics

Drug Name	Brand	Target Antigen	Target Cancer
Axicabtagene ciloleucel	Yescarta	CD19	Non-Hodgkin Lymphoma (NHL); Follicular Lymphoma;
Tisagenlecleucel	Kymriah	CD19	B-Cell Acute Lymphoblastic Leukemia (B-ALL); NHL;
Brexucabtagene autoleucel	Tecartus	CD19	Mantle Cell Lymphoma (MCL); B-ALL;
Lisocavtagene Maraleucel	Breyanzi	CD19	NHL
Idecabtegene Vicleucel	Abecma	BCMA	Multiple Myeloma (MM)
Ciltacabtagene Autoleucel	Carvykti	BCMA	MM

Source: (King & Orozco, 2019)

4. Hypoxia vs Normoxia vs Physoxia

4.1. Oxygen *in vivo*

Oxygen is a vital nutrient to sustain human life, one most eukaryotes cannot live without for extended periods. Human cells must undergo cellular respiration to produce the high energy threshold needed to maintain function. Cellular respiration breaks down glucose molecules to harvest cellular energy in the form of ATP, resulting in carbon dioxide and water byproducts. Consisting of glycolysis, the citric acid cycle, and the electron transport chain, cellular respiration spends both glycolysis and the citric acid cycle breaking down the glucose molecule to produce high-energy electrons and nominal amounts of ATP. The electron transport chain is when most ATP is produced, a process driven by oxygen. As the final electron acceptor, oxygen pulls high-energy electrons produced earlier in cellular respiration down a series of protein complexes that produce ATP. Oxygen then accepts the electrons along with two hydrogen molecules to form water, a byproduct of the process. Without oxygen, the body must rely on the nominal amounts of

ATP produced earlier in cellular respiration during glycolysis. This anaerobic function is unsustainable for long periods compared to its oxygen-reliant counterpart, aerobic function. On the other hand, anaerobic function does allow human life to be sustained without oxygen for short amounts of time without resulting in immediate death.

4.2. Defining Normoxia, Physoxia, and Hypoxia

Medical research classifies cellular oxygenation under three categories: Normoxia, physoxia, and hypoxia. Normoxia is defined as healthy oxygen levels in tissue culture research *in vitro*, with oxygenation ranging between 20-21% (McKeown, 2014). However, this number can vary depending on environmental factors such as altitude and CO2 levels. Physoxia is the "normal" oxygenation of tissues *in vivo*, which ranges from 2-7% oxygen depending on the tissue (McKeown, 2014). There is a wide gap between "normal" *in vitro* versus *in vivo (McKeown, 2014)*. Novel techniques including changing the physical state of the experiment medium (Lorian, 1989) and even computer simulation of *in vivo* experimentation (Sorguven et al., 2021) seek to narrow this gap in the lab to better stimulate environments inside the body.

Hypoxia occurs when oxygenation is low enough to result in inhibited function. Cells rely on energy to undergo crucial mechanisms such as survival, division, and even antitumor function (Nakazawa et al., 2016). In a cancerous tumor, rapid cellular growth requires more energy, and thus oxygen as a fundamental component in cellular respiration, to sustain. Hypoxia can be caused by a variety of factors such as pulmonary disease, altitude, intoxication, anemia, and the focus of our research, solid tumors (Hockel & Vaupel, 2001). Tumor hypoxia has become a popular area of research due to the negative impact hypoxia can have on anti-tumor immune cell function, but there is still confusion surrounding the classification of tissue oxygenation.

4.3. HIF Transcriptional Regulators

When cells face hypoxic environments, pathways such as hypoxia-inducible factors (HIFs), mTORC1, autophagy, ER stress responses, oxygen-dependent dioxygenases, and other oxygen-detecting mechanisms are activated (Nakazawa et al., 2016). These factors reduce cellular function to promote cell survival, which limits cytotoxicity and other immune defenses in the case of the immune system. (Zagzag et al., 2000). Under normoxia, HIF-1α undergoes degradation as opposed to its increased expression under hypoxia (Huang et al., 1998; Maxwell et al., 2001). In NK cells, multiple groups have demonstrated HIF-1α to be a potent inhibitor against anti-tumor function and cytotoxicity (Ni et al., 2020). Mouse experiments show deletion of HIF-1α receptor have improved antitumor function in the TME (Ni et al., 2020), thus making HIF-1α a viable target in NK-based immunotherapeutics. Interestingly, there is minimal literature supporting this observation in CD8+ T cells. Due to their transcriptional similarity to NK cells, it will be important for future studies to examine this pathway in T cells to determine if these cytotoxic cells are also improved by deletion of HIF-1α.

Targeting HIF-1α has been utilized in a different approach to activate CAR-T cells, specifically under hypoxic conditions. These hypoxia-responsive CAR-T cells are engineered by fusing oxygen-sensitive domains derived from HIF-1α to chimeric antigen receptor scaffolds (Juillerat et al., 2017). During hypoxia, the CAR construct is highly expressed as opposed to low expression under physoxia. As many solid tumors are hypoxic, oxygen-responsive CAR-T cells will selectively express the car to target the tumor antigen. Selectivity would prevent potential on-antigen off-target toxicity, stopping the CAR construct from being expressed in healthy tissue. Researchers hope to use this technology to engineer a new generation of CAR-T cells specific to hypoxic tumors with an increased repertoire of tumor antigens to choose from, which are also lowly expressed on healthy tissue (Smith et al., 2019).

4.4. Effect of Hypoxia on T Cells

CD8+ T cells are essential in the anti-tumor immune response for their cytotoxic function, thus making them an important topic of research regarding ICB and CAR-T. Many studies support that hypoxia lowers cell proliferation and increases cell death in naïve T cells (Atkuri et al., 2005, 2007; Caldwell et al., 2001; Conforti et al., 2003; Dimeloe et al., 2016; Gaber et al., 2013; Larbi et al., 2010; Ohta et al., 2014; Vuillefroy de Silly et al., 2016; Xu et al., 2016), although this is contested by studies that concluded hypoxia had no effect or even resulted in naïve CD8+ T cell expansion (Dziurla et al., 2010; Krieger et al., 1996; Makino et al., 2003; Naldini et al., 1997; Roman et al., 2010; Vuillefroy de Silly et al., 2016; Zuckerberg et al., 1994). CD8+ T cell-activating cytokines TNF- α, IFN-γ, and various ILs were compounding factors when interpreting these results. Some studies argued an increased expression of such cytokines under hypoxia while others observed a decrease or lack of change thereof. Although more research is needed, the field has mostly concluded that CD8+ T cells differentiated under normoxia *in vitro* tend to be more effective than those differentiated under hypoxia (Vuillefroy de Silly et al., 2016). Studies have also found reduced differentiation, proliferation, and IFN- γ production in CD4+ T cells under hypoxic conditions, thus contributing to the TME's immunosuppressive capabilities. An example of this behavior was exhibited in mice afflicted by colitis-associated colon cancer (Westendorf et al., 2017), along with an influx of Treg presence supported widely by recent literature (Kondělková et al., 2010; Ohue & Nishikawa, 2019; Paluskievicz et al., 2019).

5. Tissue-Specific Oxygenation

Many studies have explored the relationship between the physoxia of healthy tissues compared to their hypoxic tumor counterparts (Muz et al., 2015). There is a clear dependence between physoxic and hypoxic levels in various tissues, which is beneficial to explore when considering the effect on immunotherapeutics targeting solid tumors.

5.1. Comparing Oxygenation in Healthy vs. Cancerous Tissues

Healthy oxygenation ranges from 3-7.4% depending on the tissue in question, their cancerous counterparts decreasing to roughly 0.3-4.2% (McKeown, 2014). Most healthy tissue has significantly higher oxygenation than cancers in the same tissue (McKeown, 2014). For example, brain tissue has a resting average of 4.6% oxygen content under normoxia compared with 1.5% in a brain tumor's hypoxic condition. Table 2 may be consulted to compare average physoxia levels in healthy tissue to hypoxic in cancerous (McKeown, 2014; Muz et al., 2015). Further research will be important to determine if immune cells are behaving differently in between tissues based on physoxia levels. It will be of particular interest if those tissue resident immune cells found in low oxygen physoxia conditions are actually more primed to handle hypoxia during tumorigenesis and if immune cells found in high oxygen tissues are more sensitive to loss of physoxia.

Table 2: Tissue-Specific Oxygenation in Cancer Opposed to Healthy Tissue

Tissue	Physoxia % O2	Cancer	Hypoxia % O2
Brain	4.6	Brain Tumor	1.7
Breast	8.5	Breast Cancer	1.5
Lung	5.6	Non-Small Cell Lung cancer	2.2
Cervix	5.5	Cervical Cancer	1.2
Liver	4.0-7.3	Liver Cancer	0.8
Pancreas	7.5	Pancreatic Tumor	0.3
Kidney	9.5	Renal Cancer	1.3

Sources: *(McKeown, 2014; Muz et al., 2015)*

5.2. In-Silico Approach to define the role of Hypoxia on Cancer Treatments

Traditional methods of hypoxia measurement have become less prevalent in favor of an in-silico approach. Oxygen electrode is the most common direct pO2 measurement in oncologic research, a method that relies on the electrochemical reduction of oxygen to hydrogen peroxide (H202) (Jung et al., 2020). Considered to be both safe and effective, measurements are taken through an electrode inserted into the area of effect. The electrode is shifted to measure many locations sub-millimeters apart for each needle track, thus accurately determining the hypoxic signature (Walsh et al., 2014). When compiled, a histogram is created from the accumulation of the needle tracks to represent O2 pressure compared to the frequency of said pressure in the tumor (Walsh et al., 2014). Despite its success, oxygen electrodes are too invasive to test consistently and monitor progress. Measuring oxygen in this way is also demanding because there is no easy with to differentiate between living and necrotic cells, and the 3D mapping needed to visualize oxygenation is complex, despite only covering around 50-100 individual cells (Walsh et al., 2014). Phosphorescence quenching is an alternative direct pO2 measurement to oxygen electrode, which utilizes the reaction of oxygen to phosphorescent dyes. The dyes emit light when interacting with a brief illumination from an outside source. Oxygenation is proportional to the exponential rate the dyes decrease in light emission, allowing scientists to determine oxygenation in the exact moment the tissue is measured (Walsh et al., 2014). *In vivo,* molecular reporters and needle probes are used to report on the state of a tissue (Walsh et al., 2014). Other methods of oxygen measurement include electron paramagnetic resonance and F-magnetic resonance spectroscopy (Walsh et al., 2014), although oxygen electrode and phosphorescence quenching remain the most prevalent.

These methods are highly invasive and outdated, therefore current research has examined methods to predict hypoxia levels in the TME. For an in-silico approach, oxygen is not directly measured but assumed through indirect measurement of O2. The basis of the in-silico approach relies on RNA-sequencing (RNA-seq), a technique that examines the presence of different RNA strands transcribed in a sample. RNA-seq can determine the gene expression level in different experimental conditions based on differences between the RNA content in different samples (Anjum et al., 2016). Differentially expressed genes (DEGs) during hypoxia can be tested by controlling the oxygen content in different tissue types, determining tissue-specific DEGs associated with a hypoxic TME. Many groups have determined hypoxic signatures in multiple tissues and cancer types (Q.-G. Li et al., 2017; J. Liu et al., 2018; T. Long et al., 2019; Saliani et al., 2022; Zhang et al., 2019). RNA-seq in hypoxic tissues has enabled researchers to connect gene signatures to the severity of hypoxia in most solid tumors (Abbas et al., 2020; Kim et al., 2020; J. Liu et al., 2022; Yin et al., 2020). An in-silico approach uses hypoxic gene signatures to predict hypoxia levels in the tumor to assess tumor severity and survival probability. The most prevalent genes assessed through this method and their weighted influence on a patient's survival are accumulated to calculate the risk hypoxia plays in cancer survival (Anjum et al., 2016). This risk score can be used to differentiate high-risk from low-risk patients, otherwise estimating how effective different treatments may be on a patient (Anjum et al., 2016). The in-silico approach could one day be used as a biomarker of efficacy for immunotherapeutics such as CAR-T and ICB. Even further, an in-silico approach could classify a patient with a highly hypoxic tumor and identify a therapeutic strategy that targets hypoxia-induced genes known to be deleterious to cytotoxic immune cells.

6. Conclusion

Hypoxia in the TME is regarded as one of the most significant inhibitors of immunotherapeutics in solid tumors, making it a prevalent topic in scientific literature. With treatments such as CAR-T and ICB becoming more effective, hypoxic inhibition must be reduced to maximize their effect in both blood-born and solid tumors. HIF-1α is an example of a transcriptional regulator used to alter immune cell function under hypoxia. Novel treatments that remove HIF-1α in NK cells to improve their efficacy in hypoxic tumors display the promise such research may have in the future.

Removing HIF-1α in CD8+ T cells is certainly something to explore because of cytotoxic T cells' transcriptional similarity to NK. Removal may improve the efficacy of T cell-based immunotherapeutics such as CAR-T. Hypoxia's effect also highlights the importance of oxygenation in all research involving cells. Unfortunately, normoxia cannot accurately replicate oxygenation inside the human body (physoxia), making *in vitro* experiments less applicable *in vivo*. Bridging this gap may improve how effective treatments are in clinical trials. An in-silico approach can estimate how fatal a tumor's hypoxia can be, the first step to combat hypoxic inhibition against immune cells. The expression of DEGs in different tumors are identified and weighted based on their association with a patient's survival to calculate a risk score. Patients with higher risk scores may benefit from different treatments depending on their effectiveness against hypoxia, allowing doctors to determine the best approach for specific patients. Another distinction must be made between oxygenation in different tissues and how such measurements compare to the same tissue when it is cancerous. Considering such variation will make the in-silico risk score more accurate, thus maximizing treatment. Future research about hypoxia will improve our understanding of the TME and help create better treatments for patients with solid tumors.

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