Published in final edited form as:

Immunity. 2023 October 10; 56(10): 2231–2253. doi:10.1016/j.immuni.2023.09.005.

CD8+ T cells in the Cancer Immunity Cycle

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Abstract

CD8+ T cells are end effectors of cancer immunity. Most forms of effective cancer immunotherapy involve CD8+ T cell cytotoxic function. Here we review the current understanding of T cell function in cancer, focusing on key CD8+ T cell subtypes and states. We discuss factors that influence CD8+ T cell differentiation and function in cancer through a framework that incorporates the classic three signal model and a signal 4 – metabolism - and consider also the impact of the tumor microenvironment from a T cell perspective. We argue for the notion of immunotherapies as "pro-drugs" that act to augment or modulate T cells, which ultimately serve as the drug *in vivo,* and for the importance of overall immune health in cancer treatment and prevention. The progress in understanding T cell function in cancer will enable improved harnessing of the immune system across broader tumor types so as to benefit more patients.

CD8+ T cells are "end effectors" of cancer immunity. Wherry, Kaech and colleagues review the current understanding of CD8+ T cell differentiation and function in cancer, discussing recent advance within a four-signal framework that incorporates T cell metabolism and the impact of the tumor microenvironment.

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Author Contributions

All authors contributed to conceptualization, writing, and editing of the manuscript. We thank members of both labs for helpful discussions.

Declaration of Interests

EJW is a member of the Parker Institute for Cancer Immunotherapy. EJW is an advisor for Danger Bio, Janssen, New Limit, Marengo, Pluto Immunotherapeutics Related Sciences, Santa Ana Bio, Synthekine, and Surface Oncology. EJW is a founder of and holds stock in Surface Oncology, Danger Bio, and Arsenal Biosciences. JRG is a consultant for Arsenal Biosciences. SMK is on the scientific advisory boards and has equity in EvolveImmune Therapeutics, Affini-T Therapeutics, Arvinas, and Pfizer. AMG has no competing interests.

Introduction

Our understanding of the Cancer Immunity Cycle[1] has advanced substantially in the past 10 years. These advances are nowhere more evident than in our knowledge of and ability to therapeutically target T cells for enhancing anti-tumor immunity. Major drivers of this field are the unprecedented successes of immune checkpoint blockade (ICB) therapies together with breakthroughs in engineered cellular therapies. The widespread use of such T cell-based therapeutics provides an extraordinary opportunity to apply science in the clinical setting and directly link efforts from preclinical models to observed realities in human disease. Furthermore, advances in high dimensional cytometry and single cell genomics have greatly expanded our understanding of T cell differentiation states. Such studies, including in cancer patients, have highlighted the central role of exhausted $CD8^+$ T (Texh) cells and other $CD8^+$ T cell subtypes in cancer immunity and in response to ICB therapies.

One useful framework for discussing advances in T cell immunity to cancer is a three-signal model for effective T cell priming and differentiation, incorporating TCR signaling by antigens presented by MHC molecules (signal 1); co-stimulation (signal 2), and cytokines (signal 3). It is only with all three optimal signals that naïve T cells are effectively primed, differentiate into functional effector $CD8^+$ T (Teff) cells, and form high quality memory $CD8^+$ T (Tmem) cells. There have been major advances in our understanding of all three signals in cancer immunity. Considerable efforts have been directed towards understanding the nature of antigens that can activate T cell responses in cancer and harnessing such antigens for treatments like cancer vaccines and cellular therapies. The nature of the checkpoints CTLA-4 and PD-1 also reframes the concept of signal 2 and highlights the “negative” co-stimulation exploited by tumors for immune evasion. Signal 3 was initially conceptualized as the direct sensing of inflammation through cytokines necessary for full T cell activation and differentiation. The absence of such inflammatory signals may partly underly the poor priming environments of many tumors resulting in “tolerance” to tumor antigens. However, recent advances also highlight the role of chronic inflammation as a potential negative regulator of T cell immunity in cancer. Moreover, the ability to exploit cytokines to augment T cell immunity to cancer, and the expansion of this concept to include growth factor-type cytokines (e.g. IL-2), remains of considerable interest. In addition to this classic three signal model, recent work in cancer immunology has highlighted the role of a signal 4, the nutrients and metabolites that fuel T cell metabolism. We now appreciate the importance of how T cells are metabolically reprogrammed and how changes in nutrients, oxygen, and other environmental signals constrain T cell responses to tumors. Lastly, the influence of the physical structure and the other cell types within the tumor microenvironment (TME) on the T cell response is beginning to be better characterized, and will be discussed in this review. Together, these advances provide a framework for understanding the major signals that influence T cell differentiation and how these signals may be therapeutically manipulated to improve T cell functions for anti-tumor immunity.

Here, we first discuss our current understanding of $CD8^+$ T cell differentiation as it relates to cancer, then explore variations in signals 1, 2, 3 and 4 in the Cancer Immunity Cycle, and lastly consider how additional factors in the TME alter T cell state and function.

Although this review is mainly focused on $CD8^+$ T cells, work over the past decade has also highlighted a critical role for $CD4^+$ T cells in cancer biology and response to immunotherapy. $CD4^+$ T cells can have pro- or anti-tumor effects, including providing "help" for $CD8^+$ T cells, influencing myeloid and other cells within the TME, and mediating either direct cytotoxicity or regulatory effects. For further detail, we refer the reader to other excellent reviews discussing the $CD4^+$ T cell compartment [2, 3, 4].

$CD8^+$ T Cell Differentiation States

Over the past decade, an increasingly comprehensive and detailed understanding of $CD8^+$ T cell differentiation in tumors has begun to emerge. This knowledge has come from an array of human studies and mouse models of cancer and chronic infection. While technological advances have enabled deep immune profiling directly in human patients, precisely controlled mouse models have generated the foundational framework with which to interpret these new data. Here, we synthesize information across all these sources to discuss our current understanding of $CD8^+$ T cell differentiation as it relates to the Cancer Immunity Cycle.

Upon activation, $CD8^+$ T cell differentiation follows two main developmental pathways that ultimately results in the formation of either Tmem cells or Texh cells, with branches of shorter-lived effector-type cells (Figure 1). In settings with optimal signals 1-4, such as acutely-resolving infections or vaccinations, $CD8^+$ T cells initially differentiate into Teff cells or memory-precursors (Tmp) cells, and as antigen is cleared, long-lived Tmem cells emerge [5, 6, 7]. The Tmem population is heterogeneous with multiple subsets defined by surface phenotype, function, differentiation potential, and location. The most commonly used classification scheme for the circulating Tmem (Tmem-circ) population include stem cell memory (Tscm), central memory (Tcm), effector memory (Tem), and effector memory $CD45RA^+$ (Temra) [8]; however, alternative schemes have also been proposed [9]. A distinct subset of Tmem cells reside within tissues, termed "resident memory" (Trm). These Trm cells likely arise from Tmp cells that enter tissues and acquire additional programs of long-term tissue residency that include the ability to provide local protective immunity [10, 11, 12]. Among Trm cells there is additional heterogeneity, in part reflecting tissue-specific signals [13, 14]. Within the TME, it is often possible to identify cells that phenotypically resemble Tem, Temra, and Trm cells, with Tscm and Tcm cells being less common.

In contrast to the development of Tmem cells, persistent stimulation, particularly through the TCR (signal 1) [15, 16, 17] leads to the development of T cell exhaustion, a branch of $CD8^+$ T cell differentiation epigenetically coordinated by the transcription factor (TF) TOX [18, 19, 20, 21, 22]. When exactly the Texh branch diverges from the Tmem branch is an active area of investigation (Box 1). Regardless, the majority of $CD8^+$ T cells within many human tumors are likely Texh cells based on surface protein or gene expression, though the frequency can vary among cancer types and between patients [23]. Although Texh cells were originally identified in a mouse model of chronic infection [24, 25], it is now clear that this cell fate arises in many settings of persistent stimulation including infections such as HIV, HCV, and HBV, and human cancers [23, 26, 27]. Texh cells have been traditionally characterized by high co-expression of multiple inhibitory receptors (IRs), such as PD-1,

CD39 and LAG-3, as well as reduced production of effector cytokines, including IFN-γ and TNF, and also IL-2 [27, 28, 29]. We now know that Texh cells are defined by a distinct transcriptional and epigenetic program compared to naïve, Teff, and Tmem cells [30, 31, 32, 33, 34, 35, 36]. A frequent misconception is that Texh cells are non-functional. Rather, Texh cells have altered effector functions and are adapted to withstand chronic stimulation associated with persisting infections and tumors [37, 38, 39, 40]. During chronic infection, Texh cells can maintain moderate viral control while avoiding excessive immunopathology [16, 41, 42]. Indeed, loss of Texh cells in chronic SIV infection results in disease progression [43, 44], and Texh cells may drive epitope escape in persisting HIV and HCV infection [45, 46]. Furthermore, the association of ICB efficacy and Texh cells within the TME indicates functional activity of these cells in cancer [47, 48]. These data highlight the attenuated, yet biologically important functional capacity of Texh cells. Despite Texh cell activity in cancer, an immune-tumor stalemate, or equilibrium, is often unstable and eventually results in failure of immune control, tumor escape and disease progression [49]. Major regulation of this balance is through IRs. Indeed, blocking IRs through ICB therapies, such as αPD-1, enhances Texh cell effector functions, boosts anti-tumor immunity, and has produced long-lasting cures in many patients.

Within the fate-committed mature Texh population, there is additional heterogeneity. Initially, this complexity was conceptualized as a progenitor-like subset capable of responding to PD-1 blockade and a terminal subset that could not [50]. These progenitor and progeny subsets were subsequently identified by many groups in mouse models of chronic infection [51, 52, 53, 54, 55], mouse models of cancer [53, 55, 56, 57, 58], and human tumors [55, 59, 60]. Additional studies point to at least three major subtypes within the mature Texh population: exhausted progenitor cells (Texh-prog), intermediate exhausted T cells that possess some effector function (Texh-int), and terminal exhausted T cells (Texh-term) [61, 62, 63, 64, 65]. Texh-prog cells have both exhausted and stem-like qualities. This subset expresses TCF-1, has enhanced proliferative capacity, and gives rise to the other mature Texh subsets. Texh-prog are often localized to secondary lymphoid organs [51, 52, 56, 57, 58, 61, 64, 66, 67] but are also found within the TME [48, 57, 68, 69, 70] where they reside within specialized niches near antigen-presenting cells (APCs) or tertiary lymphoid structures (TLSs) [60, 71, 72, 73]. Texh-prog cells can express chemokines and/or chemokine receptors such as XCL1, CCR7, and CXCR5 that might facilitate these interactions [51, 52, 53, 54, 55, 56, 59, 74]. The Texh-int subset expresses both exhaustion-related molecules, such as TOX and IRs, as well as effector-associated molecules. This subtype exhibits functional heterogeneity that likely depends on specific environmental signals. For example, a subset expressing NK receptors (or killer-cell immunoglobulin-like receptors, KIRs) is observed in chronic infection and some human cancers [23, 74, 75, 76]. Texh subpopulations expressing interferon stimulated genes (ISGs), granzyme molecules, heat shock proteins, IL-17, the oxidative phosphorylation pathway, or other specific programs may also fall into this Texh-int subset [23, 67, 69, 74, 76]. Although this subset has been found in models of chronic infection [61, 62, 63, 74, 76] and human cancer [23, 26, 64, 65, 67, 69, 77], diverse definitions and nomenclature, combined with considerable biologic heterogeneity have made it difficult to consistently identify and compare these cells across data sets. Indeed, despite an underlying epigenetic program of exhaustion, Texh-int cells may appear as a type of effector-like population in

some settings based on high mRNA (or protein) expression of individual granzymes or other genes. Lastly, Texh-term cells have the lowest proliferative capacity and highest IR expression. Some Texh-term cells express GZMA [74] and thus may contribute to direct killing. It is also tempting to speculate whether these cells play a role in local immune regulation. They produce large amounts of beta chemokines, specifically CCL3, CCL4, and CCL5, which can contribute to both inflammation and tissue repair through recruitment of other leukocytes [74, 78]. Moreover, this subset has high expression of CD39 and may exert regulatory effects through the production of adenosine [79]. These three major Texh subsets all share a core exhaustion epigenetic program and depend on TOX [18, 19, 20, 21, 22]. Together, they form a proliferative hierarchy that resembles those observed in regenerating or self-sustaining tissues enabling the Texh population to persist even in stressful conditions, such as chronic antigen stimulation, while retaining some functionality. Defining the lineage relationships between Texh subsets remains an active area of investigation. Current data suggest that both linear (Texh-prog → Texh-int → Texh-term) and branched (Texh-prog → Texh-int and Texh-prog → Texh-term) paths exist, and these trajectories may be regulated, at least in part, by strength of TCR signaling (signal 1) [76, 80] and IFN signaling (signal 3) [80]. Furthermore, how these exhaustion trajectories intersect with those of Trm populations is also of considerable interest (see Box 2).

Immune profiling techniques, including high dimensional cytometry and next generation sequencing (NGS) assays, coupled with clinical and translational efforts to collect patient samples have revealed considerable heterogeneity of Texh and Tmem subpopulations within tumors defined by protein or gene expression. However, interpreting human profiling data poses several challenges: 1) we lack a unified nomenclature for T cell subpopulations, 2) connecting T cell phenotypes or states to antigen specificity remains difficult, and 3) it is often challenging to define temporal relationships between differentiation states. The first challenge arises, at least in part, from different naming conventions including those based on historical subpopulation names, highly expressed genes, and/or functional qualities [26]. Comprehensive comparison to existing profiles, such as “reference maps” for scRNA-seq, can help provide context [81]; however, these references may not capture the full spectrum of cell states that are possible within disease contexts. Furthermore, an important question is what constitutes a distinct “subset” of T cells? Data from our group and others[74, 82, 83] suggest that classification strategies that use epigenetic information may resolve more distinct and stable subtypes compared to transcriptional or protein information, in part because the latter may represent transient responses to environmental signals. As more studies capture multimodal data, the ability to compare and cross-validate cellular definitions using different biologic measurements should improve, enabling a potential unified and robust classification system.

A second major challenge in interpreting T cell phenotypic data from human cancer patients is the limited information regarding antigen specificity. Within the TME, TCR clonality and local expansion of the Texh population is often used to infer tumor specificity [23, 68, 70, 77, 84, 85]. However, confirming antigen specificity of individual T cell clones is technically challenging [86], and some studies indicate only a small fraction of $CD8^+$ TIL are tumor-specific [86, 87]. Recently, new strategies have profiled scRNA-seq/scTCR-seq in parallel with *in vitro* tumor-reactivity assays then use TCRs as barcodes to match differentiation

state defined by gene expression with antigen specificity determined *in vitro* [70, 88]. One study that tested the reactivity of highly expanded TCRs found that 83% of the tested TCRs within the Texh population were tumor-specific compared to only 10% from non-Texh cells [70]. Even though the sample size was small and from a classically "hot" tumor type (melanoma), this result indicates a greater proportion of $CD8^+$ TIL may represent on-target responses than originally suggested. Overall, T cell clones with confirmed tumor reactivity are often found within Texh subsets whereas virus-specific T cells (CMV, EBV, influenza virus, etc.) that can also be found in tumors reside in Tmem populations [28, 70, 87, 88, 89]. Collectively, these data raise several questions. First, these data are consistent with tumor antigen driving T cell exhaustion. However, strength of stimulation is likely to vary substantially across different microenvironments, therefore focusing on the most expanded clones or Texh-like cells may not capture all tumor-reactive T cells. For example, T cells that experience weaker stimulation may be less clonally expanded, but also less exhausted and thus potentially useful therapeutic targets. Indeed, T cells specific for sub-dominant tumor antigens may be enriched for TCF-1^+ Texh-prog cells [90]. Second, these observations suggest that bystander T cells of unrelated specificities can be recruited into the TME, potentially through inflammatory signals, such as CXCL9 and CXCL10 via CXCR3, which is expressed by most Tmem subsets [91]. Alternatively, reactivation of latent viruses such as EBV or CMV, or de-repression of endogenous retroviruses (ERVs), within tumor cells or other cells within the TME, may recruit antigen-specific Tmem subsets from circulation or adjacent tissues. In addition, tumor antigens may be molecular mimics of viral or other pathogenic peptides and provide antigenic stimulation for cross-reactive T cells [92]. Regardless, bystander T cells that become activated have the potential to exert anti-tumor effects [93]. Another T cell population that may be present in the TME are those specific for tumor antigens that have been lost by immune editing and/or loss of antigen presentation. Such tumor-specific T cells would become ignorant and, depending on their differentiation state when antigen was lost, could differentiate into Tmem or memory-like cells (e.g. if these cells where Texh-pre or possibly Texh-prog when antigen mutated). Alternatively, these clones could simply disappear if they had become fully exhausted before antigen loss. Thus, defining what is meant by tumor-specific is not necessarily straightforward.

Defining temporal relationships for tumor-reactive T cells in humans remains a third challenge. As tumors evolve, endogenous $CD8^+$ T cells can be activated at different times and in different locations. However, most patient data only captures snapshots of these trajectories. In silico techniques used to infer temporal relationships have provided some insights, but these approaches remain challenged in their ability to accurately capture T cell trajectories. T cells can undergo cycles of activation and quiescence, and quiescent cells, such as naïve and Tmem, are often predicted to be temporally related based on transcriptome despite substantial epigenetics differences [94]. Using TCR sequences as cellular barcodes and tracking individual clones through scRNA-seq data can provide additional insight regarding potential relatedness of different T cell clusters. For example, the same T cell clones have been identified in Texh-prog and Texh-term cells in human patients suggesting potential lineage relationships [67, 70]. Such data aligns with the Texh developmental relationships defined in mouse models [39, 52, 54, 57, 61, 62, 74, 76, 80]. However, this strategy depends on the number of cells sampled, biasing such analyses to more abundant clones and more frequent

cell states. In addition, existence of a TCR sequence in multiple scRNA-seq clusters is often interpreted to mean recent clonal relatedness; however, this TCR sharing may result from distal events, including the first division after priming. Indeed, without temporal data, the exact relationship between different T cell clusters or subsets may remain challenging to determine unambiguously.

Given the clinical success of ICB in the past decade, a central question has been to understand the underlying mechanisms by which these drugs work and specifically how they alter T cell differentiation states and functions. Texh cells are thought to be a primary target of ICB due to their high expression of IRs and preclinical data on PD-1 pathway blockade [27]. Indeed, pre- or early on-treatment tumor infiltration by Texh cells in humans is positively correlated with clinical benefit across diverse tumor types [95, 96, 97, 98, 99, 100]. Early studies in mice showed that PD-1 blockade "reinvigorated" Texh cells, numerically increasing antigen-specific T cells and improving function [101, 102]. However, PD-1 blockade does not substantially change the epigenetic landscape of fate committed Texh population [31]. Rather PD-1 blockade (at least temporarily) alters the balance of Texh subsets, acting primarily on Texh-prog cells and driving differentiation towards downstream subsets including Texh-int and Texh-term cells [50, 52, 54, 56, 57]. An increase in the Texh-int subset is likely responsible for much of the anti-tumor activity given the higher expression of effector molecules by this subset [61, 62, 63, 64, 65, 67, 74]. Recent work also suggests that these Texh-prog cells can "self-renew" under these conditions of PD-1 pathway blockade [103]. An increased frequency of Texh-prog cells is linked to improved clinical outcomes after ICB treatment [23, 48, 104]. Although, as noted above, phenotypic diversity or similarity, varying nomenclature, lack of antigen specificity and/or temporal information create significant hurdles for synthesizing information across studies. Regardless, these data highlight the importance of progenitor or "stem-like" cells capable of further differentiation into progeny with anti-tumor activity in ICB treatment. Although PD-1 blockade does not re-program the $CD8^+$ T cell differentiation state from Texh to Teff or Tmem cells, a large expansion of the Texh-int subset can be sufficient for substantial clinical benefit, and even cure if tumor burden is low [105]. Treatments that boost the expansion and/or function of Texh-int cells may enhance anti-tumor efficacy. Indeed, combining PD-1 blockade with other ICBs, such as αCTLA-4 or αLAG-3, improves clinical efficacy [106], and preclinical data supports combination treatments with cytokines signals such as IL-2 to further boost T cell function [107, 108, 109].

Designing rational treatment combinations that act on a cellular target of choice and elicit a specific change in differentiation state or functional capacity is becoming a reality and will be an important goal for the next decade. T cells are a prime target in the immune system for such therapies. As discussed further below, it is now possible to therapeutically intervene in many key signals that shape T cell activation and differentiation, including signals 1-4. These pathways can be targeted extrinsically with drugs that can act on the endogenous immune response and/or targeted intrinsically through cellular engineering and adoptive cell therapy.

Signal 1: TCR Signaling and Antigens

The first step of T cell activation is recognition of antigen presented by MHC on a professional APC, typically a dendritic cell (DC), through the TCR on the T cell. One of the historically challenging areas of tumor immunology has been identifying tumor antigens capable of providing signal 1 for T cell activation (Figure 2). Although somatic mutation is hallmark of tumorigenesis, not all tumors produce sufficient immunogenic antigens to induce a robust endogenous immune response. These tumors may represent a failure in the first step of the Cancer Immunity cycle due to insufficient antigen-specific priming.

Tumor antigens can be broadly classified as tumor-associated antigens (TAAs), expressed by both tumor and healthy tissues, or tumor-specific antigens (TSAs), exclusively expressed by tumor cells. The four main classes of TAAs include differentiation, over-expressed, cancer germline or testis antigens, and ERV-derived antigens [110]. As a result of thymic selection, T cells targeting TAAs may express TCRs with lower affinity [111]. On the other hand, TSAs are essentially foreign antigens and thus TSA-specific T cells are not subject to central tolerance. TSAs can be either viral proteins (e.g. from oncogenic viruses) or neoantigens. Infection with specific viruses can cause cellular transformation resulting in expression of viral proteins detectable by the immune system [112]. In contrast, neoantigens have novel amino acid sequences derived from the host. Neoantigens can be produced at any point during peptide production, including DNA mutations, transcription, such as alternative splicing or fusion events, distinct post-translational protein modifications, or changes due to proteasomal processing [110].

Not all tumor mutations produce a neoantigen. To generate signal 1 for T cell activation, a mutation must be expressed, presented by MHC, and stimulate a T cell response. Identifying such immunogenic antigens has been a major effort in the field for the past decade. DNA sequencing followed by *in silico* prediction is the most widely used approach due to its high throughput nature and is often combined with mass spectrometry and/or T cell reactivity assays [86]. Many different neoantigen prediction pipelines have been developed that incorporate different kinds of data, use different assumptions, and employ distinct algorithms. Recently, a consortium-based benchmarking study compared 25 neoantigen prediction pipelines and identified key parameters for predicting immunogenic peptides, including strong and stable MHC binding, high tumor abundance, and immunogenicity quantified as distance to self (“agretopicity”) or similarity to pathogens (“foreignness”) [113]. Such advances in neoantigen prediction are key for the successful design of many immunotherapy strategies as discussed below.

The goal of antigen-targeted therapies is to increase tumor-specific T cells by providing antigens for signal 1 or bypassing this step in vivo by directly transferring activated tumor-specific T cells. These therapies have two primary considerations: the target antigen(s) and the type of therapy. Antigen-directed therapies include cellular therapy (CAR T cells, TCR-engineered, and TIL) and vaccination. Engineered cells can introduce new specificities or higher affinities compared to the endogenous response. Cellular therapies have shown curative potential, especially in hematological malignancies [114], but can also have considerable side effects including cytokine release syndrome (CRS) arising in part

from artificially increasing the magnitude of the T cell response to a disseminated target such as CD19 for B cell malignancies. Despite substantial success in treating liquid tumors, identifying appropriate tumor antigen targets (and overcoming exhaustion) for solid cancer-targeting CAR T cells remains challenging [115]. Numerous synthetic biology engineering strategies using, for example, logic gates that require recognition of more than one antigen or other strategies have the potential to unlock other tumor targeting possibilities and enhance safety [115]. In addition to cellular therapy, TAAs are targets in vaccine trials aiming to enhance the endogenous T cell response. Results from early efforts were mixed [116], but new technologies, including mRNA vaccine platforms, have renewed interest in TAAs as therapeutic targets [117, 118].

In contrast to TAAs, neoantigens are tumor-specific and thus excellent potential targets for immunotherapy. The first evidence that T cells could recognize neoantigens was reported over 20 years ago [119, 120]. However, most neoantigens are patient-specific [86], requiring individually designed treatments. Advancements in NGS and neoantigen prediction pipelines over the past decade are making such personalized vaccine treatments a reality. Although many different types of cancer vaccines have been tested (reviewed in [117, 118]), mRNA-based platforms are attractive options in part due to advances made during the accelerated production of the SARS-CoV2 vaccines. A recently completed phase I trial for pancreatic ductal adenocarcinoma (PDAC) treated patients with personal neoantigen vaccines using uridine mRNA-lipoplex nanoparticles [121]. Half of the patients (8/16) were classified as responders. None of these responders experienced recurrence at the time of publication, whereas the non-responders had a median recurrence free survival of 13.4 months. Furthermore, a recent trial (mRNA-4157-P201/KEYNOTE-942) demonstrated that mRNA personalized neoantigen vaccines could be combined with pembrolizumab (αPD-1) to increase recurrence-free survival in patients with resected high-risk melanoma [122]. These studies highlight the feasibility of manufacturing personalized cancer vaccines in real time and the ability of such vaccines to elicit novel T cell responses, including in a cancer type that is traditionally considered immunologically cold.

One major potential consequence of strong T cell pressure on tumors, predicted by the Cancer Immunity Cycle, is immune editing. Originally defined in mouse models where T cells, largely through IFN-γ, shape the antigenicity of tumors [49], immune editing is now appreciated to have a considerable impact on the human cancer landscape. On-target T cell responses can lead to the elimination of tumor subpopulations expressing immunogenic antigens, resulting in eventual tumor escape [1]. Designing tumor antigen-directed therapies can proactively consider predictions about tumor-immune evolution. One example is targeting multiple antigens, exemplified by CD19 and CD22 CAR T cells, allowing for tumor recognition even if one antigen is lost [123]. Indeed, endogenous single TCRs have been found that recognize multiple TAAs which could also protect the host against antigen escape [124]. Another strategy is to target clonal driver mutations that control tumor cell growth or viability. Some driver mutations occur frequently at specific genomic locations called "hotspots" [125], such as amino acid 12 in the KRAS oncogenic protein [126]. These mutations in oncogenic drivers can sometimes produce shared neoantigens, providing the opportunity to generate therapies that can be used across different patients; such efforts are underway targeting KRAS mutations [127]. A third method is engineering tumor cells to express specific

antigen(s), such as CD19 encoded by an oncolytic virus [128] or membrane-inserting tags [129], that are paired with specificity matched CAR T cells. Lastly, it is also possible to target non-tumor host cells that are critical for a tumor-supportive local environment through targets such as Fibroblast Associated Protein (FAP) which is expressed by cancer-associated fibroblasts [130]. Ultimately, longitudinal tracking of both tumor evolution and the immune response can inform effective design and application of antigen-directed immunotherapy. Vaccines and cellular therapies built upon optimal antigens have the potential to introduce new T cell responses that can help overcome hurdles at the beginning or later states of the Cancer Immunity Cycle, such as T cell exhaustion.

Signal 2: Co-stimulation and Co-inhibition

Signal 2 was originally defined as co-stimulation from CD80/CD86 ligands on the APC, received through the CD28 receptor on the T cell. During infections, these co-stimulatory ligands are upregulated on APCs in response to pattern recognition receptor activation and/or inflammation. Signaling through CD28 facilitates survival, metabolism and cytokine production by T cells, ensuring full activation [131]. Since these original concepts were defined for CD28 and CD80/86, a variety of co-stimulatory molecules have been identified (e.g. CD40L, OX40, 4-1BB, ICOS, CD27, etc.) that can have a role in priming or at later stages of T cell differentiation and be delivered by many cell types including help from $CD4^+$ T cells [132]. In cancer, a non-inflammatory or suppressive TME may prevent robust upregulation of co-stimulatory molecules on APCs resulting in sub-optimal T cell activation. Several strategies have been developed to stimulate APCs and thus enhance T cell activation, such as αCD40 [133] and soluble FLT3 ligand [134]. In addition, agonists that target co-stimulatory molecules directly on $CD8^+$ T cells are currently being tested [135].

Following proper activation, T cells increase expression of CTLA-4 which eventually outcompetes CD28 for binding to CD80/CD86 and transmits inhibitory signals that regulate the extent and duration of T cell activation [131] . Beyond CTLA-4, many other "negative signal 2" molecules have been identified including PD-1, LAG-3, 2B4, TIGIT, TIM-3, and others [132]. The balance of positive (co-stimulation) and negative (co-inhibition) signals, akin to “gas” and “breaks”, regulates T cell activation and differentiation. The inhibitory aspect of the signal 2 concept has received considerable attention in the past decade, primarily due to the clinical success of ICB, first from αCTLA-4 (ipilimumab and tremelimumab), then αPD-1 and αPD-L1 (nivolumab, pembrolizumab, atezolizumab, etc.). In cancer, Texh cells express high levels of multiple co-inhibitory molecules [27]. PD-1 pathway blockade provides a temporary release from this inhibitory signal, shifting the balance toward activation and driving proliferation [136]. Notably, this αPD-1 effect requires signaling through CD28 [137], highlighting the interplay between positive and negative signal 2s for T cells in cancer biology.

Although the importance of these co-stimulatory and co-inhibitory molecules in regulating T cell function is now well-recognized, the specific downstream signaling pathways involved remain incompletely understood. It is clear that signals through different receptors can have different functional consequences. For example, CAR T cells with 4-1BB domains exhibit enhanced durability compared to those with CD28 domains that generate more effector and

exhaustion features [115]. Furthermore, blocking different IRs, such as PD-1 and LAG-3, have distinct functional outcomes [138]. However, defining proximal downstream targets, impact on cellular function, and whether these effects are conserved or context-specific remains an area of active investigation. This mechanistic knowledge should enable better rational design of next generation combination treatments. Moreover, the patterns of co-stimulatory and co-inhibitory molecules expressed by T cells change at different stages of effector, memory, or exhaustion development. In fact, CD28 is expressed on Texh-prog, but not Texh-term cells [52, 74] which may contribute to the ability of Texh-prog cells to selectively respond to ICB. Thus, it may be possible to selectively tune endogenous T cell responses by appropriate targeting of these co-stimulatory and co-inhibitory pathways.

Signal 3: Cytokines

Signal 3 was initially conceptualized as the inflammatory cytokine signal perceived by T cells during priming that instructed proper effector differentiation [139]. This paradigm linked the innate immune system directly to T cell activation (i.e. in addition to the role in activating APC) and allowed T cells to calibrate the magnitude and quality of the effector response to the severity of infection. Signal 3 was initially thought to comprise IL-12 and type I interferons. However, numerous cytokines, including IL-1, IL-18, IL-33, IL-6, and IL-27, as well as growth factors and regulatory cytokines like IL-10 and TGF-β, are now recognized to contribute to T cell responses [140]. These cytokines orchestrate aspects of T cell differentiation that are non-redundant with signals 1 and 2 and can also influence bystander memory T cells, an effect that may be relevant to anti-tumor responses in some settings.

Cytokines that can function as growth and survival factors also have a critical role as a form of signal 3, primarily members of the common γ-chain family (IL-2, IL-7, IL-9, IL-15, and IL-21). Although the effect of each cytokine varies based on receptor expression and differentiation state, these cytokines collectively regulate cell cycle, survival, and/or homeostasis. In cancer immunology, these cytokines, especially IL-2, have long been appreciated and exploited [141]. Combining PD-1 blockade with IL-2 has a potent effect on modulating T cell exhaustion and enhancing anti-tumor immunity [107, 108, 109, 142, 143] and numerous ongoing trials are aimed at exploiting the biology of IL-2 in cancer immunity [144]. In contrast, Texh cells typically express lower IL-7R than Tmem cells resulting in suboptimal responses to IL-7 compared to other competing T cells *in vivo* [27]. Nevertheless, IL-7 treatment during chronic viral infection in mice can expand the Texh population and moderately improve function [145, 146], including in combination with PD-1 blockade [31]. IL-15 may be another important common γ-chain cytokine for Texh cells, and targeting this cytokine may achieve some similar effects to IL-2 based on the shared use of IL-2Rβ. IL-21R is necessary for Texh cells to maintain functionality and control during chronic viral infection [147, 148, 149], and IL-21 can augment *ex vivo* expansion of tumor-specific CD8 T cells [150]. This effect is likely due to a requirement for IL-21 produced by CD4 T cells to promote generation of the Texh-int subset which contains more functional cells within the exhausted hierarchy [63]. Indeed, treatment with a tumor-targeting IL-21 fusion protein expanded cytokine-producing $PD1^{Int}Tim\text{-}3^{neg}$ $CD8^{+}$ T cells resulting in tumor control [151] and IL-21 combined with αCTLA-4 may have beneficial effects in patients [152]. Cellular

engineering approaches have also exploited common γ-chain cytokines to influence T cell state. The use of IL-7, IL-15, and IL-21 during CAR T cell production *in vitro* can promote memory differentiation and maintain proliferative capacity in infusion products [153]. In addition, some CAR T cells are being designed to secrete specific cytokines to augment their function in an autocrine manner but also enhance anti-tumor immunity through effecting in the local TME [154]. Despite the potential of common γ-chains to modulate (and improve) T cell function and differentiation, consistent clinical benefit has been challenging to achieve on a broad scale, likely due to complexities in drugging these cytokines and their receptors selectively on the T cell subsets of interest. Nevertheless, common γ-chain cytokines remain prime targets for therapeutic manipulation.

Particularly important for T cell biology in cancer are cytokines with regulatory properties, such as TGF-β and IL-10. These cytokines can be produced by a variety of cells, including Treg cells and suppressive myeloid cells, and are often found at high levels within the TME. Although TGF-β and IL-10 are generally considered to suppress $CD8^+$ T cell proliferation and function[140], they can also have potentially pro-immune/anti-tumor effects. For example, in chronic infection Texh cells express high levels of TGF-β and downstream phosphorylation of SMADs. Attenuation of TGF-β results in decreased expression of the proapoptotic protein BIM resulting in increased cell numbers[155]. In addition, TGF-β can promote Texh-term differentiation[156] and enhance PD-1 expression on $CD8^+$ TIL via SMAD3 [157]. These data demonstrate a generally suppressive effect of TGF-β. However, TGF-β is also required for maintaining the stem-like capacity of Texh-prog cells through suppression of mTOR signaling and preservation of cellular metabolism [158]. TGF-β also plays a major role in the formation and maintenance of $CD103^+$ Trm cells [10, 12]. Thus, tumor-derived TGF-β could enhance tissue-residency programs which has the potential to have pro-immune and anti-tumor effects. For example, *ex vivo* treatment of CAR T cells with TGF-β increased stem-like and residency properties resulting in increased tumor infiltration [159]. However, TGF-β also promotes the retention of Texh-prog cells in secondary lymphoid organs by regulating migration-related proteins [160] which can prevent migration from tumor-draining lymph nodes (TDLNs) to tumor after vaccination [161]. Paradoxical roles for IL-10 have also been observed. IL-10 is typically considered to be immunosuppressive, but treatment with PEGylated IL-10 can activate Texh cells and induce tumor regression in cancer patients [162]. This effect may be due, at least in part, to promoting OXPHOS in Texh-term subset [163]. These data highlight the complex effects of IL-10 and TGF-β beyond traditional roles in regulation and draw attention their influence altering metabolism and ultimately T cell function. Nevertheless, there are also strategies to block these regulatory cytokines [164] or convert them to positive activation signals [165].

Overall, it is now clear that signal 3 is required for optimal T cell differentiation. However, the net effect of cytokine signaling on $CD8^+$ T cell differentiation is complex, temporally regulated, and likely interactive with other signals. This complexity is highlighted by the example of IFN signaling. Although IFN-α/β serves as a critical positive signal for $CD8^+$ T cells during priming, in Texh cells IFN-α/β may drive the differentiation to terminal exhaustion and prevent tumor clearance [55, 80]. In addition, IFN-α/β can promote cell death through the Fas/FasL pathway [166]. Furthermore, persistent IFN-α/β and IFN-γ signaling induces negative regulators of T cell function, such as PD-L1, on various cell types

including tumor cells, Treg cells, and suppressive myeloid cells [167]. As a result, type I and II IFN represents a double-edged sword with both positive and negative effects on tumor immunity [168]. These complexities highlight the need for a nuanced understanding of the role of signal 3 and cytokines on $CD8^+$ T cells to optimize immunotherapeutic strategies and overcome resistance mechanisms.

Signal 4: Nutrients

Over the past decade, the existence of a crucial fourth set of signals that regulate T cell function and differentiation has become increasingly apparent – metabolic fuels and nutrients, like glucose, amino acids (AAs) and lipids. The importance of this set of signals becomes particularly evident in the TME. Here, the metabolic activities of many cells, especially the tumor cells, alter the nutrient landscape, and these changes can metabolically suppress T cell function and enhance exhaustion. This signal 4 also presents a new opportunity for therapeutic intervention, and treatments that metabolically rewire T cells, tumor cells, or other cells in the TME have the potential to improve anti-tumor immunity.

In a healthy setting, the nutrients that a T cell consumes and the metabolic pathways it employs change throughout differentiation to meet its energetic needs. These changes not only affect T cell bioenergetics, but also alter gene expression via epigenetic and post-translational protein modifications that, in turn shape T cell effector function and differentiation state. Naive T cells primarily rely on mitochondrial respiration driven by IL-7 dependent glucose use [169]. As T cells become activated, they need to generate biomass to support activation, proliferation, and logarithmic increase in cell numbers, as well as effector functions. This metabolic switch is driven largely by the combined effects of signals 2 and 3 that stimulate signaling pathways, such as PI3K → AKT→ MTORC1, and is regulated by TFs, including HIF1, MYC and STAT5 [170, 171, 172]. This anabolic phase is characterized by robust glycolytic activity, one-carbon metabolism, fatty acid synthesis and mitochondrial respiration and biogenesis, and is fueled by the large consumption of nutrients like glucose, glutamine, leucine, arginine and imported fatty acids [173]. As T cells return to quiescence and develop into Tmem cells, they switch metabolic activities to synthesizing and storing triglycerides and burning fatty acids and ketones [174, 175, 176]. Mitochondrial fitness therefore is a key feature of long-lived Tmem cells.

In contrast, Texh cells have an altered metabolic profile. These alterations include decreased mitochondrial mass, membrane potential, and oxygen consumption, whereas ROS production is increased [177, 178, 179, 180, 181]. Texh-prog cells, however, have improved mitochondrial function compared to Texh-term cells, consistent with the lack of proliferative capacity of the latter subset [158]. Mitochondrial cristae in Texh cells also appear damaged, diffuse and swollen, but increasing mitochondrial biogenesis via PGC-1α can overcome some of the poor metabolic features associated with T cell exhaustion [178, 179, 182]. The decreased mitochondrial function of Texh cells is coupled to PD-1 driven impairment of glycolysis, an effect that is associated with a greater reliance of Texh cells on lipids as they become more catabolic and autophagic [183, 184]. In tumors, TILs can exhibit increased lipid import, particularly of oxidized lipids, through the expression of the scavenger receptor CD36 [185, 186, 187]. This increased lipid uptake leads to increased lipid peroxidation, p38

signaling and ferroptosis, effects that can be reversed by overexpression of glutathione peroxidase 4 leading to improved TIL survival and function [186, 187]. Additionally, pharmacologically improving fatty acid use by TILs with peroxisome proliferator-activated receptor agonists enhances anti-tumor immunity and responses to ICB [188, 189]. There is also evidence that T cells can use the microbiota-derived metabolites inosine or acetate, which can improve anti-tumor efficacy and responsiveness to ICB [190, 191, 192].

The Tumor Metabolic Landscape

The metabolic states of different cell types and their fuel preferences can create different metabolic ecosystems within the TME (Figure 3). Tumor cells have high nutrient demands to support their rapid proliferation, and their aberrant consumption and production of metabolites alters the metabolic composition of the TME. Although recent work suggests that primary tumors might not be as hypermetabolic as once thought [193], the metabolic cross-talk between tumor cells, TILs, and other cell types within the TME can further influence the metabolic state of $CD8^+$ TILs, that in turn, affects their differentiation and function. In healthy organs, resident immune cells including Trm cells likely develop cooperative or complementary metabolic relationships with neighboring tissue cells. In contrast, in tumors, a metabolic tug-of-war occurs between the tumor cells, stromal cells, and immune cells with competition for key nutrients, such as glucose, that forces TILs to adopt metabolic states that limit tumor control. As tumors evolve, specific metabolic states may be selected for that not only promote tumor growth and invasion, but also suppress or evade T cell attack. Indeed, nutrient availability, oxygen levels, pH, and immunosuppressive metabolites in tumors can all affect $CD8^+$ T cell infiltration, survival, and antitumor functions, presenting challenges for rejuvenating immune responses through immunotherapy.

Historically, considerable attention was directed towards glucose use by tumor cells, largely due to the seminal work of Otto Warburg. However, glucose partitioning in tumors based on FDG-PET-tracing reveals that tumor associated macrophages (TAMs) also consume substantial amounts of glucose [194], suggesting a rethinking of glucose use in tumors. Nonetheless, TMEs with high glycolytic activity are often associated with reduced T cell infiltration, reduced IFN-γ production, and poor response to TIL therapy [195, 196, 197, 198, 199]. One possibility is direct competition between cancer cells, TAMs, and T cells for the limited supply of glucose in the TME [196, 197]. Indeed, deprivation of glucose and/or poor metabolism downstream of glucose uptake are hallmarks of Texh cells and poor TIL functionality. In contrast to Teff or Texh cells, Treg cells remain functional (ie., suppressive) under the low glucose conditions in the TME [200, 201]. This imbalance in Treg:Teff functions in highly glycolytic tumors likely contributes to their noteworthy resistance to ICB and TIL therapy [199, 202, 203]. Notably, hypoxia is another tumor feature linked to ICB resistance [204]. In addition to increasing glycolysis, hypoxia also increases expression of PD-L1 on both immune and tumor cells, and restricts T cell infiltration into hypoxic zones [205, 206, 207, 208]. Finding ways to overcome immunosuppressive hypoxic ecosystems in tumors to increase ICB responsiveness will be key. Mitochondrial toxins, like metformin, or anti-angiogenics, like VEGF-inhibitors, aim to achieve this goal and have demonstrated beneficial outcomes

with increased TILs in certain cancer types like kidney, liver, and lung cancer, but not in others [209].

A byproduct of increased glycolysis is the production of lactate and lactic acid. Lactic acid secretion acidifies the TME, an effect that may exacerbate T cell exhaustion [199, 210, 211, 212, 213]. However, tumors and T cells can also use lactate as a carbon source for oxidative metabolism, and LDH-A is necessary for Teff function [214, 215, 216]. Quantifying the contribution of different TME cell populations to tumor glycolysis together with other approaches such as spatial metabolomics may provide new opportunities to better understand the glycolysis-lactate-hypoxia axis in solid tumors.

Tumors further consume large amounts of AAs like glutamine, and the regulation of other AA availability and/or metabolism in the TME can impact T cell biology. For example, excessive uptake of glutamine by cancer cells can interfere with optimal T cell responses since T cells require glutamine to provide carbon and nitrogen for anabolic metabolism upon activation [217]. However, whereas cancer cells tend to be detrimentally affected by glutamine blockade, T cells display metabolic plasticity and can shift their metabolism to retain anti-tumor function [218, 219]. Glutamine and glucose metabolism are linked, and increased glutamine uptake can suppress glucose metabolism whereas under glutamine restriction, T cells increase glucose uptake [194]. The link between these metabolic pathways highlights a potentially important immunoregulatory metabolic axis in the TME. Additionally, immunoregulatory immune cells also shape the intratumoral metabolic environment. For example, TAMs import AAs and express enzymes like arginase or indoleamine 2,3-dioxygenase (IDO) that hydrolyze AAs into T cell suppressive derivatives [220]. Thus, both tumor cells and other immune cells can contribute to a metabolically hostile immune environment through AA-dependent processes including AA consumption and conversion into immunosuppressive molecules.

While some nutrients are limited in the TME, others like lipids, especially oxidized lipids, appear more abundant [186]. Increased lipid uptake and peroxidation can limit T cell function and survival, yet foster Treg activity in tumors [186, 187, 221, 222, 223]. On the other hand, tumors that excessively burn lipids through fatty acid oxidation may be sensitized to ICB [224], and perhaps such activities limit the availability of oxidized lipids to T cells. The lipid Prostaglandin E2 signals via the receptors EP2 and EP4 to suppress survival and function of T and NK cells. Indeed, reducing PGE2 production via Celecoxib synergizes with ICB[225, 226, 221, 228]. Notably, PGE2 production can be affected by dietary lipids, suggesting a potential mechanism contributing to the increased risk of cancer caused by obesity and diets rich in sugars and fats[229].

Tumor-Immune Ecosystems Within the TME

In addition to the diverse metabolic ecosystems, microenvironments of cellular interactions exist within the TME (also discussed in this issue [230]). Besides cancer cells and T cells, the TME is comprised of many other immune and non-immune cells, including stromal cells (like cancer associated fibroblasts (CAFs)), blood vessels, neurons, and extracellular matrix (ECM). The interactions between these diverse cell types give rise to unique ecosystems,

niches within tumors, which will likely emerge as regulators of tumor growth, invasion, and T cell suppression. It is becoming increasingly clear, that pathological landmarks such as fibrosis, hypoxia, necrosis, vascularization, tumor cell heterogeneity, and TLSs are associated with differences in local TIL composition and functions. High resolution spatial mapping of cell types and non-cellular structures within the TME is revealing conserved cellular networks and transcriptional programs and these insights will be crucial for advancing our comprehension of tumor immunology and tailoring effective therapeutic strategies.

Distinct tumor “archetypes”, “ecosystems”, or “hubs” which are organized cellular neighborhoods composed of variations in T cells, APCs, and stromal cells have been proposed that collectively impact antitumor T cell responses and consequently patient survival and response to therapy [231, 232, 233, 234, 235, 236, 237]. Immune-rich archetypes are dominated by $CD8^+$ T cells and further divided into macrophage-enriched (associated with Texh cells) or NK and conventional type 1 dendritic cell (cDC1)-enriched subtypes (associated with Teff cells) or $CD4^+$ T cell and conventional type 2 dendritic cell (cDC2)-enriched archetypes [85, 231, 237, 238]. Tumors can exhibit multiple ecosystems simultaneously, that can communicate with each other [231], and can change over time and in response to therapeutic interventions. While the dominant ecosystem in tumors is typically immunosuppressive, ecosystems that are immuno-stimulatory, such as TLSs, can exist within the same tumor. ICB and other immune-based interventions aim to shift the TME from a pro-tumorigenic environment to a more immunosupportive environment. Collectively, these data indicate that aside from the frequency of certain immune cell types, it is their spatial localization within the TME that is relevant for the generation of productive anti-tumor immune responses. Understanding the spatial dimensions of the TME will allow us to move beyond simplistic classifications of “hot” and “cold” tumors based solely on the quantity of intratumoral TILs.

APC-T cell interactions within the TME determine the efficiency of T cell responses by influencing signals 1 and 2 (and perhaps also 3 and 4), and the type of APC can determine the resulting T cell response [239]. APCs exist along a spectrum, ranging from T cell suppressive TAMs to specific subsets of conventional dendritic cells (cDCs) (also discussed in this issue [240]) that support anti-tumor T cells in some cases and tolerize in others. APC-T cell interactions can be geographically categorized into three primary locations: (1) local interactions within the tumor bed, (2) interactions within peritumoral areas including TLSs, and (3) distal interactions in the TDLNs.

Within solid tumors, the most prevalent APCs are typically TAMs derived from either tissue-resident macrophages or circulating monocytes that mature intratumorally[241]. TAMs are heterogeneous and can adapt to their surrounding environment. Antigen-specific T cells generally reside in TAM-rich regions of the TME and T cell-TAM interactions contribute to T cell exhaustion programs or prevent TIL infiltration into the tumor nests [242, 243, 244] In contrast, cDCs, although more rare, are a prognostic marker and correlate with the number of stem-like Texh-prog cells [60, 245, 246]. cDCs can be divided into cDC1s that predominantly promote $CD8^+$ T cell responses and cDC2s that support $CD4^+$ T cell responses, though cDCs can likely support either T cell type [247, 248]. Although both TAMs and cDCs express

PD-L1, it appears that cDC-derived PD-L1 suppresses TILs more dominantly than TAM-derived PD-L1, making cDCs an important target of PD-L1 blockade[249]. cDC1-T cell interactions also help maintain Texh-prog, and NK cells can help recruit cDC1s via Flt3, with both cell-cell interactions linked to ICB responsiveness [250, 251]. In addition to cDC1 interactions, intratumoral Texh-prog differentiation into Texh-int subsets can be enhanced by $CD4^+$ T cells and mreg DCs (dendritic cells enriched in maturation and regulatory molecules) in distinct intra-tumoral niches [63, 65, 104]. Structural cells like $MHCII^+$ CAFs can also serve as APCs. Other CAF subtypes including myofibroblastic CAFs and inflammatory CAFs can develop different spatio-functional relationships around tumor nests [252]. CAFs can impair T cell responses via multiple mechanisms, such as altering ECM density and composition, preventing TIL trafficking into tumor beds (often called TIL-'excluded' tumors), and promoting T cell exhaustion and apoptosis [253, 254, 255, 256, 257].

Peritumoral regions can often be more immune rich than deeper intratumoral areas. TLSs in these regions appear to be critical for facilitating intratumoral immune responses. Indeed, TLSs support Texh-prog biology and may enable Texh-prog cell activation and differentiation upon ICB. In fact, these structures often correlate with better prognosis and/or response to immunotherapy. Specifically, the presence of $PD\text{-}1^+CXCL13^+$ CD8 T cells is associated with responses to PD-1 blockade [70, 95, 236, 258, 259]. In addition to TLSs, proliferating Texh-prog-like cells can co-cluster with APCs in semi-organized structures, and this clustering can be associated with better disease outcome [60, 260]. It is likely that these organized or semi-organized lymphoid aggregates require additional support from stroma. Indeed, some CAFs may be involved in orchestrating formation of TLSs [261].

Immunotherapy can also activate immune responses distally in TDLNs [262]. Texh-prog-like cells that are clonally related to Texh cells in the tumor migrate from the TDLNs to the tumor, sustain the anti-tumor T cell response, and eventually undergo differentiation to terminal exhaustion [58, 64, 66, 263, 264]. Although some data from animal models using FTY720, a drug that blocks T cell migration, suggest that responses to ICB can occur without contribution from TDLNs [265], in most settings, it appears that some contribution from regional LN is important [64, 262, 264, 266]. TDLNs are often surgically removed due to the risk of metastasis. Given this emerging geographic understanding of Texh-prog biology and the role of TDLNs, re-evaluating how to maintain optimal anti-tumor T cell responses from TDLNs during the initiation of immunotherapy may be warranted in some cases [64, 267]. Tumor metastases in the TDLNs, however, impair ICB responses by altering the TDLN architecture and promoting the generation of Texh-term cells [64, 268]. Targeting TDLNs suggests new therapeutic opportunities including strategies to promote migration of tumor-specific T cells from TDLNs, or cancer vaccines that target TDLNs to improve activation of the lymphoid anti-tumor immune response. Thus, the appreciation that the systemic immune response might contribute to anti-tumor immunity not only broadens the opportunities for therapeutic interventions, but is also consistent with the ability to detect anti-tumor T cell responses in blood in many settings [239]. During the last decade, we have significantly improved the geographic and temporal resolution of our understanding of the TME and note the importance of incorporating space and time into the concept of signals 1-4.

New concepts for CD8 T cells in the Cancer Immunity Cycle

Over the past decade, in part due to the unparalleled progress in immunotherapy, significant strides have been made in understanding the role of the immune system role in cancer. The successes of T cell-based immunotherapies provoke three new concepts that could frame the next advances in harnessing T cells for treatment, cure, and prevention of cancer.

First, the increased mechanistic knowledge regarding the actions of many immunotherapies suggests that the therapies delivered to patients are “pro-drugs” (Figure 4). The T cells (and perhaps other immune cells) that are reinvigorated, induced, or otherwise manipulated are the actual *in vivo* drug. For engineered cellular therapies, the “pro-drug” phase has been skipped - or at least performed *ex vivo.* Such a concept of pro-drug and drug has implications for immune-pharmacodynamics. This concept highlights the need to examine and define pharmacological principles (pharmacokinetic/pharmacodynamic, PK/PD) for the T cell targets themselves in addition to the pro-drug molecules (i.e. ICB antibodies). Defining the kinetics, magnitude, and duration of the target T cell population (such as Texh cells) as well as specific anti-tumor effector functions (cytokine production, etc.), can then be used to further optimize properties of the pro-drugs. For example, the burst of $CD8^+$ T cell reinvigoration that occurs following PD-1 pathway blockade typically peaks at ~2-4 weeks. Such a pharmacodynamics suggest that combination therapies intended to capitalize on changes to the *in vivo* drug (i.e. the reinvigorated $CD8^+$ T cells) should be delivered during this window of response. Dose regimens and combination therapies could be re-imagined if immune-pharmacodynamics were known in detail.

Second, we have witnessed a major shift in human immunology research from primarily observational studies to attempting true mechanistic science in patients and healthy donors. This transformation can be traced, in part, to major investments and advancements made during the height of the HIV crisis in the last century but has also been accelerated by the ever-increasing capacity for high dimensional analysis, and more recently advancements spurred on by the SARS-CoV-2 pandemic. Studies in human cancer patients receiving immunotherapies have significantly contributed to these advances in human immunology by providing opportunities to capture temporal immune changes associated with therapeutic interventions, such as ICB. The ability to capture the window of immune dynamics upon initiation of immune-based treatments has been a key to some of these insights, for example, in neoadjuvant ICB studies where changes in the intratumoral T cell response can be examined shortly after treatment initiation. These data have led to transformational insights into the mechanisms of CTLA-4 and PD-1, the action of CAR T cells and TILs. Thus, a tremendous opportunity for the future is to continue to capture these interventions and deeply interrogate the mechanisms not only of clinical response to the intervention, but the broader impact of these immunotherapies on the immune system.

Third, a major emerging concept is that overall “immune health”, including immune history and lifestyle, plays a substantial role in shaping potential immune responsiveness to future interventions. It is becoming increasingly clear that biologic sex, hormones, and age can have a substantial impact on baseline immune health status and ability to respond to perturbations such as cancer or infections. In addition, we now appreciate that many

other non-genetic factors influence future immune responses. There is strong evidence for major immunological events including previous infections [269], chemotherapy treatment, or immunosuppression impacting responses to vaccines or other immune-based therapies. There is also emerging data that concomitant medication may shape immune responses to cancer. Similarly, the immunological role of the microbiome in cancer immunotherapy has received considerable attention, provoking obvious questions about the use of antibiotics with cancer immunotherapy [270, 271]. Such data also highlight the role of diet, and emerging evidence supports dietary influences, including fiber intake, on ICB outcomes through effects on the microbiome [272]. The human immune system is shaped by a lifetime of interactions with the microbial and external antigen world, many of which form durable imprints (i.e. immunological memory) that shape future responses. More broadly, however, lifestyle factors including obesity, fasting behavior, circadian biology, chronological versus biological age and many other variables are likely to influence immune fitness. Together, these durable and/or malleable features of individual immune systems form the basis of how an individual patient will respond to a future intervention. A major opportunity for the future is to assess how immune health characteristics of patients relate to T cell responses to immunotherapy and use this information to tailor our interventions to the needs of individuals.

Concluding Remarks

Our ability to deeply profile both the immune and the non-immune cells in tumors can help identify which part(s) of the Cancer Immunity Cycle require intervention to improve tumor control. Examining such information from individual patients in the context of the signals 1-4 that control T cell differentiation and function should provide guidance for personalized immune-based therapies. For example, a dearth of activated T cells in a tumor might suggest that providing additional signal 1 through antigen-targeted therapies such as vaccination could be useful. Absence of inflammatory DCs could indicate αCD40L, perhaps in combination with vaccination, could help activate APCs and provide more optimal signals 1,2 and even 3 to induce an endogenous tumor-specific T cell response. In contrast, if a tumor is highly infiltrated with Texh cells, ICB such as αPD-1 alone or in combination with other therapies, such as αLAG-3 or IL-2 treatment (further decreasing inhibitory signal 2 or augmenting signal 3) could stimulate these tumor-specific T cells to enhance tumor cell killing. Moreover, therapeutically addressing signal 4 from metabolism and nutrients provides additional opportunities for individual or combination immunotherapy. A major goal for the future will be to integrate broader data about overall immune health and immune fitness with detailed tumor immunity and tumor genetics data, at baseline and on-treatment to guide treatment choices.

Thus, the last decade has seen a true transformation of our understanding of $CD8^+$ T cells not only as key targets for our immunotherapies, but as critical *in vivo* drugs. Our knowledge of $CD8^+$ T cell differentiation and developmental biology, while still incomplete, has improved our understanding of how immunotherapies like ICB function, and has also enabled considerable progress in engineering CAR T cells and TCR engineered cellular therapies. A conceptual framework of a 4-signal model of T cell activation together with an

ever improving map of the differentiation trajectories of $CD8^+$ T cells should continue to provide a foundation for understanding and improving cancer immunity.

Acknowledgments

This work was supported by grants from the NIH AI105343, AI082630, AI108545, AI155577, and AI149680 (to EJW); HL143613, T32 CA009140, Cancer Research Institute-Mark Foundation Fellowship, and the Parker Institute for Cancer Immunotherapy (to JRG, EJW); R01 CA230275, P01 AG073084, and the Mark Foundation (to SMK), P30 CA014195 (R. Shaw); NOMIS Center Postdoctoral Fellowship and German Research Foundation (Deutsche Forschungsgemeinschaft, GL 991/1-1, to AMG). Work in the Wherry laboratory is supported by the Parker Institute for Cancer Immunotherapy.

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Box 1:

When does exhaustion start?

When exactly development of Texh cells diverges from that of Teff/Tmem cells, when the Texh differentiation state becomes irreversibly established, and whether there are early changes before the Texh epigenetic commitment to exhaustion occurs are all central questions with implications for immunotherapy. $CD8^+$ T cell exhaustion is progressive, becoming more severe with higher concentrations and/or a longer duration of antigen stimulation [15, 27, 58, 273, 274, 275]. Many studies have shown that once the mature Texh population is formed, these cells cannot be de/re-differentiated into the Teff/Tmem states [15, 31, 274, 276, 277, 278]. Mouse models of cancer and chronic infection have revealed a potential early divergence (~5-8 days) of exhausted precursors (Texh-pre) from cell states such as Tmp and conventional Teff that arise during acute-resolving infections [15, 18, 34, 74, 279]. Recently, it has been suggested that this bifurcation may occur as early as the first cell division [273 280]. However, the “inflection” point for this transition to irreversible exhaustion appears to be ~2-3 weeks of chronic stimulation [15, 276, 277, 278]. This switch is associated with major epigenetic and transcriptional changes [32, 34, 41, 74].

One key reason that a progressive model of T cell exhaustion has emerged is that the early Texh-pre cells in mouse models of cancer and chronic infection retain fate-flexibility whereas later Texh do not. If Texh-pre are removed from chronic antigen stimulation and adoptively transferred into an antigen-free host, these cells can recover and differentiate into Tmem cells [15, 277]. In the setting of chronic stimulation, these early fate-flexible Texh-pre cells seed the fully committed Texh population by differentiating into exhausted progenitors (Texh-prog) [21, 41, 74, 279]. Within the mature Texh population, these Texh-prog give rise to the other Texh subsets. Committed Texh-prog cells are transcriptionally and epigenetically distinct from the fate-flexible Texh-pre cells [74]; however, both Texh-pre and Texh-prog express TCF-1 and CXCR5 [74]. Numerous human studies have used TCF-1 and/or CXCR5 to identify Texh-prog [51, 52, 53, 54, 55, 56, 59], but the distinction between Texh-pre and Texh-prog is unclear based on these two markers alone. Indeed, not all $TCF\text{-}1^+$ $CD8^+$ T cells are the same. This TF is also expressed by naïve T cells, as well as Tmem and Tmp cells during acute-resolving infection. Furthermore, additional complexity within the Texh-prog population has been proposed, with high expression of CD62L marking cells with enhanced stem-like capacity [281] similar to a $CD62L^+$ Tmp population[282]. In contrast, CCR6 is expressed by $TCF\text{-}1^+$ Texh-prog cells that respond poorly to ICB [90]. Identifying developmentally “young” Texh-pre within the TME through the discovery of additional robust biomarkers could have considerable utility because these cells may be more amenable to therapeutic manipulation compared to fate-committed mature Texh cells. However, disentangling these relatively similar cell types in heterogenous and evolving human tumors is challenging without knowing when each T cell clone was initially activated.

During chronic infections, in addition to the Texh-pre subset, a second population of short-lived effector-like (Teff-like) cells is generated that is distinguished from bona fide Teff cells in acute infection [21, 41, 55, 74, 279]. This population is terminal, dies off quickly after the first week in the presence of persisting stimulation, and does not

contribute substantially to the mature Texh population [41]. It is currently unclear whether an analogous subset arises in developing cancers.

The early bifurcation between the Texh and Teff/Tmem branches suggests alterations during priming such as increased signal 1 (TCR stimulation), suboptimal signal 2 (low co-stimulation/high co-inhibition), altered signal 3 (low or high inflammatory cytokines), or different signal 4 (nutrients and fuel) may drive these distinct $CD8^+$ T cell fates. A potentially relevant aspect of cancer is TME evolution during tumorigenesis which may provide different contextual settings for T cell priming over the course of cancer development. However, direct comparison of mature Texh populations in cancer and chronic infection has revealed central transcriptional and epigenetic principles, pathways, and specific drivers of Texh differentiation, while also revealing potential disease-specific features related to microenvironment, such as inflammatory signals and diverse regulatory pathways [56]. Indeed, differences between cancer types, such as cold/non-inflamed versus hot/inflamed tumors, are also likely to influence $CD8^+$ T cell priming and differentiation.

Box 2:

Texh or Trm?

There is increasing interest in defining the relationship between Texh and Trm cells because both populations can be present in non-lymphoid tissues, and these cell types can display partially overlapping phenotypes [14, 275, 283]. Texh-term cells display features of residency, such as CD69 expression [61], and Trm cells share some characteristics with Texh cells, including IR expression even in healthy antigen-free tissues [284, 285, 286]. However, Trm cells can also form in tissues where antigen persists [287, 288, 289], but they may have distinct phenotypes in these settings compared to in healthy tissues [290]. Indeed, it is currently unclear whether restimulated Trm cells can migrate from adjacent tissues into the TME and become exhausted, or whether Texh cells (or Texh-pre or Texh-prog cells) migrate from circulation and upregulate residency programs upon entry to the TME. Trajectory analyses from human cancer suggest that both differentiation pathways can occur with some tumor types predominated by T cells employing one pathway over the other [23, 291]. It is also likely that the stage of tumorigenesis effects which pathway is most dominant. Early stage tumors that have less vascularization and exhibit less inflammation may only recruit Trm from nearby tissues, whereas later stage highly vascular tumors can draw in more $CD8^+$ T cells from circulation with chemokines and other inflammatory cues.

Increased Trm cells and/or Trm signatures are associated with better prognosis in several cancer types [292, 293, 294, 295, 296]. These data may indicate increased infiltration of Trm cells from adjacent tissues or increased expression of residency programs from circulating cells. However, mouse models have demonstrated better protection against tumor challenge when both tumor-specific tissue-resident and circulating T cells are present [297, 298, 299, 300]. Regardless, enhancing migration into the TME and/or improving retention within the TME may be beneficial to enhance tumor immunity. As a result, therapeutic targeting of Trm is currently an active area of drug development. Although, it is also possible that retention within the TME could drive antigen-specific $CD8^+$ TIL towards terminal exhaustion differentiation due to chronic stimulation and suppressive elements within the TME – if the tumor is not eradicated. The intersection between T cell exhaustion and tissue residency and how these biologic programs can be manipulated to improve tumor control will be key research areas in the future.

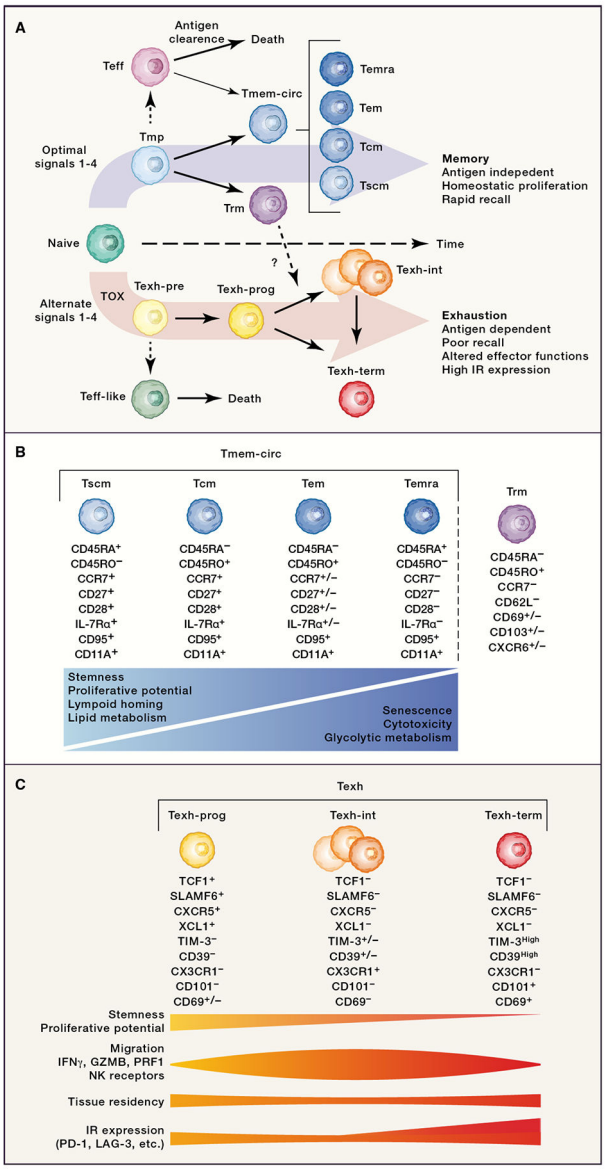

Figure 1: CD8$^+$ T cell differentiation: Two pathways

A. Upon activation, naïve CD8$^+$ T cells differentiate down two main developmental pathways which are regulated, in part, by four signals. **B**. Conventional classification scheme for human memory CD8$^+$ T cells. Below each subset are expression profiles commonly used for identifying these subsets within Tmem population. Key functional characteristics are depicted by relative expression across the memory CD8$^+$ T cell differentiation spectrum. These comparisons focus on later stage memory fate-committed subsets. **C**. The three major Texh subtypes with expression profiles commonly used for identification within Texh population. The relative expression of various traits, functions, and expression profiles across subsets within Texh population are represented below. These comparisons focus on later stage exhaustion fate-committed subsets.

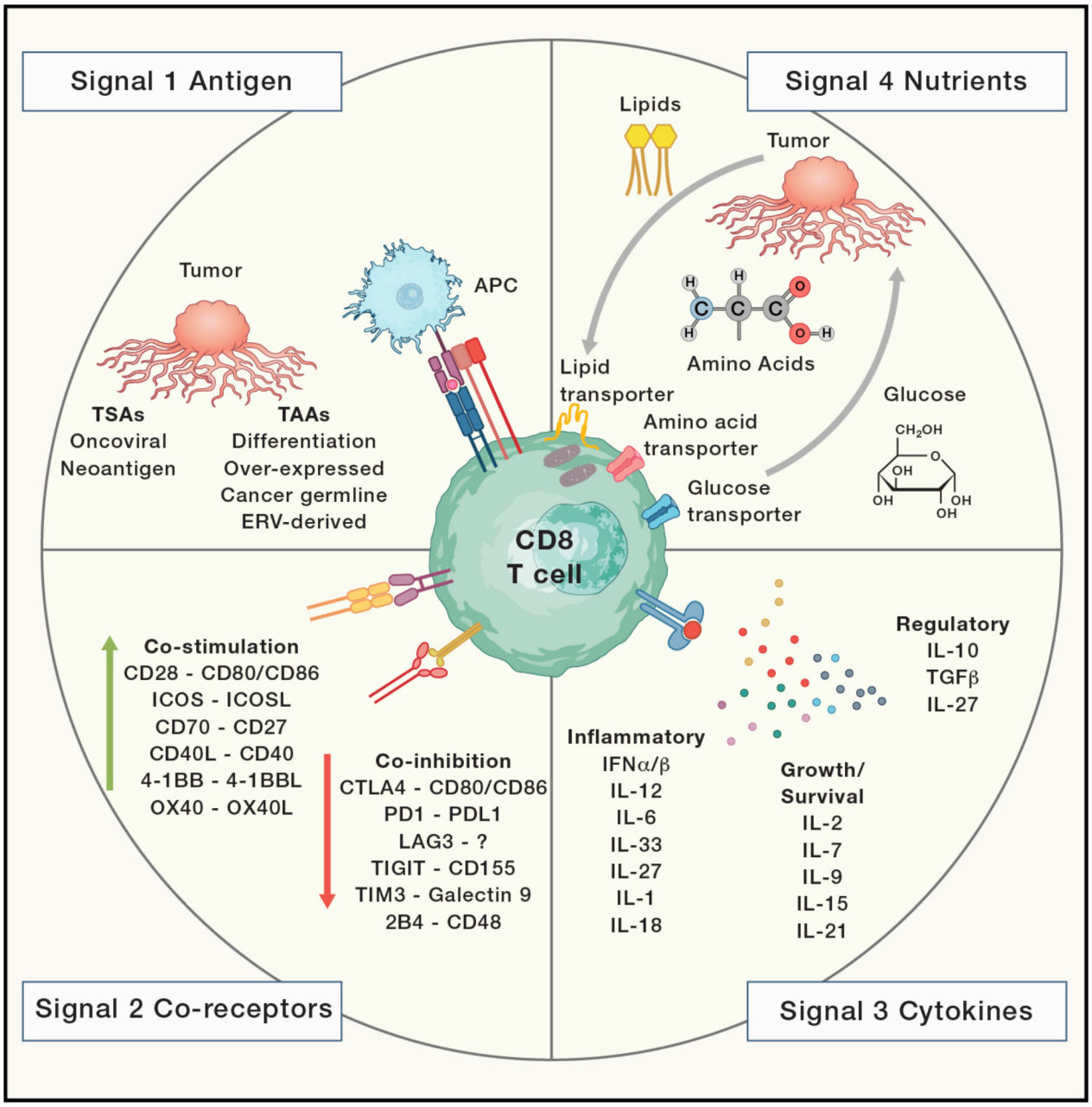

Figure 2: Four signal model of T cell activation and differentiation

Signal 1 is TCR recognition of peptides presented on MHC molecules. In cancer, these antigens are classified as tumor-associated antigen (TAAs) which can be expressed by both tumor and healthy tissue or tumor-specific antigens (TSAs) which are exclusively expressed by cancer cells. Signal 2 encompasses co-stimulatory and co-inhibitory signals, the balance of which influences T cell activation status and effector functions. Signal 3 includes signaling received through cytokines. The 3 main classes of cytokines include inflammatory, growth/survival, and regulatory. Signal 4 includes nutrient sensing and usage as well as internal changes in metabolic lifestyle.

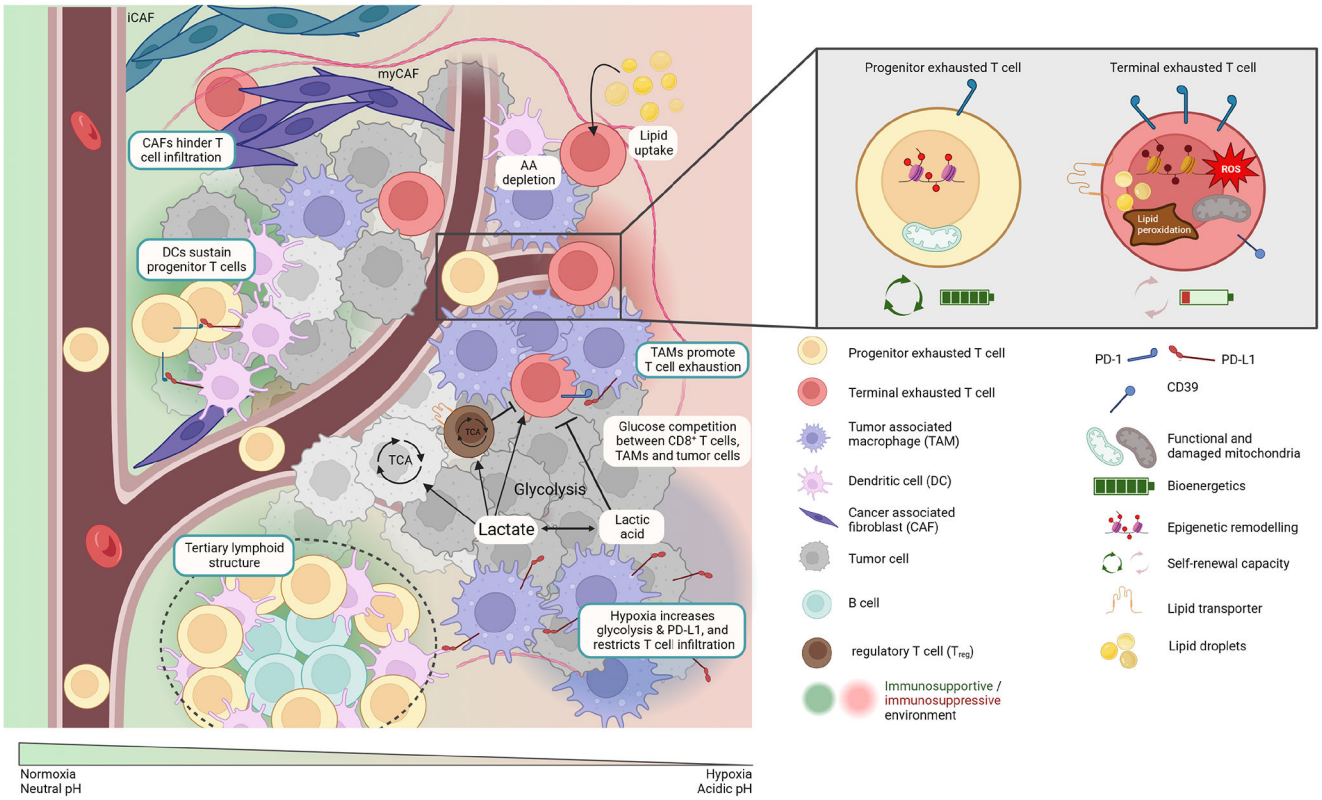

Figure 3: Metabolic ecosystems in the TME

The metabolic states of different cell types and their fuel preferences, as well as histological landmarks in the tumor microenvironment (TME) such as vasculature can create metabolic niches. These niches in combination with the cellular interactions of T cells with other immune and non-immune cell types generate immunosupportive (green) or immunosuppressive (red) conditions, that can influence T cell differentiation and function. Progenitor exhausted and more terminal exhausted T cells display unique metabolic characteristics.

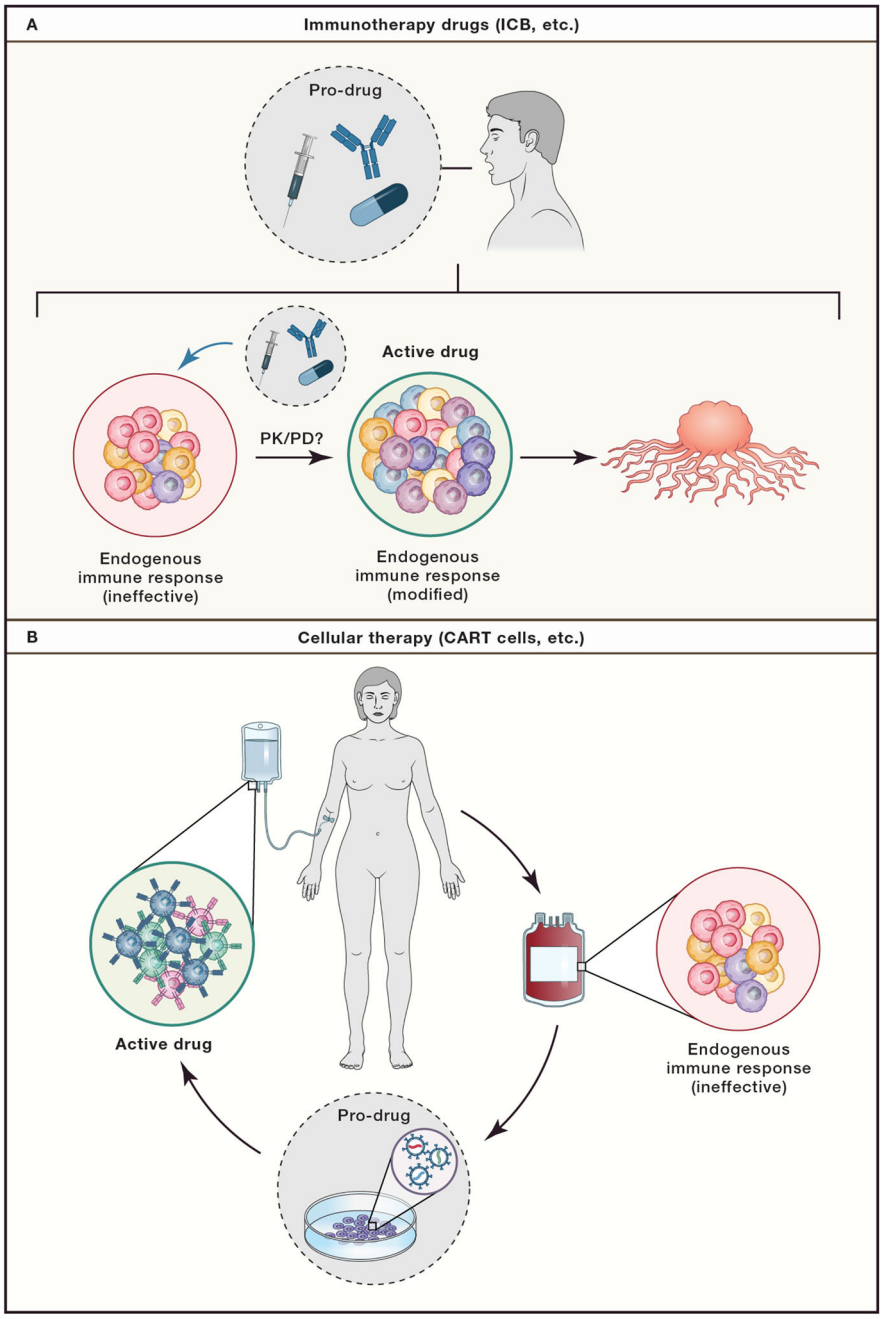

Figure 4: The immune system is the active drug component of immunotherapy

A. Immunotherapies, such as ICB, represent pro-drugs that are taken by patients. These pro-drugs (i.e. αPD-1) act on cells in the endogenous immune system, such as ineffective Texh cells. These modified endogenous cells (such as "reinvigorated" Texh cells) then represent the active drug which mediates the anti-tumor activity. In this scenario, traditional pharmacology concepts such pharmacokinetic/pharmacodynamic (PK/PD) measurements and modeling can be applied to the active drug, the modified immune cells, instead of the pro-drug. In this case, the kinetics, magnitude, and duration of the T cell response would be treated as the measurements of interest. **B.** Cellular therapy, such as CAR T cells, or

other engineered cells including TIL, are manufactured from a pro-drug (ineffective and unmanipulated endogenous $CD8^+$ T cells) to an active drug in vitro (i.e. CART19) which are then transferred in vivo to mediate tumor killing.