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The Road Less Taken: Less Appreciated Pathways for Manipulating CD8$^+$ T Cell Exhaustion

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Exhausted CD8$^+$ T (Tex) cells are a distinct cell population that arise during persistent antigen exposure in the context of chronic infections and cancers. Although characterized by progressive loss of effector functions, high and sustained inhibitory receptor expression and distinct transcriptional and epigenetic programs, Tex cells are heterogeneous. Among these, a self-renewing TCF-1$^+$ Tex population, having unique characteristics and the ability to respond to immune-checkpoint blockade, gives rise to TCF-1$^-$ terminally Tex cells. These TCF-1$^+$ cells have stem cell-like properties similar to memory T cell populations, but the signals that regulate the developmental pathways and relationships among exhausted cell populations are still unclear. Here, we review our current understanding of Tex cell biology, and discuss some less appreciated molecules and pathways affecting T cell exhaustion. We highlight two co-stimulatory receptors, CD226 and CD137, and their role in inducing or restraining T cell exhaustion, as well as signaling pathways that may be amenable to pharmacological inhibition with a focus on Phosphoinositide-3 Kinase and IL-2 partial agonists. Finally, we discuss novel methods that may increase TCF-1$^+$ populations and therefore improve immunotherapy responsiveness. Understanding features of and pathways to exhaustion has important implications for the success of immunotherapy, including checkpoint blockade and adoptive T-cell transfer therapies.

Keywords: CD8$^+$ T cell exhaustion, CD226, CD137, TCF-1, PI3 Kinase delta, IL-2, metabolism

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INTRODUCTION

CD8$^+$ T cells play critical roles in both fighting infection and restraining tumor growth. Activation of CD8$^+$ T cells occurs upon the engagement of the T cell receptor (TCR) complex that recognizes foreign or tumor antigens presented by MHC Class I molecules, in conjunction with co-receptors that enhance or diminish TCR signaling.

During acute infection, CD8$^+$ T cells can adopt several fates: they can become cytolytic short-lived or long-lived effector cells that help clear infections; alternatively, they can differentiate into memory-precursor cells that form long-lived central and effector memory cells poised for future protection (1). In

contrast, during chronic infections and cancer, chronically stimulated antigen-specific T cells progressively decrease in quantity and function as they enter a state of hyporesponsivness called "T cell exhaustion", characterized by the loss of cytokine production and proliferative potential, development of metabolic dysfunction and increased expression of inhibitory receptors (IRs), including PD-1, Tim-3 and CTLA-4. Targeting these IRs has been validated as a promising therapeutic strategy against cancer, and potentially chronic infection, as illustrated by clinical success achieved with immune checkpoint blockade (ICB) using monoclonal antibodies (mAbs) against PD-1 and CTLA-4 in metastatic melanoma (2, 3). Although exhausted T (Tex) cells display impaired responses to TCR engagement, this hyporesponsive state enables Tex cells to persist under conditions of chronic stimulation (4). Extensive efforts have focused on understanding cellular and molecular mechanisms that drive T cell exhaustion, and finding potential strategies to recover and maintain effector T cell function under conditions of exhaustion.

Nonetheless, heterogeneity has been observed among Tex cells, related to the progressive nature of this process. A specific subset of Tex cells, defined as 'precursor exhausted' or 'stem-like' progenitor (pTex) cells, retains some effector function and shares characteristics with memory cells (**Figure 1**). pTex cells are defined by and require the expression of the transcription factor TCF-1, which is critical for T cell 'stemness' (5–7) and is essential for the development of central memory T cells during acute infection (7, 8). TCF-1$^+$ pTex cells both self-renew and, upon persistent antigen stimulation, convert into more 'terminally exhausted' states, as well as cytolytic effector-like cells (9). Thus, pTex cells are critical to maintain CD8$^+$ T cells under conditions of exhaustion. Data argue that pTex cells, as opposed to the bulk of Tex cells that do not express TCF-1, play an indispensible role in immunotherapy, since this population is required for and correlates with efficient responses to ICB (5–7, 10, 11).

Recent studies have revealed that Tex cells also acquire a distinct epigenetic state. The transcription factor Tox is central to this process *via* its role in epigenetic remodeling and transcriptional cascades that orchestrate Tex cell development by directing histone acetylation (12–14). These conserved epigenetic features in terminal exhaustion become fixed and can persist independently of chronic antigen stimulation and inflammation (15–17): this exhausted state cannot be rescued. In contrast, the transcriptional repressor BACH2 is transcriptionally and epigenetically active in TCF-1$^+$ pTex cells and has been shown to suppress the molecular program driving terminal exhaustion (18, 19). Since only a fraction of cancer patients' respond to current ICB such as anti-PD-1 mAbs, therapeutic efforts to recover Tex effector functions may require new approaches including those that increase epigenetic plasticity of Tex cells and promote pTex cells.

In this review, we focus on some less-appreciated pathways affecting the development of T cell exhaustion. We discuss two co-stimulatory receptors, CD226 and CD137, as well as signaling pathways that may be amenable to pharmacological inhibition with a focus on Phosphoinositide-3 Kinase (PI3K) and IL-2 partial agonists and their complex roles in T cell function and exhaustion. Finally, we discuss novel methods that may promote TCF-1$^+$ populations and potentially enhance immunotherapy responsiveness. Understanding molecular pathways that contribute to exhaustion has important implications for improving successful immunotherapy, including both ICB and adoptive T cell transfer approaches.

THE ROLE OF ACTIVATING RECEPTORS IN T CELL EXHAUSTION

The original two signal model for lymphocyte activation states that T cells require both antigen-receptor engagement and co-stimulatory signals to achieve appropriate activation following interaction with activated antigen presenting cells. Since this original hypothesis and early experiments supporting this concept were published, several decades of work have elucidated an important diversity not only in positive co-stimulatory pathways that increase lymphocyte activation, but also IRs that counterbalance these activation signals (20, 21).

While most strategies for countering T cell exhaustion focus on IRs restraining effector functions of CD8$^+$ T cells, activated T cells constitutively express or upregulate numerous activating co-stimulatory molecules that can fine tune CD8$^+$ T cell activation and are important for regulating CD8$^+$ T cell responses to persisting infections and cancer. These receptors present potential therapeutic targets. However, analyses of these pathways also highlight the complex nature of co-stimulation in the development and function of Tex cells, where promoting activation can invigorate cells but may also paradoxically promote exhaustion.

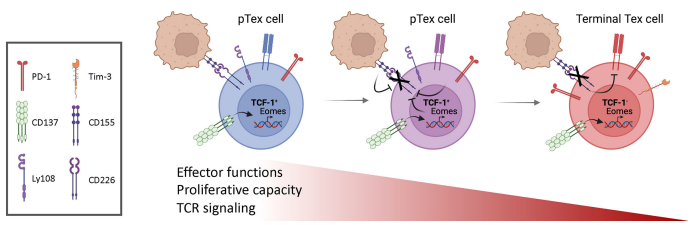

FIGURE 1 | Progressive changes in T cell exhaustion. Stem-like or progenitor exhausted cells (pTex) self-renew and, in the presence of continual antigen stimulation, give rise to more exhausted T (Tex) cells, characterized by expression of inhibitory receptors, loss of effector cell functions, proliferative capacity and their ability to respond to ICB. CD137 stimulation of early pTex cells induces high expression of Eomes, which, together with posttranslational mechanisms induced by CD155 expressed on cancer cells, drives CD226 downregulation. Loss of CD226 further impairs TCR signaling and contributes to T cell dysfunction. Figures drawn using Biorender.

The Importance of Being There: CD226 in T Cell Exhaustion

CD226 (DNAX accessory molecule 1, DNAM-1) is expressed on T cells and contributes to cytotoxic lymphocyte (CTL) activation (22, 23). Studies examining CD226-deficient mice indicated that CD226 serves as a co-stimulatory receptor that amplifies CTL and NK cell-mediated cytotoxicity against targets expressing its ligands CD112 and CD155 (24, 25).

In chronic infection with HIV-1 and HIV-2, downregulation of CD226 has been observed in the peripheral blood: increased $CD226^-PD\text{-}1^+$ antigen-specific $CD8^+$ T cells correlated with increased viral load (26, 27). CD226 downregulation also occurs in antigen-specific $CD8^+$ T cells in mice chronically infected with LCMV clone 13, a common model used to evaluate T cell exhaustion. These studies suggest that Tex cells lose CD226 expression; however, the functional consequences remained unclear.

Through complementary experiments involving human samples and mouse tumor models, two groups showed that loss of CD226 induces hypo-responsiveness in $CD8^+$ T cells, and limits both TCR signaling and responsiveness to anti-PD-1 mAbs (28, 29). $CD226^-CD8^+$ T cells failed to proliferate and produce effector cytokines upon CD3/CD28 mAbs stimulation, whereas ectopic re-expression of CD226 in $CD226^-CD8^+$ cells rescued responsiveness. Single-cell RNA sequencing (RNAseq) of tumor-specific $CD8^+$ T cells isolated from murine melanomas revealed that $CD226^+$ Tumor Infiltrating Lymphocytes (TILs) exhibited an enrichment for genes associated with T cell activation and immune synapse formation compared to $CD226^-$ counterparts (28). Of note, decreased TCR signaling is observed in $CD8^+$ Tex cells during chronic infection, as evidenced by low expression of the *Nr4a1*-GFP reporter and RNAseq analysis of TCR signaling-associated genes (30).

However, RNAseq comparing $CD226^+$ and $CD226^-$ $CD8^+$ T cells post CD3/CD28 mAbs stimulation revealed that activated $CD226^-CD8^+$ T cells exhibited increased expression of genes associated with pTex cells including *Tcf7*, *Slamf6* (encoding TCF-1 and Ly108, respectively), as well as increased *Tox*. Nonetheless, one principal characteristic of pTex cells, the ability to response to ICB, is not shared by $CD226^-$ cells. Anti-PD-1 mAbs failed to restore effector functions of TILs lacking CD226 expression in a transplantable melanoma model, suggesting that this Tex population resembles a more terminally exhausted population: a recent study confirmed that CD226 expression is required on TILs for efficient ICB responsiveness (31). Indeed, a continuum of low, intermediate, and high CD226 expression within $PD\text{-}1^+CD39^+CD8^+$ exhausted TILs has been described. High CD226 expression correlated with increased ability of $CD8^+$ TILs to secrete IFN-γ, suggesting that CD226 expression defines functional states among Tex and potentially more cytotoxic cells (28). These studies provide support that the absence of CD226 represents an unappreciated mechanism limiting TIL responsiveness independently of IR expression.

How is CD226 expression regulated? Post-translationally, the CD226 ligand, CD155, within the tumor microenvironment has been implicated in the downregulation of CD226 on TILs. Furthermore, mice with a Y319F mutation in CD226, which abrogates recruitment of the E3 ubiquitin ligase CBL-B for ubiquitinylation and proteasomal degradation, exhibited increased CD226 expression on $CD8^+$ T cells and suppressed tumor growth (28). Additionally, CD226 downregulation was found to be dependent on the transcription factor Eomesodermin (Eomes), which is associated with T cell exhaustion (**Figure 1**) (32–34); increased Eomes was observed in $CD226^-CD8^+$ cells compared to $CD226^+$ counterparts upon *in vitro* activation. In contrast, the absence of CD226 on $CD4^+$ T cells is associated with decreased T-bet, a closely related transcription factor associated with IFN-γ and effector function; similarly, in NK cells, CD226 induces T-bet (35). Whether CD226 directly affects these transcription factors in $CD8^+$ T cells is unclear. It is therefore of interest that another co-activating receptor, CD137 (4-1BB, TNFRSF9), has been implicated in the induction of Eomes and downmodulation of CD226 (29).

CD137 – Too Much of a Good Thing

CD137 was initially described as a co-stimulatory member of the tumor necrosis factor receptor (TNFR) superfamily that enhanced T cell proliferation and cytokine secretion, as well as protected T cells from activation–induced cell death (36–38). Injection of CD137 agonist mAbs expanded $CD8^+$ effector T memory cells and promoted tumor regression in a variety of mouse tumor models in a $CD8^+$ T cell-dependent manner (39, 40), suggesting CD137 is a promising target to increase T cell function. In mouse tumor models, CD137 stimulation increased cytotoxicity of $CD8^+$ TILs through increased Eomes expression (41). However, anti-CD137 mAb stimulation also promoted accumulation of dysfunctional $CD226^-CD8^+$ T cells in C57BL/6 WT mice (**Figure 1**).

Similar to hypofunctional $CD226^-CD8^+$ T cells within tumors, $CD226^-CD8^+$ T cells induced by anti-CD137 mAbs in C57BL/6 WT mice failed to proliferate and secrete TNF-α and IFN-γ in response to TCR stimulation. $CD226^-CD8^+$ TCR transgenic T cells generated in response to CD137 stimulation were devoid of effector functions after antigen stimulation *in vitro* and had significantly weaker anti-tumor properties than $CD226^-CD8^+$ T cells *in vivo*. Thus, hypofunctional $CD226^-CD8^+$ T cells are generated both in a tumor context and following CD137 stimulation in mice. In view of the expression of CD137 ligand (CD137L) by dendritic cells (42) and by numerous tumor lines (43, 44), CD137 may down-modulate CD226 both in early phases of anti-tumor immune responses and in later phases at the tumor site, respectively.

While the functions of CD137 may seem contradictory, CD137 ligation, similar to chronic infection, increases T cell activation, which may promote T cell exhaustion. These observations may provide insight into why anti–CD137 agonists decrease clinical symptoms in mouse models of autoimmunity including collagen-induced arthritis (45), experimental autoimmune uveoretinitis (45), EAE (46) and systemic lupus erythematosus (47). In addition, anti-CD137 mAbs can also activate NK cells (48, 49), macrophages (50) and inhibit Treg cells (51, 52), further complicating interpretation of their actions. Thus, these studies highlight the

paradoxical roles of CD137 signaling in T cell exhaustion and the need for caution in clinical trials.

PHARMACOLOGICAL APPROACHES TO MANIPULATE EXHAUSTION

Pharmacological interventions, particularly those increasing or boosting TCF-1$^+$ populations present alternative approaches to countering exhaustion, which are not focused on directly activating or inhibiting co-stimulatory molecules. In this regard, recent data on Phosphoinositide 3 Kinase delta (PI3Kδ present intriguing possibilities.

PI3Kδ and the Regulation of CD8$^+$ T Cell Function

PI3Ks are lipid kinases that catalyze the addition of phosphate to the D3 position of phosphoinositides, most notably the ubiquitous membrane phospholipid PI$(_{4,5})$P$_2$, to generate PI$(_{3,4,5})$P$_3$ (PIP$_3$). PI3Kδ is highly expressed in hematopoietic cells and participates in signaling downstream from the TCR, CD28, ICOS, as well as chemokine and cytokine receptors. PIP$_3$, in turn, recruits proteins containing Pleckstrin homology and other PIP$_3$-binding domains to the membrane where they can interact with other proteins and/or be phosphorylated. Among its effectors, AKT kinases are key components of PI3K-activated pathways, phosphorylating downstream targets including transcription factors such as FoxO1 and BACH2, the chromatin modifier Ezh2, and regulators of mTOR (53). AKT-mediated phosphorylation leads to nuclear exclusion and inactivation of FoxO1 and BACH2. These signaling cascades can be counterbalanced by PD-1-mediated SHP1 recruitment which limits PI3K activation (54) as well as lipid phosphatases such as SHIP and PTEN (53).

Recent data suggest that PI3K plays a major role in regulating expression of *Tcf7*, which is a FoxO1 transcriptional target (55). Evaluation of asymmetric T cell division *ex vivo*, revealed a bifurcation of TCF-1 expression: daughter cells that inherit robust PI3K activity inactivate FoxO1 and silence *Tcf7* expression. Daughter cells with reduced PI3K activity maintained TCF-1 and generated a self-renewing memory population (8, 56–58). Antagonism of PI3K activity *in vitro* limited repression of *Tcf7* and induction of differentiation markers (58). Conversely, recent work from our laboratory showed that expression of an activated PI3Kδ allele suppressed the maintenance of a TCF-1$^+$CD8$^+$ T cell population and the development of central memory cells during acute viral infection. Instead, activated PI3Kδ-expressing CD8$^+$ T cells were driven to a long-lived effector cell fate with increased expression of effector cytokines IFN-γ and TNF-α (8). Similarly, T cells from patients with the immunodeficiency, Activated PI3K Delta Syndrome, failed to maintain a TCF-1$^+$ population when expanded *in vitro* (8, 56). Together these studies raise the possibility that inhibition of PI3Kδ could promote expansion of TCF-1$^+$ pTex, thereby increasing the population that can respond to ICB (**Figure 2**). Inhibition of PI3K would also be expected to prevent BACH2 inactivation, which also promotes pTex cells.

Nonetheless, PI3Kδ also plays an important role in both effector T and B cell differentiation (8, 59). Intriguingly, CD226-mediated induction of T-bet and cytotoxicity in NK cells occurs *via* FoxO1-mediated regulation (35); it is intriguing to speculate that CD226 engagement increases CD8$^+$ T cell effector function *via* PI3K-mediated pathways. Thus, while PI3Kδ inhibition may increase TCF-1$^+$ pTex, this may come at the expense of the ability to develop effector cells and even to

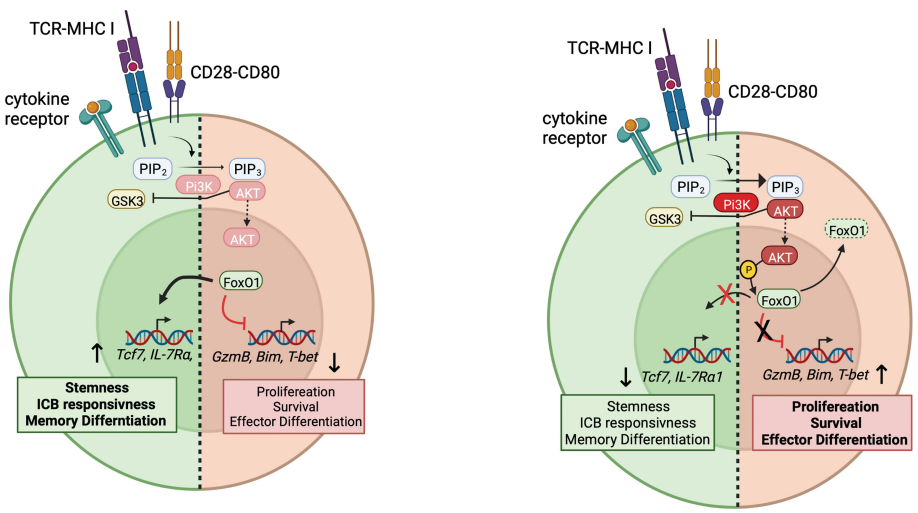

FIGURE 2 | PI3K and regulation of stem-like pTex cells. Right: Upon T cell activation, PI3K generates PIP$_3$, which recruits AKT, leading to its activation at the plasma membrane, which then leads to the phosphorylation and nuclear exclusion of FoxO1 and BACH2. Downstream genes associated with stemness and memory differentiation, such as *Tcf7* are no longer induced by FoxO1, whereas effector genes such as T-bet and GranzymeB, usually suppressed by FoxO1, can be transduced. How CD226 integrates in this model is not yet clear. Left: Under conditions of low PI3K activity, FoxO1 remains active, and *Tcf7* and other genes associated with pTex cells are expressed.

respond to ICB (60). Manipulation of PI3Kδ pathways therefore raises multiple issues, including how to appropriately balance the promotion of TCF-1$^+$ populations, while allowing development of effective cytolytic CD8$^+$ T and other immune cell responses. Whether PI3Kδ inhbitors are useful during *ex vivo* expansion of TILs or CAR-T cells to maintain a TCF-1$^+$ population that then can be transfered *in vivo* or further expanded without PI3K inhibition remains to be seen.

IL-2: A Key Player in T Cell Differentiation and Function

Another approach that has been considered is therapeutic administration of cytokines such as IL-2, to reinvigorate Tex cells; however, this has yielded disparate results. *In vitro*, IL-2 drives the differentiation of cytolytic effector CD8$^+$ T cells and the acquisition of effector functions (61, 62). Accordingly, following LCMV chronic infection, *in vivo* IL-2 treatment boosted the number of antigen specific Tex cells and improved viral control (60, 63). Interestingly, IL-2 treatment combined with PD-1 blockade had synergistic effects, perhaps through anti-PD-1 effects on pTex cells and IL-2 effects on promoting effector cells (60). However, IL-2 treatment can also result in the expansion of immunosuppressive Tregs, as well as vascular leakage syndrome and thus may have undesirable secondary effects (64, 65). IL-2-anti-IL2 complexes and stabilized forms of IL-2 also can have distinct effects on different cell-types.

While the effects of IL-2 and IL-2-complexes are beyond the scope of this review, recent data on IL-2 variants have yielded some intriguing results (63–66). An engineered pegylated-IL-2 variant, THOR-707, was found to selectively engage the IL-2R β/γ complex and have a longer half-life, leading to tumor reduction without Treg expansion (67). Another engineered IL-2 partial agonist, H9T, that also activates IL-2Rβ/γ, promoted CD8$^+$ T cells with sustained TCF-1 expression and maintenance of a stem-like state, with higher spare respiratory capacity indicative of improved mitochondrial fitness (68). Although pAKT was unaffected under the conditions examined, it is interesting to speculate that H9T and other IL-2 variants may indirectly affect PI3K and thereby, FoxO1-mediated regulation of *Tcf7*.

These engineered IL-2-variants unveil promising strategies for boosting immunotherapeutic treatment regimes *via* promoting TCF-1$^+$ pTex cells (65). Interestingly, data argue that following antigen stimulation, TCF-1 is required for the induction of glycolytic capacity of central memory T cells, which rely on fatty acid oxidation during memory phases in acute infection (69–71). TCF-1 expression in Tex cells may therefore be critical to maintain a state that can meet the continued bioenergetic demands in response sustained antigen exposure. Indeed, metabolic profiling revealed the importance of metabolism in Tex cell fate. Tex cells display metabolic insufficiency, including diminished glucose uptake and OXPHOS (72–74), resulting at least in-part from decreased expression of the transcriptional coactivator peroxisome proliferator-activated receptor gamma co-activator 1-alpha, PGC1α, which has critical roles in mitochondrial biogenesis and anti-oxidant responses (74). An *in vivo* CRISPR–Cas9 mutagenesis screen found that targeting the ribonuclease REGNASE-1 reprogrammed CD8$^+$ T cells into long-lived effector cells with improved mitochondrial fitness and anti-tumor responses. These findings highlight the importance of mitochondrial quality, and potentially mitochondrial activity, in orchestrating T-cell function and fate during exhaustion (72, 74).

Nonetheless, Tex cells originating from a TCF-1$^+$ population during chronic infection following antigen elimination ('recovered' cells) likely still remain compromised: these cells maintain features of an exhausted chromatin landscape and have been referred to as 'epigentically scarred' (15, 16, 75). Whether this contributes to the failure of checkpoint blockade in many cases is unknown. Thus, increasing the TCF-1$^+$ population may not be sufficient as a therapeutic strategy; approaches to increase epigenetic plasticity of TCF-1$^+$CD8$^+$ T cells remain an important area for further investigation. Such approaches could involve dampening chronic inflammation (16) as well as preventing nutrient deprivation and/or elimination deleterious metabolities (73, 76, 77).

NOT ALL ROADS LEAD TO ROME – ROUTES TO PREVENT EXHAUSTION

In a striking report, intravenous vaccination using a TLR7/9 agonist in conjunction with nanopartical presentation of a neoantigen induced a higher proportion of stem-like CD8$^+$ T cells compared to subcutaneous immunization. Moreover, the stem-like TCF-1$^+$ cells generated were able to differentiate into effector CD8$^+$ T cells to elicit anti-tumour responses (78). Similarly, the duration of antigen presentation by specific dendritic populations in the draining lymph node and spleen may help augment and/or maintain the reservoir of TCF-1$^+$CD8$^+$ T cells required for optimal immunity during chronic antigen exposure (79–81). Such reports suggest that routes and types of immunizations can elicit responses that facilitate the development of successful tumor vaccines to overcome limitations of exhaustion. Whether these approaches can work in combination for chronic infection and cancer, whether they affect some of the pathways described above, including activation of PI3K, IL-2 or costimulatory pathways and what other techniques may be used to boost the long-lived potential of adoptive cell therapies remain important questions.

SUMMARY

The study of T cell exhaustion and the development of ICB has moved rapidly in recent years. However, while the success of immune-based therapies has been striking, the successful implementation in only a fraction of cancers, argues that new approaches are needed. Here, we reviewed several novel approaches including stimulation of activating receptors and pharmacological methods to increase TCF-1$^+$ pTex cells. Nonetheless, these data highlight the complexity of T cell exhaustion and the fragile balance between T cell activation and exhaustion. Combinations that recognize these limitations

while taking advantage of distinct features of these approaches may ultimately help improve the success of immunotherapy.

AUTHOR CONTRIBUTIONS

ACP, JLC and PLS wrote and edited the manuscript. ACP designed and generated the figures with feedback from JLC and PLS. All authors contributed to the article and approved the submitted version.

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