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REVIEW
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CD8$^+$ T Cell Exhaustion in Cancer

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A paradigm shift in the understanding of the exhausted CD8$^+$ T cell (T_{ex}) lineage is underway. Originally thought to be a uniform population that progressively loses effector function in response to persistent antigen, single-cell analysis has now revealed that CD8$^+$ T_{ex} is composed of multiple interconnected subpopulations. The heterogeneity within the CD8$^+$ T_{ex} lineage is comprised of immune checkpoint blockade (ICB) permissive and refractory subsets termed stem-like and terminally differentiated cells, respectively. These populations occupy distinct peripheral and intratumoral niches and are characterized by transcriptional processes that govern transitions between cell states. This review presents key findings in the field to construct an updated view of the spatial, transcriptional, and functional heterogeneity of anti-tumoral CD8$^+$ T_{ex}. These emerging insights broadly call for (re-)focusing cancer immunotherapies to center on the driver mechanism(s) underlying the CD8$^+$ T_{ex} developmental continuum aimed at stabilizing functional subsets.

Keywords: T cell exhaustion, PD-1/PD-L1, T cell trafficking, tumor immunity, cancer immunotherapy, CXCR3, co-stimulatory/inhibitory receptors, stem-like CD8+ T cells

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INTRODUCTION

T cell exhaustion is a blanket term covering all of the dysfunctional states that exist within antigen-specific CD8$^+$ T lymphocytes as first described in the framework of chronic viral infection, where these cells persist but are unsuccessful in clearing a pathogenic threat (1). Blockade of surface co-inhibitory receptors such as programmed death 1 (PD-1) expressed by CD8$^+$ T_{ex} was shown to reinvigorate cytolytic cell-mediated immune responses leading to the eradication of some persistent viruses (2). Later found in cancer, CD8$^+$ T_{ex} are found to be equally hyporesponsive to anti-tumor immunotherapies (3). Cells expressing PD-1 were thought to be rescued by ICB *via* simple unidirectional reversion from the unresponsive, exhausted state (2). In cancer, this was also believed to involve dysfunctional CD8$^+$ T_{ex} expressing high levels of PD-1, primarily residing in the tumor microenvironment (TME) (3).

Recent advances in single-cell transcriptomics and genome-wide epigenetic profiling comparing normal tissue, peripheral blood, and the lymphoid compartment to tumor parenchyma have challenged this view. New insights have been made regarding the spatial arrangement and heterogeneity of CD8$^+$ T_{ex} and their modulation by ICB (3). We now understand that PD-1 expression is not an absolute measure of cellular dysfunction and senescence. Instead, PD-1 intensity reflects a complex heterogeneity existing within CD8$^+$ T_{ex} (4). Emergent data now casts CD8$^+$ T_{ex} as a developmental continuum, where the lineage is comprised of stem-like PD-1^{lo}CD8$^+$ T_{ex} precursors/progenitors that ultimately give rise to terminally dysfunctional PD-1^{hi}CD8$^+$ T_{ex} (3). In cancer, these CD8$^+$ T_{ex} subsets appear to be unevenly spread amongst normal peripheral versus tumoral tissues and are differentially responsive to ICB (3).

This review discusses the original works that first identified $CD8^+$ T_{ex} and more contemporary reports describing this population as a developmentally distinct lineage using chronic viral infection. We draw on these data as a basis to further our understanding of $CD8^+$ T_{ex} function during anti-tumor immune responses and elucidate the cellular dynamics and molecular pathways underlying the success and limitations of ICB. Throughout this review, we highlight fundamental knowledge gaps regarding the factors underlying control over $CD8^+$ T_{ex} heterogeneity.

TRANSLATING $CD8^+$ T CELL EXHAUSTION FROM CHRONIC INFECTION TO CANCER: A COMMON ROLE OF PERSISTENT ANTIGEN

The origin of the term T cell exhaustion goes back to the notable decay of T cell responses first documented in human immunodeficiency virus (HIV)-infected patients (5). It was speculated that viral persistence was linked with loss of function observed in these declining T cell subsets. $CD8^+$ T cell functionality (the ability to rapidly expand after priming, produce effector cytokines and cytolytic molecules, and contract to form memory) characterizes acute recognition of cognate antigen during vaccination or natural, but eventually cleared, viral/bacterial infections (6, 7). Throughout the expansion phase, naïve $CD8^+$ T cells differentiate into short-lived effector cells (SLEC) or memory precursor effector cells (MPEC) (6). Upon contraction and antigen clearance, most SLECs die while MPECs survive to form memory $CD8^+$ T cells for long-term protective immunity (**Figure 1A**) (6, 8). The existence of a $CD8^+$ T_{ex} counterpart to the conventional acute immune response was formally realized at the height of the HIV pandemic when Zinkernagel et al. exposed mice to acute (Armstrong and WE) versus chronic (Clone 13 and DOCILE) strains of lymphocytic choriomeningitis virus (LCMV), a rodent-borne negative-stranded RNA arenavirus (9). In this seminal work, Clone 13 and DOCILE strains persisted in infected mice for greater than 200 days at high inocula while transferred T cell receptor (TCR) transgenic virus-specific $CD8^+$ T cells disappeared or crashed without contraction to memory (9). Initial exposure of select viral strains and doses thus appeared to scale cellular immunity towards protection or completely 'exhausted' the response, as it was coined.

This finding was later examined by two teams [Zajac, Wherry, and Ahmed et al. (10, 11) along with Gallimore and Rammensee et al. (12)] concurrent with the advent of major histocompatibility complex class I (MHC I) tetramer staining technology to track endogenous antigen-specific $CD8^+$ T cells. It was found that initially dominant cytolytic $CD8^+$ T cell responses against LCMV-derived peptides with high MHC I affinity ($NP_{396-404}$ and GP_{34-42}) were rapidly deleted, just as Zinkernagel initially observed (9). However, functionally inadequate responses against low/moderate affinity peptides (GP_{33-41} and $GP_{276-286}$) persisted for greater than 60 days post-infection (**Figure 1B**) (10–12). These results showed that constantly elevated viral load and peptide affinity for MHC I strongly correlated with the degree of exhaustion and determined deletion versus persistence of $CD8^+$ T_{ex} (10, 11). Low avidity persisting cells exhibited a hierarchical loss of functionality at relatively low viral loads, which manifested as a dramatic decrease in proliferation, cytotoxicity, and cytokine production (2, 10, 11). Interleukin-2 (IL-2) and tumor necrosis factor (TNF) were lost early, whereas interferon-γ (IFN-γ) production persisted longer after infection (2, 10, 11). At elevated viral doses or with depletion of $CD4^+$ T cell help, these gradual losses of functionality (or dysfunction) resulted in a nearly complete reduction in effector function followed by cell death/deletion (9–11). This process translated to HIV infection and other chronic or latent viral infections in humans, including hepatitis B and C viruses (HBV/HCV), herpes simplex virus (HSV), cytomegalovirus (CMV), human papillomaviruses (HPV), Epstein-Barr virus (EBV), and others (2, 13).

A common feature of chronic viral infection and cancer is that both are prolonged diseases characterized by an overt persistence of antigen (4). $CD8^+$ tumor-infiltrating lymphocytes (TILs) are similarly hyporesponsive as those found during chronic viral infection but are instead caught in an *in vivo* détente against the progressively growing tumor (14). Patient TILs are also tumor antigen-specific and MHC-

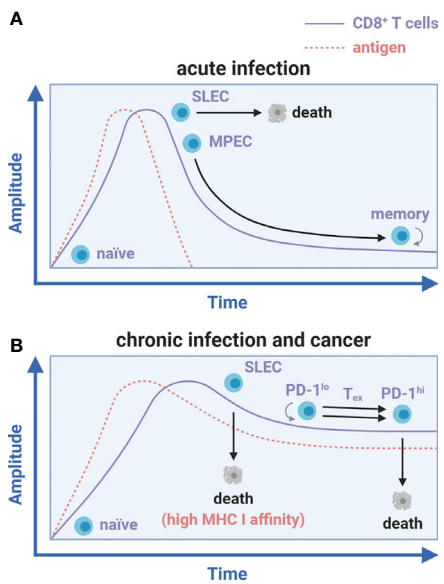

FIGURE 1 | Antigen load differentially influences $CD8^+$ T cell memory and exhaustion fates. $CD8^+$ T cell differentiation during acute infection versus chronic infection and cancer. **(A)** Activation of naïve $CD8^+$ T cells during acute infection leads to SLEC and MPEC differentiation. Upon antigen clearance, SLECs undergo apoptosis while MPECs survive and differentiate into long-lived, self-renewing memory $CD8^+$ T cells. **(B)** With chronic infection and cancer, SLEC specific to peptides of high MHC I affinity develop and prematurely die while the MPEC subset does not form. Instead of memory formation, $CD8^+$ T cells against peptides of low MHC I affinity expand, exhaust (in a unidirectional $PD\text{-}1^{lo}$ to $PD\text{-}1^{hi}$ transition), and die in a continued stalemate against persistent antigen.

restricted, supporting the role of chronic antigen persistence in driving T cell exhaustion (15, 16). Importantly, antigen displayed in the TME appears to fully drive CD8+ TIL exhaustion towards completion, whereas the periphery does not, as shown in preclinical models (17, 18). These data imply that the periphery may be an active reservoir of functional precursors to CD8+ T_{ex} (**Figures 2A, B**) before the physical invasion of tumors and chronic exposure to tumor-derived antigen (**Figure 2C**)—a spatial feature distinct from Clone 13 infection. Although persistent antigen plays a significant role in sustaining CD8+ T_{ex} for terminal differentiation in the tumor, other early events in CD8+ T cell activation may also be critical for the initial programming of exhaustion in the periphery or specialized tumor niches, including TCR signal quality/strength (NFAT versus NFAT/AP-1 signaling, discussed below), co-stimulation, IL-2 availability (with associated CD4+ T cell helper signals), and inflammatory cues in the first few divisions (**Figure 3A**) (1, 10, 19–22).

Contemporary studies comparing chronic viral infection to cancer have sought to identify common CD8+ T_{ex} transcriptional signatures. At first glance, both tumor- and chronic virus-specific CD8+ T cells possess significant enrichment of genes related to recent TCR signaling (*Batf*, *Egr2*, *Ezh2*, *Irf4*, *Nfatc1*, *Nfatc2*, *Nr4a1*, *Nr4a2*, and *Nr4a3*) (17, 18, 23–25). This observation reinforces that constant engagement of persistent antigen is a dominant driver of exhaustion. These dominant transcriptional features are notably shared in a direct comparison of CD8+ T_{ex} isolated from HIV-infected and melanoma patients. They can also be recapitulated in CD8+ T cells given repeated cognate peptide stimulations *in vitro* (26, 27). However, significant disparities in CD8+ T_{ex} transcriptional phenotypes also exist between cancer and viral settings. These appear to be unrelated to exhaustion *per se*, where TIL uniquely retain gene ontologies associated with the suppressive TME and are devoid of pathways linked with virally-induced inflammation (3, 18, 28).

REVERSING T CELL EXHAUSTION: LESSONS LEARNED FROM IMMUNE CHECKPOINT BLOCKADE

The onset of exhaustion coincides with the surface expression of co-inhibitory receptors, which control CD8+ T cell function (2). It has been considered that these immune checkpoints, which include PD-1 (among others), evolved to constrain T cell activation, preventing excessive adverse inflammatory and autoimmune events (29, 30). They also seem to function throughout exhaustion and not merely correlate with loss-of-function, as blocking interactions between PD-1 and its ligand (PD-L1) can restore the function and survival of CD8+ T_{ex} (2, 31). With Clone 13 infection, ICB of PD-(L)1 was initially shown by Barber and Ahmed et al. to reinvigorate CD8+ T_{ex} (31). Importantly, restoration of the response originated from PD-1+CD8+ T_{ex} and not from *de novo* naïve PD-1−CD8+ T cell priming (31). This early study led to the idea that reinvigoration

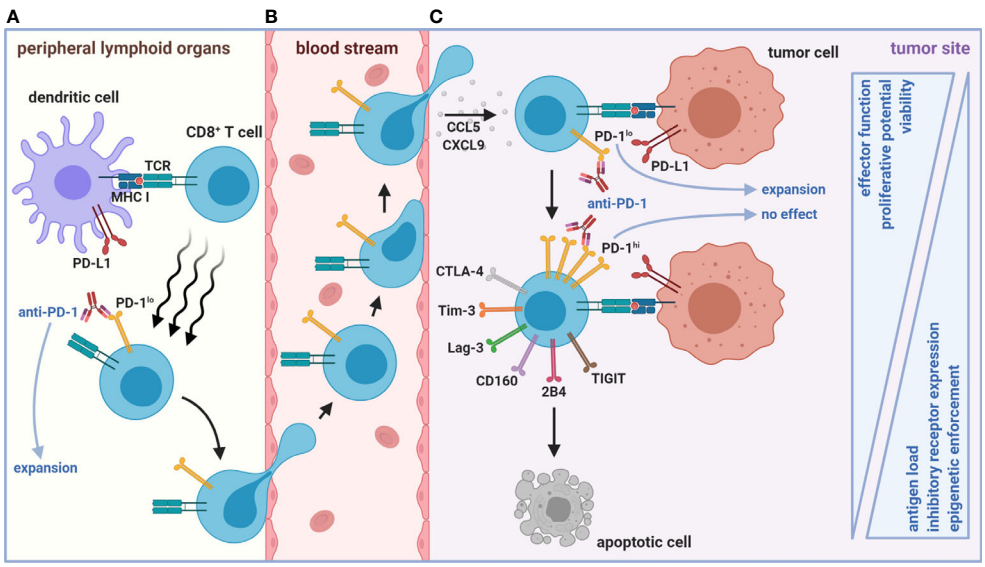

FIGURE 2 | Spatiotemporal organization of early versus late stages of tumor-mediated CD8+ T cell dysfunction. **(A)** Naïve CD8+ T cell priming against tumor antigen in peripheral LNs (or intratumoral TLS, not depicted) results in the formation of a stem-like PD-1loCD8+ T cell population with self-renewing properties. **(B)** This population represents an active reservoir of cells that can give rise to effector-like PD-1loCD8+ T_{ex} after chemokine-mediated trafficking to and positioning within the TME via CCL5 and CXCL9. **(C)** However, persistent antigen load in the TME eventually forces continued differentiation of these cells into terminally dysfunctional PD-1hiCD8+ T_{ex}. The PD-1hi state is accompanied by heightened co-inhibitory receptor expression (including Tim-3, Lag-3, CD160, 2B4, TIGIT, and CTLA-4) and progressive loss of effector functions. Once CD8+ T_{ex} enter a PD-1hi state, epigenetic enforcement prevents de-differentiation back to functional stem-like and effector-like PD-1lo states. Anti-tumoral responses facilitated by ICB (*e.g.*, anti-PD-1) arise from expansion from only lymphoid or intratumoral PD-1loCD8+ T_{ex} subsets. The functionally inferior, ICB-resistant PD-1hiCD8+ T_{ex} fate ultimately culminates in apoptosis.

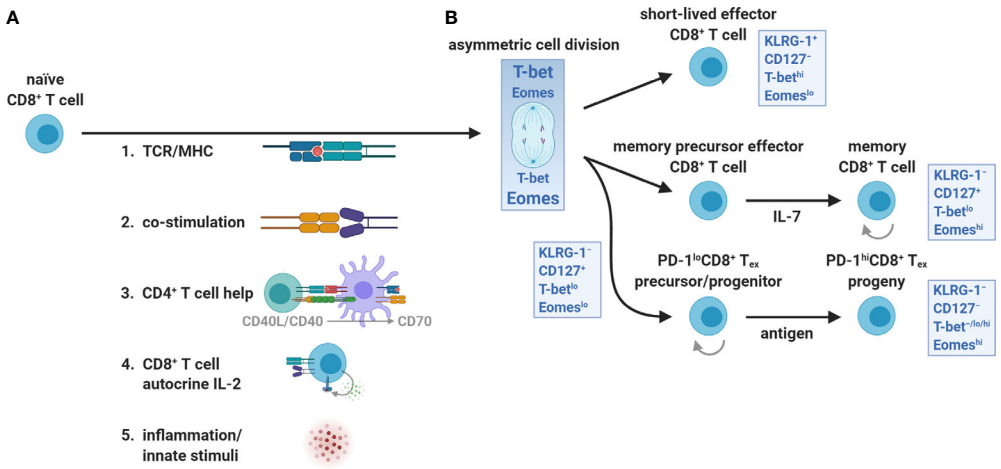

FIGURE 3 | T-bet and Eomes partitioning during CD8+ T cell priming and expansion. **(A)** The orientation and strength of TCR/MHC ligation, co-stimulation (*e.g.*, CD28 interaction with CD80 and CD86), CD4+ T cell help (CD40L/CD40 licensing of DCs including up-regulation of MHC I, CD80/CD86, CD70, and third signal cytokines), autocrine IL-2 exposure, and innate inflammatory stimuli (danger- and pathogen-associated molecular patterns) all influence the activation, survival, and differentiation of naïve CD8+ T cells. **(B)** CD8+ T cells integrate these input events at priming and during the first division. The uneven partitioning of T-bet and Eomes favors SLEC (effector) versus MPEC (memory) differentiation early after activation, respectively. In contrast, the CD8+ T_{ex} lineage requires both transcription factors and retains some features of memory cells including self-renewal of PD-1^{lo} subsets and expression of memory-associated transcription factors and survival molecules. The reliance on homeostatic cytokines (predominantly IL-7) versus persistent antigen for development and self-renewal distinguishes memory from PD-$1^{lo/hi}$ exhaustion lineages, respectively.

of CD8+ T_{ex} was practically synonymous with 'reversal' of exhaustion. At this time, ICB was rapidly advanced into the clinic and established a new paradigm for cancer treatment, leading to durable responses in a limited set of patients (32, 33). Despite its early success and first recordings of tails in long-term endpoint survival curves, the mechanism of action behind ICB remained elusive.

Blackburn and Wherry et al. uncovered an underlying complexity within the presumed homogenous PD-1^{+}CD8+ T_{ex}, where this population could be further separated into PD-1^{lo} and PD-1^{hi} subsets (34). A hypothesis emerged from this that proposed PD-1^{lo} cells differentiate into the PD-1^{hi} subset as CD8+ T cells exhaust. Inherent in this theory, reinvigoration did not equate to the reversal of exhaustion (herein defined as a PD-1^{hi} to PD-$1^{lo/-}$ transition). Beneficial responses rather arose solely from the mobilization of less exhausted, permissive PD-1^{lo} cells instead of PD-1^{hi} terminally exhausted counterparts. After transferring day 30 Clone 13-generated PD-1^{lo} and PD-1^{hi} sorted cells into naïve mice subsequently re-challenged with Clone 13 in the presence or absence of anti-PD-L1, Blackburn and Wherry et al. showed that only PD-1^{lo}CD8+ T_{ex} could proliferate in response to ICB (34). Similar transfer experiments also revealed that PD-1^{lo}CD8+ T_{ex} were more effective at controlling viral load and remained less apoptotic compared to PD-1^{hi}CD8+ T_{ex} (34). The PD-1^{hi} subset was later associated with expression of additional co-inhibitory receptors, including T cell immunoglobulin domain and mucin domain protein 3 (Tim-3), lymphocyte activation gene 3 (Lag-3), natural killer cell receptor BY55 (CD160), signaling lymphocytic activation molecule 4 (2B4), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), and T cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT), where cells having heightened co-expression appeared more exhausted (**Figure 2C**) (24, 35–38).

DAWN OF STEM-LIKE PRECURSORS AND PROGENITORS OF EXHAUSTED CD8+ T CELLS

It also became apparent that heterogeneity existed at a deeper level than surface PD-1, where CD8+ T_{ex} appeared to use the same T-box family transcription factors, T-box expressed in T cells (T-bet) and eomesodermin (Eomes), for SLEC and MPEC lineage commitment, respectively, but with different expression patterns, nuclear localization, and developmental connectivity (4, 39–41). In response to TCR/MHC ligation and orientation of the immune synapse, a naïve CD8+ T cell will asymmetrically divide and unequally partition T-bet and Eomes, separately dictating effector versus memory fates from the first division (22, 42–45). Distinct from SLECs and MPECs, T-bet and Eomes were shown to be dually required for CD8+ T_{ex} development (41). In addition, these transcription factors appeared to arise at different stages of CD8+ T_{ex}, where PD-1^{lo}T-bet^{lo}CD8+ T_{ex} were found to increase Eomes expression and sustain its nuclear localization, divide, and differentiate into PD-1^{hi}T-bet$^{-/lo/hi}$ Eomes^{hi}CD8+ T_{ex} (**Figures 3B** and **4A**) (40, 41, 46). This differential usage of T-bet and Eomes also suggested that CD8+ T_{ex} was a distinct lineage.

Ahmed et al., therefore, reexamined the Clone 13 model and the underlying CD8+ T_{ex} transcriptional heterogeneity at the

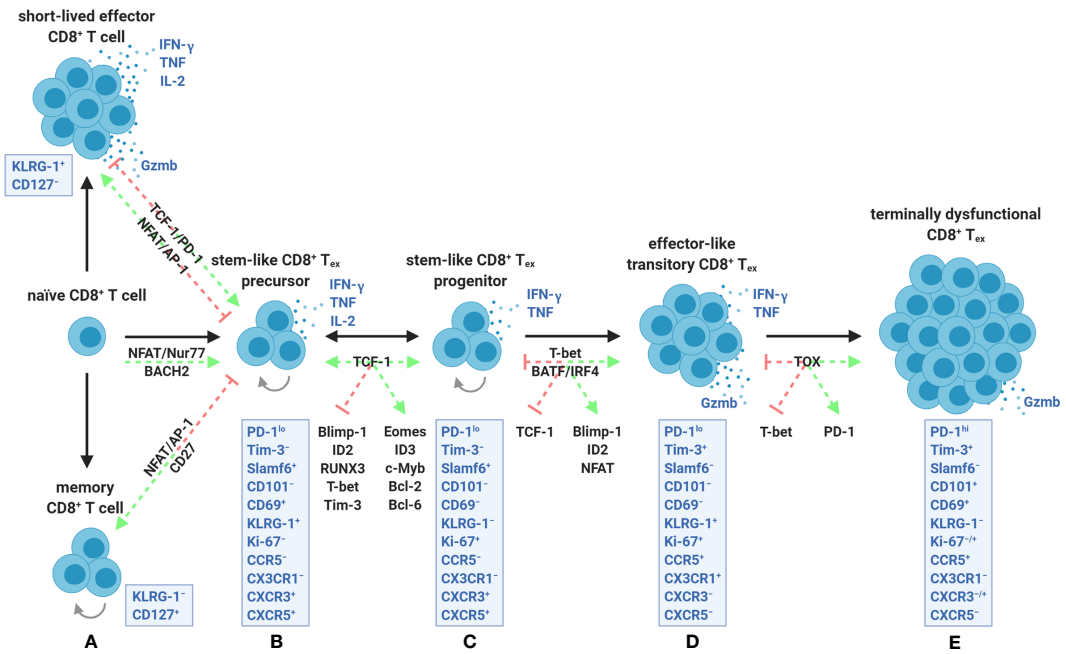

FIGURE 4 | The CD8$^+$ T cell exhaustion lineage is comprised of a continuum of transcriptionally and epigenetically controlled states. **(A)** Activation of naïve CD8$^+$ T cells for SLEC and MPEC/memory differentiation is optimally driven by transcription factors such as NFAT/AP-1 and sufficient co-stimulation (*e.g.*, CD27). Early development of stem-like CD8$^+$ T_{ex} precursors instead involves partnerless NFAT, lack of co-stimulation/help, constant Nur77 activity, and/or strict dependence on BACH2. These events appear to be stabilized by TCF-1 activity and PD-1 dampening of chronic TCR ligation. **(B, C)** TCF-1 further supports stemness (ability to survive, self-renew, and proliferate) by promoting Eomes, ID3, c-Myb, Bcl-2, and Bcl-6 expression while antagonizing effector-associated transcription factors including Blimp-1, ID2, RUNX3, and T-bet. Stem-like CD8$^+$ T_{ex} precursors **(B)** and progenitors **(C)** are collectively marked by a PD-1^{lo}Tim-3$^-$Slamf6$^+$ surface profile and varied expression of CXCR3 and CXCR5 in specific tumor settings. Although equally stabilized by TCF-1, precursors can be distinguished from progenitors as being quiescent, LN-resident, less reliant on antigen, and having a CD69$^+$KLRG-1$^+$Ki-67$^-$ profile. **(C, D)** TCF-1 down-regulation coupled with ongoing exposure to persistent antigen drives constant BATF and IRF4 signaling (which positively feedback on partnerless NFAT activity) and T-bet expression. T-bet additionally overrides a TCF-1 memory-like program by supporting Blimp-1 and ID2 activity leading to an effector-like transitory PD-1^{lo}Tim-3$^+$ state marked by initiation of granzyme B production. **(D, E)** Continued NFAT activity ultimately leads to TOX upregulation within this subpopulation, which epigenetically enforces terminal exhaustion, inhibits T-bet-mediated effector programming, and promotes heightened PD-1 expression. Transitory cells **(D)** are discriminated from terminally dysfunctional cells **(E)** by a PD-1^{lo}Tim-3$^+$CD101$^-$KLRG-1$^+$CX3CR1$^+$ surface phenotype, remnant IFN-γ and TNF production, and having high proliferative potential. Terminally dysfunctional PD-1^{hi}Tim-3$^+$CD8$^+$ T_{ex} co-express multiple co-inhibitory receptors (not depicted), cannot proliferate, have diminished polyfunctionality, but retain granzyme-based cytolytic potential. PD-1^{lo} precursors, progenitors, and transitory CD8$^+$ T_{ex} subpopulations are amenable to ICB **(B–D)**, whereas terminally dysfunctional PD-1^{hi}CD8$^+$ T_{ex} are not **(E)**. Precursors and progenitors may interconvert, whereas differentiation into transitory and terminally dysfunctional subsets is unidirectional.

core of the PD-1$^{lo/hi}$ dichotomy (47). Transcriptional analyses of the PD-1^{lo} population revealed an association with *Icos* (inducible T cell co-stimulator; ICOS), *Cxcr5* (C-X-C motif chemokine receptor 5; CXCR5), *Bcl6* (B cell lymphoma 6; Bcl-6), and *Tcf7* (T cell factor 1; TCF-1) expression reminiscent of CD4$^+$ T follicular helper cells (T_{fh}), which is why CD8$^+$ T_{ex} are sometimes referred to as T_{fh}-like (47, 48). TCF-1 acts as the main transcription factor downstream of Notch receptors as part of the evolutionarily conserved Wnt signaling pathway, known to be critical for T cell thymic development and memory formation (49). TCF-1, together with forkhead box protein O1 (FOXO1), promotes stemness in CD8$^+$ T cells by inhibiting expression of effector-associated genes including *Prdm1* (B lymphocyte-induced maturation protein-1; Blimp-1), *Runx3* (Runt-related transcription factor 3; RUNX3), *Id2* (inhibitor of DNA binding 2; ID2) and *Tbx21* (T-bet) and favoring central memory by promoting *Eomes* and *Bcl6* expression (50, 51). Blimp-1, in particular, is known to act as a rheostat balancing the promotion of cytolytic granzyme B production and terminal dysfunction in CD8$^+$ T_{ex}—events directly countered by TCF-1 (**Figures 4B, C**) (49, 51, 52). Other associations of PD-1^{lo}CD8$^+$ T_{ex} with the high affinity IL-7 receptor chain (IL-7Rα), L-selectin (CD62L), and mitochondrial β-oxidation (fatty acid metabolism) pathway enrichment suggested shared common features with self-renewing CD8$^+$ T memory precursors (47). Moreover, PD-1^{hi}Tim-3$^+$CD8$^+$ T_{ex} did not produce effector cytokines but did retain cytolytic *Gzma* (granzyme A), *Gzmb* (granzyme B), and *Prf1* (perforin) expression (47, 53, 54). Sorting and transferring PD-1$^{lo/hi}$ subsets into infection-matched hosts based upon CXCR5 positivity validated that PD-1^{lo}Tim-3$^-$CXCR5$^+$CD8$^+$ T_{ex} marked a self-renewing population that gave rise to PD-1^{hi}Tim-3$^+$CXCR5$^-$CD8$^+$ T_{ex} (47). Further and

more critical, anti-PD-L1 blockade triggered a proliferative burst only within the stem-like PD-1^{lo}Tim-3^{-} subset and facilitated transitions to the treatment-refractory PD-1^{hi}Tim-3^{+} fate (47).

Since TCF-1 expression was generally known to maintain stemness in hematopoietic stem cells, its role in the PD-1$^{lo/hi}$ T_{ex} progenitor/progeny relationship was determined (47). In *Tcf7*$^{-/-}$ mice, PD-1^{lo}CD8^{+} T_{ex} fail to develop and cannot seed the exhaustion lineage (47). In contrast, transgenic overexpression of *Tcf7* was found to stabilize PD-1^{lo} stem-like cells and lead to more durable CD8^{+} T cell responses during Clone 13 infection and within the B16-GP$_{33-41}$ melanoma models, implicating TCF-1 as a critical factor for the inception of T_{ex} (55). TCF-1 was later shown to support the expression of *Id3* (ID3), *Eomes*, *Myb* (transcriptional activator Myb; c-Myb), and *Bcl2* (Bcl-2), allowing PD-1^{lo}CD8^{+} T_{ex} to survive negative downstream signals from PD-1 early after priming (**Figures 4B, C**) (56, 57).

The factors governing the expression of TCF-1 within stem-like PD-1^{lo}CD8^{+} T_{ex} have only recently been investigated. During chronic DOCILE infection of mice, the amount of antigen but not inflammation rapidly promotes the formation of the TCF-1^{+} population (57). Inconsistent with the need for chronic antigen during its establishment, some elements of the exhaustion program (maintenance of a PD-1^{hi} dysfunctional profile) were paradoxically shown to be stable after CD8^{+} T_{ex} transfer to antigen-free conditions (58). This suggests that the CD8^{+} T_{ex} lineage has some component(s) shared with memory CD8^{+} T cells, including slow homeostatic self-renewal by IL-7 and IL-15 (4, 58). In support of this, GP$_{33-41}$-specific CD8^{+} T cells deficient in BACH2 (a transcription factor that promotes memory cell development by limiting TCR-mediated transcriptional changes) fail to form any stem-like PD-1^{lo}TCF-1^{+}CD8^{+} T_{ex} (57, 59). Conversely, the progression of the stem/memory-like PD-1^{lo}TCF-1^{+} state to the TCF-1^{-}PD-1^{hi} terminally exhausted fate is halted by deleting BATF and IRF4 (two transcription factors linked with constant TCR signaling and known to destabilize TCF-1) (**Figures 4C, D**) (25, 57). Therefore, T cell-intrinsic TCF-1 expression appears to rely on a low but brief TCR signaling threshold compromised by ongoing antigenic exposure.

Other studies have oppositely shown that PD-1^{hi}CD8^{+} T_{ex} generated from Clone 13 infection are less stable without antigen, where these cells inevitably decline and cannot mount a recall response (21, 60). Discrepancies regarding CD8^{+} T_{ex} stability in the presence/absence of antigen may be due to the frequency and quality of TCF-1^{+} stem-like cells at hand. A unified atlas of 12 studies spanning cancer and chronic viral infection has recently revealed that bifurcation of memory commitment from a dysfunctional program occurs early (in less than 7 days following antigen encounter) (61). With preclinical cancer models, the time of initial antigen encounter is less controlled for compared to viral infection. Nevertheless, it has been shown that PD-1^{lo}CD8^{+} TIL removed early after tumor injection (likely containing an increased frequency of TCF-1^{+} cells) followed by transfer into naïve hosts and infection with *Listeria monocytogenes* 3-4 weeks later can mount a memory response whereas fully exhausted PD-1^{hi}CD8^{+} TIL isolated at later time points cannot (18). In addition, stem-like PD-1^{lo}TCF-1^{+}CD8^{+} T_{ex} can be divided into CD69^{+}Ki-67^{-} precursor cell and CD69^{-}Ki-67^{+} progenitor cell subsets (and are thus differentiated as such in this review) (**Figures 4B, C**) (46). Precursors are lymph node (LN)-resident, speculated to depend less on antigen for a low baseline level of proliferation, and remain quiescent compared to a circulating progenitor pool (46). In healthy human subjects, TCF-1^{+} precursors specific to common chronic diseases such as latent EBV and CMV were shown to be present in the periphery and co-express PD-1, TIGIT, and granzyme K (62). These precursors are also embedded within steady-state stem-like/central memory CD8^{+} T cell populations traditionally defined as CCR7^{+}CD45RO$^{+/-}$CD95^{+} (62). Yet, no known mediator has been identified to date which controls functional memory versus stem-like PD-1^{lo}CD8^{+} T_{ex} precursor differentiation (**Figures 4A, B**) (62). Precursors and progenitors have also been documented to reside in TIL fractions of murine B16 tumors and human melanoma (46). However, it remains to be determined if these small populations are biased in tumor versus LN organization and if CD69 positivity/negativity within the bulk TCF-1^{+}PD-1^{lo} population determines true stemness and reactivity to ICB and/or antigen.

TRANSCRIPTIONAL AND EPIGENETIC EVENTS CRITICAL FOR THE ESTABLISHMENT OF TERMINAL EXHAUSTION

Complementing these approaches, total CD8^{+} T_{ex} were shown to possess a fixed chromatin state distinct from effector and memory cells by ~6,000 open chroman regions before or after exposure to anti-PD-L1 (21, 63, 64). This reinforces that terminal CD8^{+} T_{ex} represents a distinct lineage unable to differentiate into *bona fide* memory cells. Second, ICB-mobilized stem/effector-like PD-1^{lo} populations themselves exhaust and eventually mirror pre-treatment PD-1^{hi}CD8^{+} T_{ex}. The unique epigenetic signature of CD8^{+} T_{ex} in Clone 13-infected mice was also shown to be conserved in HIV-infected and melanoma patients (26, 63). Although both acutely activated CD8^{+} T cells and CD8^{+} T_{ex} generally express PD-1, assay for transposase-accessible chromatin sequencing (ATAC-Seq) distinguishes these populations, with CD8^{+} T_{ex} possessing many unique features, including *de novo* accessibility of the region at −22.4 kb upstream of the murine *Pdcd1* (PD-1) locus containing a *Nr4a1* (Nur77) binding motif (17, 63).

Downstream from TCF-1-mediated subsistence of PD-1^{lo}CD8^{+} T_{ex}, thymocyte selection-associated high-mobility group (HMG) box protein, TOX, becomes co-upregulated alongside PD-1 and is associated with the epigenetic signatures demarcating terminal lineage commitment within PD-1^{hi}CD8^{+} T_{ex} (24, 56, 65–67). TOX is a nuclear protein that binds DNA in a structure-dependent manner (not sequence-dependent) (64). TOX directly interacts with histone acetyltransferase binding to ORC1 (HBO1) and indirectly coordinates activity with DNA

methyltransferases 3A (DNMT3A), 3B (DNMT3B), and enhancer of zeste homolog 2 (EZH2) to epigenetically fix $CD8^+$ T_{ex} towards terminal exhaustion (64). Ectopic TOX expression is sufficient to induce a full exhaustion transcriptional program in effector $CD8^+$ T cells *in vitro* (65). In contrast, deletion of *Tox* in $CD8^+$ TIL prevents exhaustion *via* decreased chromatin accessibility and expression of *Pdcd1*, *Havcr2* (Tim-3), *Cd244* (2B4), and *Tigit* (TIGIT) in the SV40-Tag-driven autochthonous liver cancer model (65). In the Clone 13 system, $Tcf7^{flox/flox}Cd4^{cre}$ and $Tox^{flox/flox}Cd4^{cre}$ mice (lacking TCF-1 and TOX in all T cells, respectively) results in favored development of effector-like KLRG-1^+CD8^+ T cells over the formation of PD-$1^{hi}CD8^+$ T_{ex} (**Figures 4A, D**) (24, 57, 68).

Recent findings by Ahmed (69) and Wherry (46) jointly demonstrate that stem-like cells are initially stable during Clone 13 infection. However, upon ICB treatment, these cells rapidly enter a T-bet-driven effector-like transitory state marked as CX3CR1^+KLRG-1^+CD101^-PD-1^{lo}Tim-3^+ (**Figure 4D**), which rapidly proliferate, temporarily produce granzyme B, and eventually digress to fully exhausted CX3CR1^-KLRG-1^-CD101^+PD-1^{hi}Tim-3^+CD8^+ T_{ex} (**Figure 4E**) (46, 69). CD101 is not expressed at baseline in $CD8^+$ T cells from healthy humans (70). Conversely, terminally differentiated $CD101^+$PD-$1^{hi}CD8^+$ T_{ex} have recently been observed to correlate negatively with tumor grade and regional LN metastasis within epithelial ovarian cancer patients (70). Transcriptional analyses of murine and human TIL corroborate these results linking changes in naïve-like PD-1^-Tim-3^-CD8^+ TIL before and after ICB (71). ICB appears to bifurcate PD-1^-Tim-3^-CD8^+ TIL into a self-renewing stem-like state (expressing *Tcf7*, *Lef1*, and *Sell*) and an effector-like program (expressing *Klrg1*, *Cx3cr1*, *Slamf7*, and *Ifng*) farther downstream along a developmental trajectory to full exhaustion (71). These phenotypic changes (stem-like > effector-like transitory > terminal exhaustion) coincide with chromatin accessibility shifts controlled by multiple transcription factors including NFAT, Nur77, BATF, IRF4, TCF-1, T-bet, and TOX that appear to be coordinated with PD-1-mediated TCR dampening (**Figures 4A–E**) (25, 46, 69, 72). In other words, $CD8^+$ T_{ex} seem to represent a lineage with limited differentiation capacity, existing within a series of fixed sequential epigenetic landscapes. Although reinvigoration of PD-$1^{lo}CD8^+$ T_{ex} can result in a detectable wave of transcriptionally 're-wired' effector-like activity, the cells appear to be limited because they eventually exhaust in response to ICB and are unable to de-differentiate into *bona fide* effector or memory cells present during acute infection (21, 46, 63). In the context of tumor immunity, understanding where these transitions occur *in vivo* (LN versus TME) and how to stabilize the transitory effector-like state is key to maximizing the cytolytic potential of stem-like $CD8^+$ T_{ex}.

What governs late-stage cell fate decisions of stem-like PD-$1^{lo}CD8^+$ T_{ex} progenitors to commit to a terminally exhausted PD-$1^{hi}CD8^+$ T_{ex} fate is partially clear at best. Constant TCR signaling is likely involved as enforced nuclear factor of activated T cells (NFAT) activity in antigen-specific $CD8^+$ T cells directly leads to *Tox* transcription (20, 24). Conversely, lack of *Nfatc1* (NFAT2) phenocopies loss of TOX (24). Further, TCR-responsive transcription factors, including BATF and IRF4, appear to positively feedback on *Nfatc1* transcription promoting PD-1^{hi}Tim-3^+ T_{ex} development (25). In contrast to NFAT1, NFAT2 itself is also known to favor the development of MPECs over SLECs (73). Imbalanced NFAT1 versus NFAT2 may also relate to skewing early T-bet and Eomes segregation in a primed $CD8^+$ T cell to seed TCF-1^+ stem-like progenitors even before ongoing direct downstream effects on TOX, and other exhaustion-associated genes are enforced. At a higher level, the overall balance between NFAT and CD28/AP-1 activity upon original and/or continued antigen encounter may be critical as anergic $CD8^+$ T cells and $CD8^+$ T cells primed in the absence of $CD4^+$ T cell help or co-stimulation mirror many of the major transcriptional and epigenetic events that occur in PD-$1^{lo/hi}CD8^+$ T_{ex} in both chronic viral infection and cancer (19, 20, 26, 66, 74–80). Exposure to microenvironmental stressors (low glucose, high lipid) in the TME may also orchestrate the TOX-centric epigenetic program that characterizes the PD-1^{hi} dysfunctional phenotype by disrupting metabolic/mitochondrial fitness (81–83). Mitochondria tend to produce elevated amounts of reactive oxygen species (ROS) in $CD8^+$ T_{ex}, which was shown to facilitate nuclear entry of NFAT downstream of a Ca^{++} flux in both $CD4^+$ and $CD8^+$ T cells (81–84). How constant PD-1 signaling, TCR engagement, and altered metabolism control the transition from a TCF-1^+ to TOX^+ state *via* constant NFAT activity in $CD8^+$ T_{ex} is an area where current knowledge is limited and is only starting to be investigated.

THERAPEUTIC POTENTIAL OF $CD8^+$ T CELL PROGENITORS IN CANCER

The significance of stem-like TCF-1^+PD-$1^{lo}CD8^+$ T_{ex} in governing ICB outcomes may lie in their pre-treatment frequency and crosstalk between other immune cell types during cancer. Surveys of TIL heterogeneity using single-cell RNA sequencing (scRNA-Seq) have indicated that activated, expanded, and exhausted $CD8^+$ T cell subsets are variably present in different tumor samples and effectively cluster based on *Tcf7* expression (53, 85). For instance, Sade-Feldman et al. profiled 48 metastatic melanoma tumor biopsies, comprising 17 responder and 31 non-responder patients receiving ICB (85). scRNA-Seq phenotyping of $CD8^+$ T cell clusters identified 6 clusters that were putatively annotated as belonging to early-activated, memory, effector, and exhausted lineages based upon cell surface marker expression profiles (85). All $CD8^+$ T cell populations were observed in most patients, albeit to differing degrees (85). However, the relative frequency of intratumoral $Tcf7^{hi}$ versus $Tcf7^{lo}$ TIL clusters was predictive of patient responsiveness to ICB (85). It has since then been confirmed in preclinical models that small populations of stem-like PD-1^{lo}Slamf6^+TCF-1^+CD8^+ T_{ex} (with Slamf6 being a surrogate for TCF-1) and PD-$1^{hi}TOX^+CD8^+$ T_{ex} indeed exist in the TME (86). In murine B16 melanoma, TILs retained some features of the

epigenetic profile seen in $CD8^+$ T_{ex} following Clone 13 infection, and anti-PD-1 treatment specifically drove stem-like PD-1^{lo} TILs to divide and convert into terminally exhausted PD-1^{hi} T_{ex} (86). In humans, stem-like TCF-1^+CD8^+ T_{ex} progenitors and terminally exhausted TCF-1^-CD8^+ T_{ex} have similarly been observed in multiple tumor indications (71, 86, 87).

As noted, Ahmed initially found that stem-like PD-$1^{lo}CD8^+$ T_{ex} express CXCR5; however, these cells co-express high amounts of *Ccr7* transcripts, migrate in response to a CCL19/21 gradient *in vitro*, and localize to the splenic T cell zone *in vivo* after Clone 13 infection (47). In this system, CXCR5 is expressed by both stem-like $CD69^+$Ki-67^- precursors and $CD69^-$Ki-67^+ progenitors (46). The function of CXCR5 is less well known in cancer immunology but may relate to stem-like $CD8^+$ T_{ex} positioning. Stem-like PD-$1^{lo}CD8^+$ TILs have been found to sporadically express CXCR5 depending on the tumor type (86, 88). In murine and human melanomas, CXCR5 positivity has thus far not tracked with stem-like PD-$1^{lo}CD8^+$ TIL (86). In contrast, $CXCR5^+$ TILs can be found in non-small-cell lung carcinoma (NSCLC) tumors and may uniquely associate with intratumoral tertiary lymphoid structures (TLS) (88). More work is needed to understand any potential association between $CXCR5^+$ TILs and tumoral TLS. It is tempting to speculate that CXCR5 facilitates localization within these structures, similar to the role of CXCR5 in positioning $CD4^+$ T_{fh} within secondary LNs (48). While only a minority of intratumoral stem-like cells express CXCR5, TCF-1^+PD-$1^{lo}CD8^+$ T_{ex} also seem to localize as crude clusters in the TME, implying that there may be additional niche microenvironments within the tumor that support anti-tumor immunity (87). In a histological analysis of prostate, bladder, and kidney cancer biopsies, TCF-1^+CD8^+ TILs were predominantly observed within MHC II dense regions, whereas the presumably exhausted TCF-1^-CD8^+ TIL appeared to be dispersed (87). Little is known about the role of these MHC II dense niches, which may influence stem-like T cell recruitment and/or dendritic cell (DC) Wnt signaling, thereby maintaining TCF-1 expression and stemness. Stem-like PD-$1^{lo}CD8^+$ T_{ex} are also preferentially found within tumor-draining secondary LNs over non-draining LNs (89). In contrast, terminally exhausted PD-$1^{hi}CD8^+$ T_{ex} are predominantly confined to the TME (89). Regardless, if TCF-1^+PD-$1^{lo}CD8^+$ T_{ex} infiltrate or expand locally within tumors after systemic delivery of ICB, the intratumoral frequency of these cells can serve as a valuable biomarker to discriminate responders against non-responders (and/or survival within the responder cohort) (85, 90).

TISSUE DISTRIBUTION AND INTRATUMORAL POSITIONING OF EXHAUSTED $CD8^+$ T CELLS

Tumor PD-L1 expression would logically seem to be a relevant prognostic factor to rationalize the usage of PD-(L)1-based ICB. PD-$L1^+$ tumors tend to respond more frequently to anti-PD-(L)1; however, there is only a weak correlation with overall treatment efficacy (33, 91, 92). A significant number of PD-$L1^+$ tumors do not respond to ICB, and durable responses are observed in PD-$L1^-$ tumors (33, 91). In other analyses, ICB was found to closely align with the raw amount of neoantigens broadly amongst cancers regardless of PD-L1 expression (91, 93–95). Following the completion of The Cancer Genome Atlas (TCGA), a strong correlation was observed between ICB-responsiveness and a T_h1/IFN-γ inflammatory signature, tumor mutational burden (TMB), and leukocyte infiltration (96). Thus, a combination of a T cell-inflamed gene signature with TMB may currently be the best predictor of ICB-responsiveness (91). PD-L1 expression in the tumor (known to be upregulated by IFN-γ) may reflect tumor inflammation status and thus rather passively indicate an overall immune system status rather than mechanistically predict the response of the tumor to ICB (91).

If inflammation and TMB underlie the response, does ICB act directly in the TME or periphery (97)? Immuno-positron emission tomography (immuno-PET) coupled with blockade of LN egress shows a large portion of effector-like $CD8^+$ TIL are derived from the periphery in mice bearing MC38 colorectal tumors systemically treated with anti-PD-1 (98). In the AC29 mesothelioma preclinical model, blockade of LN egress likewise severely compromises the number of $CD8^+$ TIL after systemic anti-PD-L1 (89). In the absence of ICB and irrespective of primary tumor PD-L1 expression, enhanced PD-1/PD-L1 contacts between stem-like PD-$1^{lo}CD8^+$ T_{ex} and migratory PD-$L1^+$ DCs entering the paracortex of tumor-draining LNs negatively correlates with survival of mice exposed to AC29 tumors and non-metastatic melanoma patients following resection (89). Localized delivery of anti-PD-L1 to tumor-draining LNs is sufficient to block these interactions and mobilize stem-like $CD8^+$ T_{ex} from the lymphatics for proliferation, migration to the TME, and preservation of stemness, leading to an increase in host survival comparable to systemic delivery (89). Further, LN-primed $CD8^+$ T_{ex} seem better able to respond to model antigen and proliferate upon *ex vivo* re-stimulation than systemically primed cells (89). These data suggest that LN-primed stem-like $CD8^+$ T_{ex} are a critical component of the response to ICB.

Additional studies involving scRNA/TCR-Seq have allowed a more in-depth look at the intratumoral versus peripheral counterparts of immune responses underlying ICB in patients. In a study by Yost et al., scRNA/TCR-Seq analysis of metastatic basal/squamous cell carcinoma patient TIL before and after ICB indicated that clonal replacement dominated the response where upwards of 84% of $CD8^+$ T cell clonotypes (having a single TCR specificity) present after treatment were novel (*i.e.*, not present in the tumor before treatment) (72). Intratumoral stem-like TCF-1^+CD8^+ T_{ex} did contribute a minor fraction to the population of ICB-activated, tumoricidal clonotypes; however, all cells that attacked tumors again eventually became exhausted (72). Therefore, ICB seems to predominantly mobilize functional $CD8^+$ T cells from the periphery into the tumor. A comparison of tumors to normal adjacent tissue (NAT) and peripheral blood *via* scTCR-Seq corroborated these findings across various cancers (99). Patients displaying extratumoral-intratumoral linked clonal expansion across blood/NAT and tumor responded more favorably to ICB (99). However, the action of

ICB on stem-like or effector like CD8$^+$ T_{ex} inside the tumor cannot be dismissed. Despite the lack of a significant correlation between intratumoral PD-L1 expression and survival, PD-1/PD-L1 interactions in the tumor as measured by immune-Förster resonance energy transfer (iFRET) is more predictive of survival in metastatic melanoma and NSCLC patients receiving ICB, in line with findings in draining LNs (89, 100).

Emergent data suggest that the CCR5 and CXCR3 chemokine receptor pathways are needed for anti-PD-(L)1-mediated CD8$^+$ T_{ex} tumor recruitment and/or intratumoral positioning (101–104). Heightened dual expression of the ligands for CCR5 and CXCR3 (CCL5 and CXCL9, respectively) positively correlates with the amount of tumor *CD8a* transcripts and patient survival in cancers of the ovary, breast, lung, colon, as well as melanoma (103). CCL5 from tumor cells or tumor-associated myeloid cells appears to license CXCL9 production almost exclusively from inflammatory CD68$^+$ macrophages and CD11c$^+$ DCs within the TME (98, 103, 105, 106). Genetic deletion or antibody-mediated blockade of either CCL5 and CXCL9 significantly compromises CD8$^+$ T cell recruitment to the TME; however, only CXCL9 correlates with ICB efficacy in multiple preclinical models (102–105). Revisiting CD8$^+$ T_{ex} CCR5 and CXCR3 progenitor/progeny expression patterns in the Clone 13 and preclinical tumor models may clarify this. In both settings, CXCR3 is predominantly expressed on stem-like PD-1^{lo}CD8$^+$ T_{ex} (**Figures 4B, C**), whereas CCR5 is oppositely elevated on terminally exhausted PD-1^{hi}CD8$^+$ T_{ex} (**Figure 4E**) (46, 69, 102). These axes may be necessary for the positioning and stability of stem-like PD-1^{lo}CD8$^+$ T_{ex} in the previously mentioned intratumoral MHC II dense clusters by undescribed mechanisms or serve as markers for recent PD-1^{lo} versus PD-1^{hi}CD8$^+$ T_{ex} CXCR3-mediated trafficking (87, 107, 108). With LN egress blocked, anti-PD-1 was shown to directly increase the expansion of intratumoral wild type but not *Cxcr3*$^{-/-}$ CD8$^+$ T cells, which may be related to localization of these cells within the TME or intrinsic effects (102). CXCR3 itself is known to support T-bet expression and favor SLEC differentiation during acute infection and may play a direct role in dictating stem-like to transitory CD8$^+$ T_{ex} differentiation (109, 110). Therapies centered on CXCR3 agonism may augment CD8$^+$ T cell trafficking, positioning, and priming/expansion depending on the exact intersection with the T_{ex} lineage.

CLINICAL PERSPECTIVES

Despite the undisputed success of ICB in the clinic, it may one day be replaced or combined with other immunotherapies due to its inherent failure in preventing exhaustion. Our increasingly granular understanding of CD8$^+$ T_{ex} and the underlying regulatory mechanisms may present novel therapeutic avenues that include alternative ways to stimulate and stabilize stem/effector-like states along the exhaustion continuum or enhance memory cell lineage commitment. Simple amplification of CD8$^+$ T cell responses by modulating trafficking and tumor positioning may stabilize stem-like and effector-like transitory CD8$^+$ T_{ex}. Durable responses may also be possible if effector-like CD8$^+$ T cells can instead be directly coerced to persist in the transitory cytolytic state, for instance, by pharmacologically antagonizing TOX or related mediators of exhaustion. In addition, as stem-like PD-1^{lo}CD8$^+$ T_{ex} exhibit heightened expression of several members of the immunoglobulin and tumor-necrosis factor receptor (TNFR) superfamily, including *Tnfrsf4* (OX40) and *Tnfrsf9* (4-1BB), combining ICB with TNFR superfamily member agonism may further support long-lived CD8$^+$ T cell reinvigoration by preferentially targeting the stem-like subset (47). Inhibiting other known or unknown transcriptional components or downstream effector pathways of the exhaustion program may offer other therapeutic avenues.

If maintaining stabilized anti-tumoral CD8$^+$ T cells is impossible, maximal amplification of the response *via* focused neoantigen vaccination or repetitive infusions of adoptive cellular therapies (ACT) may be warranted. Today, it is possible to administer autologous CD8$^+$ T cells genetically engineered to express neoantigen-specific TCRs or chimeric antigen receptors (CARs) (111, 112). This may allow for an unlimited source of artificially generated anti-tumoral CD8$^+$ T cells, thus bypassing the challenge that exhaustion may be unavoidable. ACT may also be designed to be exhaustion-resistant or to maintain stemness through gene-editing technologies (112). Alternatively, neoantigen vaccination might be a more promising strategy, either as part of a patient-shared or fully personalized therapeutic approach (113, 114). Neoantigen vaccines carrying both CD4$^+$ and CD8$^+$ T cell epitopes as long peptides, RNA/DNA vectors, or within viral constructs may better support robust, helper-primed CD8$^+$ T cell responses able to resist exhaustion upon repeated antigen encounter (115–121). Neoantigen vaccination can also strategically address tumor immunoediting. Even if persistent antigens are effectively cleared, some residual tumor cells can unavoidably become resistant to first-line ICB and/or neoantigen vaccination by altering MHC I-displayed tumor antigens *via* deletion or mutation (122). Neoantigen vaccination can solve this by applying booster regimens modified in real-time against resistant tumor cell clonal outgrowth.

CONCLUSION

Understanding how tumors shape CD8$^+$ T cell exhaustion is needed to effectively program the immune system to destroy cancer—the professed 'emperor of all maladies' (123). An exciting parallel journey between chronic viral infection and cancer has thus been embarked upon to bypass exhaustion and identify the causative molecular cues, new cell types/lineages permissive to ICB, and innovative paths for immunotherapeutic strategies. It is currently clear that reversing exhaustion in PD-1^{hi}CD8$^+$ T_{ex} is unlikely. Selective mobilization of stem-like CD8$^+$ T_{ex} is instead called for and lies at the crux of generating functional and stable anti-tumor immune responses. Besides re-shaping the CD8$^+$ T_{ex} developmental continuum, scientists are dually challenged with directing specificity of the responding population as ICB also relies on the endogenous immune system for spontaneous

recognition of select neoantigens from an initially broad TCR repertoire (90, 124, 125). Can stem-like and effector-like $CD8^+$ T_{ex} fates be stabilized to act as a continuous source to deliver an unending supply of tumoricidal $CD8^+$ T cells? Can exhaustion itself be prevented in response to ICB? Can chemokine receptor pathways be exploited to control TME positioning and differentiation status of intratumoral $CD8^+$ T_{ex}? Or should immunologists accept the demise of $CD8^+$ T_{ex} and deploy patient-tailored neoantigen and ACT strategies? The answers to these outstanding questions undoubtedly lay forth the path of future clinical trials.

AUTHOR CONTRIBUTIONS

All authors conceived, discussed content, and contributed to researching data for the article. JD produced the primary drafts of the manuscript and designed the figures. NB-B, GT, and SS-A. provided writing and editorial contributions. All authors contributed to the article and approved the submitted version.

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