

Receptor signaling, transcriptional, and metabolic regulation of T cell exhaustion

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ABSTRACT

Exhaustion cripples T cell effector responses against metastatic cancers and chronic infections alike. There has been considerable interest in understanding the molecular and cellular mechanisms driving T cell exhaustion in human cancers fueled by the success of immunotherapy drugs especially the checkpoint receptor blockade (CRB) inhibitory antibodies that reverses T cell functional exhaustion. The current understanding of molecular mechanism of T cell exhaustion has been elucidated from the studies utilizing murine models of chronic viral infections. These studies have formed the basis for much of our understanding of the process of exhaustion and proven vital to developing anti-exhaustion therapies against human cancers. In this review, we discuss the T cell exhaustion differentiation pathway in cancers and chronic viral infections and explore how the transcription factors expression dynamics play role in T cell exhaustion fate choices and maturation. Finally, we summarize the role of some of the most important transcription factors involved in T cell functional exhaustion and construct exhaustion specific signaling pathway maps.

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Introduction

T cell exhaustion is at the heart of success of checkpoint receptor blockade (CRB) inhibitory drugs (i.e., Opdivo® [nivolumab], Keytruda® [pembrolizumab]). These drugs function by reversing functional exhaustion in antigen-specific T cells inducing remissions in stage III and IV melanoma, non-small lung cancer (NSLC), and urothelial cancers.^{1–3} Despite the success of CRB therapies, the clinical data have also highlighted limitations with these therapies as only 20% to 40% of patients respond to single agent CRB therapies, others have shown resistance with progression, indicating a huge scope for improvement.^{4–7} One of the reasons impeding improvements in these therapies otherwise possible may be due to lack of understanding of the molecular and cellular mechanisms underlying T cell exhaustion lineage growth and maturation in human cancers. One among many means to improve CRB therapies could be investing resources in next generation immunotherapies. For example, developing new immunotherapy drugs that does not rely entirely on antibodies to inhibit checkpoint receptor blockade but also target signaling pathways regulating T cell exhaustion growth, and epigenetic regulators of exhaustion. Therefore, in-depth understanding of molecular mechanisms of T cell exhaustion is of great interest both from a clinical and a basic science perspective. Several papers have been published recently that present new data on the mechanisms underlying T cell exhaustion developmental fates and maintenance in murine chronic viral infection and human cancer models. These papers elegantly describe the role of novel transcription factors, transcription regulators, epigenetic remodeling enzymes, and metabolic regulators involved in the development and stability of T cell exhaustion subsets {Reviewed in ref.^{8–11}}. Table 1

summarizes the role of some of these transcription factors in T cell exhaustion development and maturation. As noted in the table, almost all transcription factors implicated in T cell exhaustion have overlapping functions in T effector and/or T cell memory differentiation. Therefore, it can be strongly argued that molecular mechanisms operational in T cell exhaustion can be expected to be operational at some level in T effector and T memory cells as well. In the following sections, we discuss how the transcription factors expression dynamics play instructive roles in T cell exhaustion, T effector, and T cell memory differentiation. We first begin with defining CD8 T cell exhaustion.

Defining CD8 T cell exhaustion in the context of chronic viral infections and cancer

Several studies have provided critical insights into the developmental fates of CD8 T cells.^{12–14} One established view in the context of murine model of acute infections that resolve successfully is that the naïve CD8 T cells become activated after interacting with professional antigen presenting cells that present viral antigens via canonical endogenous pathway or non-canonical cross presentation. This reaction occurs in draining lymph nodes.¹⁵ These activated T cells react with virally infected tissues and differentiate into short-lived effector T cells generating alongside a long-lived memory T cell pool.¹⁶ The short-lived effector T cells, according to the linear differentiation model, become terminally differentiated and subsequently undergo apoptosis with the elimination of acute infections (Figure 2). As Figure 2 demonstrates, 3-signals, the TCR, costimulatory, and cytokine signals are vital for CD8 T cell differentiation process.

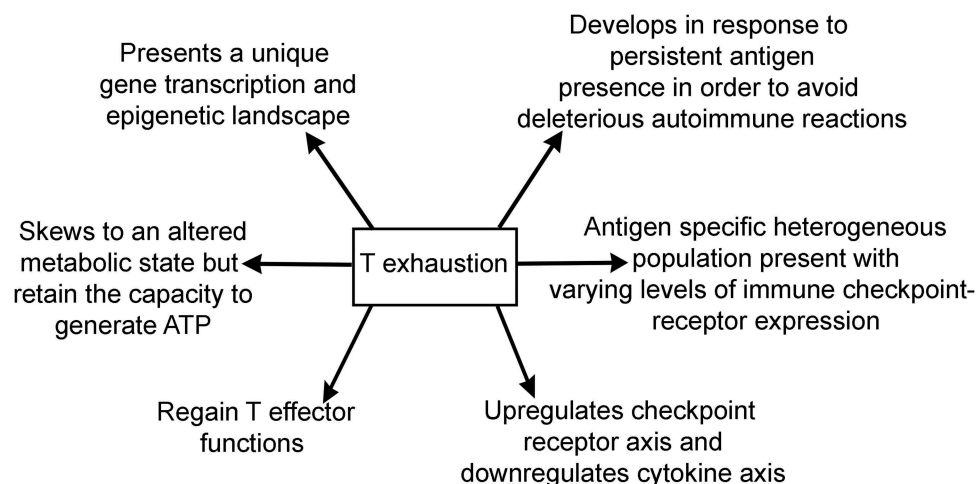
Table 1. Transcription factor activities in various T cell subtypes.

| | T _{Effector} | T _{Memory precursors} | T _{Exhaustion} |
|--------|---|--------------------------------|--------------------------|
| NFAT1 | Moderately expressed | Expressed and has a role | Highly expressed |
| BLIMP1 | Moderately expressed | Not expressed | Highly expressed |
| Eomes | Moderately expressed | Highly expressed | Highly expressed |
| T-bet | Highly expressed | Expressed and has a role | Expressed and has a role |
| IRF4 | Essential for effector functions | Not expressed | Highly expressed |
| BATF | Moderately expressed and essential for effector functions | Expressed and has a role | Highly expressed |
| YY1 | Moderately expressed | Expressed and has a role | Highly expressed |
| FOXO1 | Opposes effector functions | Expressed and has a role | Highly expressed |

The developmental fates of CD8 T cells in chronic viral infections and cancers have begun to be understood only recently. In murine models of chronic actively replicating viral infections and in immunogenic cancers, the naïve/resting CD8 T cells also follow the linear differentiation model and generate effector T cell pool. But, due to the presence of persistent antigens T effector cells fail to properly activate terminal differentiation program instead differentiate into a hypofunctional quasi-differentiated chronically stimulated state termed exhaustion. The memory precursors generated in chronic viral infections are unstable and not properly maintained in vivo.^{17,21,22} The antigen-presenting cells (APCs) engaged in antigen presentation of viral antigens via canonical endogenous pathway or cross presentation have been identified. For example, in chronic HIV macrophages harbor HIV viruses and also scavenge HIV proteins from plasma thereby presenting antigens to T cells.^{23,24} Likewise, in human cancers, several studies have established that APCs especially migratory dendritic cells that immigrate from tissues, and lymphoid resident conventional dendritic cells process and present tumor antigens to CD8 T cells via cross presentation in the draining lymph nodes.^{15,25-28} The activated viral antigen or tumor antigen-specific T cell subsequently react with virally infected tissues or tumor tissues, respectively (Figure 3a,B). Accordingly, exhausted T cells have been found to

be present in lymphoid tissue T cell zones and at the sites of infection and cancer tissues with features ranging from partial to terminal exhaustion phenotypes.²⁹⁻³¹ It is noteworthy, in chronic viral infections the antigen-specific effector T cells lose specificity for immunodominant epitopes instead develop specificity for subdominant epitopes.^{32,33} Moreover, using lineage tracing techniques, it has recently become clear that during early chronic viral infections, a precursor population exist within T terminal-effector population that become lineage committed to T cell exhaustion fate and maturation^{34,35} (Figure 3b). T cell exhaustion precursors with stem cell line properties have also been identified in cancers, but if they commit to T cell exhaustion fate is unclear.³⁶ Taken together, these studies provide strong evidence that T cell exhaustion development follows a linear differentiation model.

The hallmarks of T cell exhaustion are presented in Figure 1. One of the hallmarks of exhausted T cells is the upregulation of Checkpoint Receptor (CR) Axis, i.e., PD1, Lag3, Tim3, TIGIT, and downregulation of Cytokine Axis, i.e., IL2, IFN γ , TNF α . These represent two vital axes of exhaustion. By addressing therapeutically the checkpoint receptor blockade (CRB), the quasi-differentiated T cell exhaustion pool in cancers can reverse some aspects of functional exhaustion such as cytokine secretion, proliferation, and killing activities generating alongside the memory precursors.^{29,37-39} The origin of CRB therapy-related T cell exhaustion memory precursors in vivo is poorly understood (Figure 4). The notion that T cell exhaustion subsets are quasi-differentiated and chronically stimulated is supported by the following: 1) Their partial restoration of effector functions after therapeutic interventions such as antibody-mediated blockade of PD1:PD-L1 interaction or through attenuation of regulators that sustain exhaustion;⁴⁰⁻⁴² 2) The upregulation of CD69 activation marker and CR axis in vivo is an evidence that mature exhausted T cells are chronically activated. The upregulation of CR axis in exhausted T cells could be a negative feedback mechanism for preventing generation of deleterious autoimmune responses.^{37,43} This view is supported by the cases of toxic autoimmune responses reported in patients receiving CRB therapy, as much as the toxic responses in patients are correlated with the regression of tumors.^{44,45}

**Figure 1.** Summary of hallmarks of T cell exhaustion.

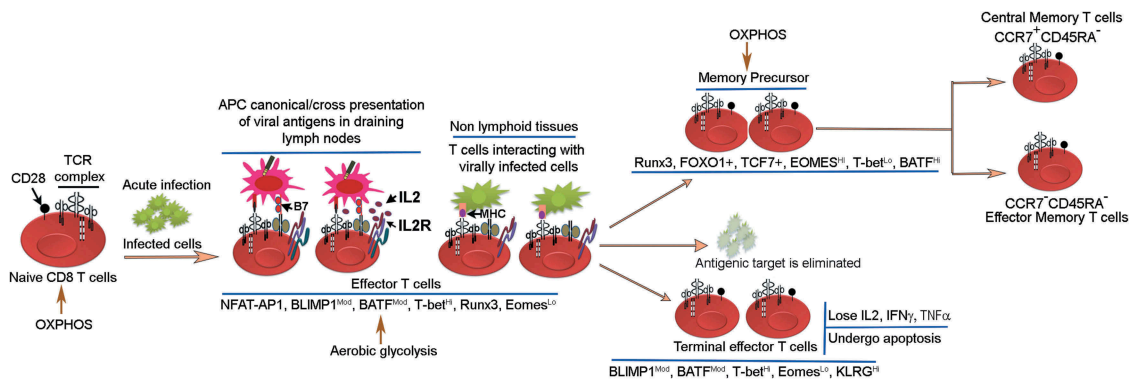


Figure 2. The CD8 T cell linear differentiation model. Naive T cells in acute viral infections become activated in lymphoid tissues via canonical and cross presentation of viral antigens by antigen-presenting cells. The activation process ensues with the delivery of signal 1 + 2 and IL2 production. IL2 subsequently diffuses locally and binds IL2 receptor to generate high affinity IL2-R that promotes IL2-R mediated signaling pathway, which is important for proliferation and survival of antigen-specific CD8 T cells. Activated CD8 T cells react with virally infected cells and undergo proliferative expansion and differentiate into terminal T effector cells generating alongside memory precursors that differentiate further into central memory and T effector memory subsets. These memory subsets persist at various sites in vivo.^{17–20} The transcription factor expression pattern in CD8 T effector, T cell exhaustion and T cell memory subsets are shown.

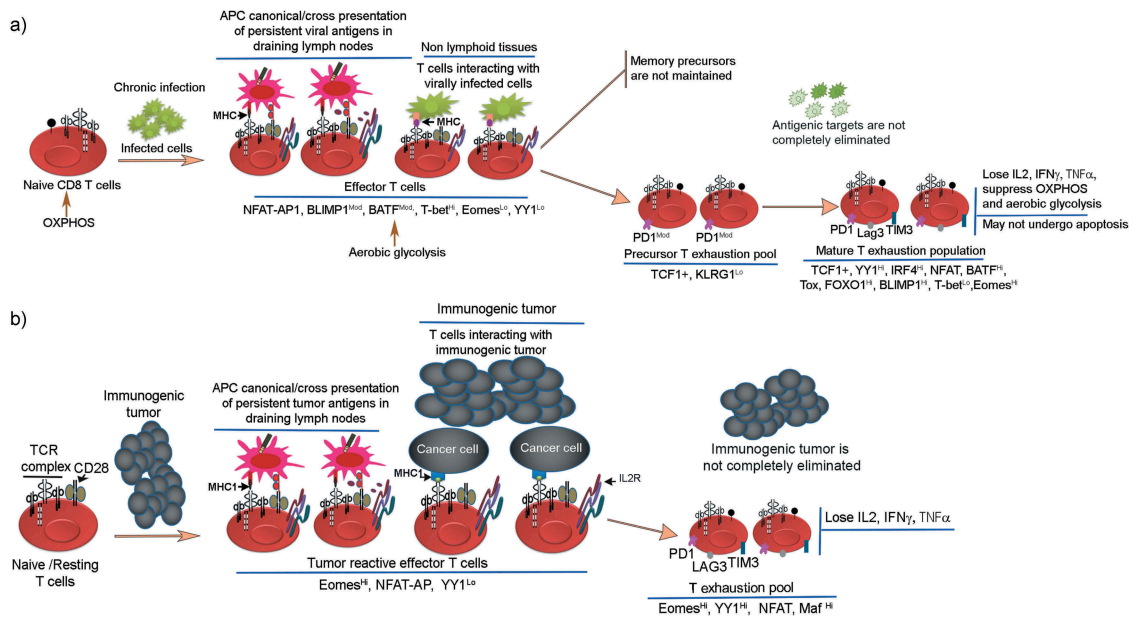


Figure 3. Transcription factors involved in regulating lineage differentiation of naive CD8 T cells into effector and T cell exhaustion fates in chronic viral infections and immunogenic cancers. (a) Illustrates naive CD8 T cells upon exposure to chronic viral infections initially differentiate into effector T cells, but with persistent antigen presence, effector T cells fail to activate terminal T effector program instead generate a PD1^{Mod}TCF1+ precursor T exhaustion population. This precursor population gives rise to mature pool of exhausted T cells that are hyporesponsive, undergo cytokine failure, and upregulate exhaustion specific markers. Exhausted T cells lose the capacity to eliminate chronic infections. (b) Illustrates the role of antigen-presenting cells especially tissue resident conventional dendritic cells in acquiring tumor antigens, processing and presenting to CD8 T cells. The reaction occurs in draining lymph nodes. The list of transcription factors involved in T cell exhaustion to tumors is provided.

The heterogeneity of T cell exhaustion pool

Exhausted T cells present at the sites of immunogenic tumors and chronic viral infections have been recognized to be heterogeneous population of cells. A specific subset of T cells within the larger pool of heterogeneous subsets of T cell exhaustion have been shown to respond vigorously to the anti-exhaustion therapies,⁴¹ and even without therapy, a subset of T cells have also been shown to retain effector functions. Therefore, examining the heterogeneity of T cell exhaustion pool is vital not only from a clinical perspective but also for our understanding of the mechanism of T cell exhaustion. Several subsets in T exhaustion pool have been

identified expressing varying levels of exhaustion-specific markers alongside the markers of memory and progenitor T cell lineage. Among the heterogeneous CD8 T cell exhaustion pool, a short-lived proliferating and long-lived non-proliferating subsets were discovered to be present in chronic human HIV1 and LCMV infections.^{21,46,47} The short-lived proliferating CD8 T cells subsets were confirmed to be Eomes^{hi} whereas long-lived non-proliferating subsets were confirmed to be T-bet^{hi}.⁴⁷ Another subset of cells found to be present within the T cell exhaustion pool include TCF1+ memory T cell like precursors, and CXCR5 + follicular cytotoxic CD8 T cell like that expresses exhaustion-specific inhibitory receptors notably PD1 albeit at lower

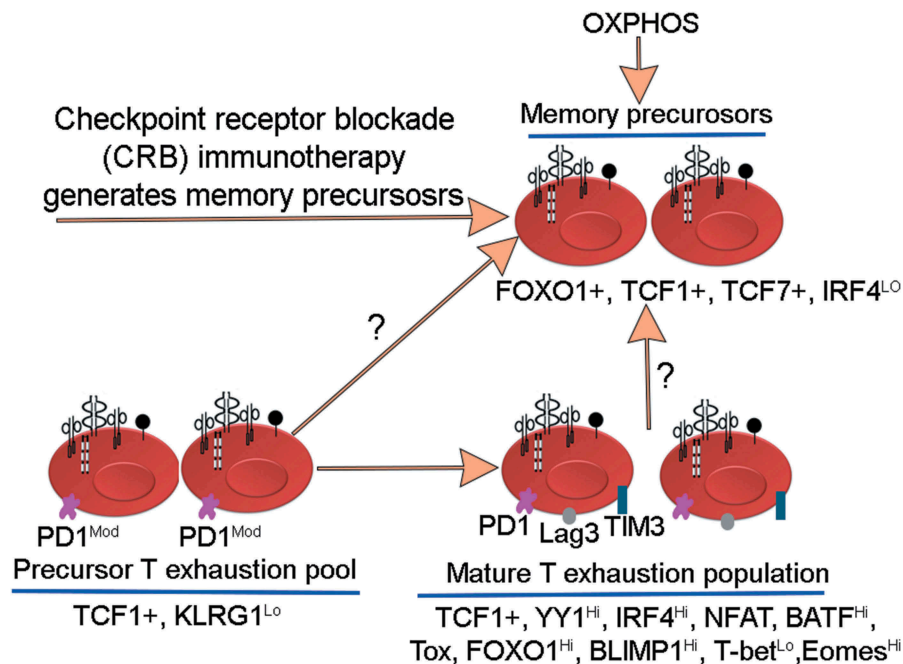


Figure 4. Illustrates differentiation of memory precursors in vivo after checkpoint receptor blockade (CRB) immunotherapy. The origin of these memory precursors is unknown. For example, it is not known if these memory precursors arise from T exhaustion precursors or from mature T-exhausted pool.

levels.^{30,42,48,49} Importantly, the Runx-family transcription factor Runx-3 has been shown to be important for repressing development of CXCR5⁺ CD8 T cells with follicular T helper cell-like phenotypes, and for regulating T-bet/Eomes functions as well as for promoting effector and tissue-resident memory CTL differentiation.^{50–52} The development of heterogeneous subsets of T cell exhaustion in the settings of cancer and chronic viral infections also seem to determine the response to therapy. The two subsets of T cells within the heterogeneous T cell exhaustion pool were identified to respond differently to the anti-PD1/PD-L1 therapy. For example, the PD-1^{Int} CD44^{Hi} population were shown to respond vigorously whereas PD-1^{Hi} CD44^{Int} respond poorly to the anti-PD1/PD-L1 therapy.⁴¹ In a recent study, Thommen et al., utilizing a gene expression profile reported a remarkable heterogeneity in the intratumoral CD8⁺ TILs population in human non-small lung cancer patients.⁵³ Thommen et al. demonstrated the distinct functional and metabolic gene signature existed for intratumoral CD8⁺PD1^{Hi}, CD8⁺PD1^{Int} and CD8⁺PD1^{Lo} T cell populations. Importantly, the differential gene signature could predict the response to anti-PD1 therapy in a small cohort of NSLC patients with PD1^{Hi} being the strong predictor of response and survival.⁵³ As to the origin of CD8 T cell exhaustion subsets, little is known so far. For example, it is unclear if it is the effector CD8 T cells that after experiencing persistent chronic viral antigens or immunogenic tumor antigens in vivo differentiate into different subsets of exhausted CD8 T cells or whether these subtypes arise following a linear differentiation program.

Reversal of T cell exhaustion

T cell exhaustion is partially reversible process, i.e., subset of cells within T cell exhaustion pool regain the antigenic target killing functions with therapeutic interventions. That makes T cell exhaustion subsets distinct from the terminal T effector

cells which cannot regain effector functions and are programmed to undergo apoptosis. In a sharp contrast to T cell exhaustion, memory T cell differentiation and for generating strong central memory recall responses both require the downregulation of immune checkpoint receptors.^{54,55} Another marked distinction with the T cell memory is that the latent memory T cells become reactivated if reexposed to the same strain of infectious pathogens. In contrast, exhausted T cells can regain antigen-specific effector functions especially cancer-killing functions in vivo with the drug interventions such as checkpoint receptor blockade (CRB) inhibitory antibodies^{56,57} or with high doses of IL2.^{58–62} In chronic viral infections and cancer, IL2 has been shown to reduce the expression of PD1 and enhance the proliferation of resting memory T cell pool.^{38,63} The need for extrinsic interventions to regain killing functions is one of the hallmarks of exhausted T cells. The key molecular, phenotypic, and functional differences between various CD8 T cell subtypes are provided in the Box.

Metabolism in exhausted CD8 T cells

Altered metabolism is a hallmark of exhausted T cells. Choice of metabolic pathways plays an important role in the differentiation and activation of antigen-specific T cells. T cells adopt two choices for ATP generation: 1. Glycolysis and mitochondrial mediated oxidative phosphorylation, 2. Aerobic glycolysis. Naïve, memory, effector, and exhausted T cells have different bioenergy demands and substrate requirements. The quiescent T cells, i.e., naïve and memory T cells under normal oxygen levels metabolize glucose through glycolysis and via oxidative phosphorylation (OXPHOS) generates ATP utilizing electron transport chain complexes I–V in the mitochondria, which is an efficient

mechanism for generating ATP.^{64,65} The antigen-activated T cells synthesize rapidly effector molecules and effector cytokines (e.g., IL2, IFN γ), and have increased proliferation rates. Accordingly, effector T cells under normal oxygen levels switch to aerobic glycolysis, a process also referred to as Warburg effect.^{66,67} In aerobic glycolysis, glucose is converted into lactic acid and even though only fewer molecules of ATP are generated, it represents a rapid mechanism of ATP and metabolic substrate intermediate synthesis including nucleotides, non-essential amino acids and fatty acids. Therefore, aerobic glycolysis helps to catch up with the demand for enhanced anabolism, proliferation, and effector T cell activities.^{65,66,68} It is presently unclear how the quiescent memory T cells switch to aerobic glycolysis after antigen rechallenge and maintain OXPHOS after antigen clearance. It appears that memory T cells unlike naïve or effector T cells maintain mitochondrial functions especially fatty acid oxidation, and IL15 seem to play a vital role in promoting mitochondrial biogenesis and respiratory capacity.⁶⁹ The metabolic requirements of T cell exhaustion subsets may be distinct in the hyporesponsive state versus reactivation phases, i.e., during reactivation of T cell exhaustion subsets induced by CRB inhibitory antibodies. Exhausted T cells, as reported in both chronic viral infections and cancers present with significant suppression of glycolysis, OXPHOS, and glucose deprivation due to GLUT1 downregulation, and repression of mitochondrial biogenesis.^{70–72} There may be additional candidates for how the change in metabolism in this transition works, such as downregulation of mTOR or activation of the AMPK pathway, although it is unclear how the choice for such pathways occurs, some works suggest asymmetrical division of effector T cells may play a role. In the LCMV exhaustion model, the metabolic dysfunction of T cells can be detected immediately after the infection and appears to be PD1 regulated and driven by the enhanced mTOR activity. Among the metabolic changes that were noted in chronic LCMV, specific CD8 T cells include reduced glucose uptake and glycolysis, reduced OXPHOS and mitochondrial respiration, but enhanced mitochondrial mass, membrane depolarization, ROS production, and ultrastructural changes such as elongated mitochondria cristae.⁷¹ Part of the metabolic dysregulation driven by PD1 was found to be through repression of the key metabolic regulator, the peroxisome proliferator-activated receptor γ (PPAR γ) coactivator 1 α (PGC1 α).⁷³ PGC1 α is a transcriptional activator that induces expression of genes important for mitochondrial biogenesis, and when reexpressed in exhausted T cells recovers glucose uptake, lowers mitochondrial biomass and depolarization, and improves effector functions.⁷¹

The metabolic dysfunctions such as the reduced glucose uptake and mitochondrial biogenesis as seen in chronic LCMV specific CD8 T cells were also noted in the CD8 TILs in B16 melanoma mouse model and metastatic melanoma genetic mouse model, Braf^{V600E}/Pten^{-/-} with the exception of mitochondrial mass that was found to be reduced.^{72,74,75} The intratumoral TILs remain exposed to glucose deprivation in the tumor microenvironment that restricts aerobic glycolysis and also negatively affect T cell effector functions. In glucose sufficient environment, the glucose gets transported via GLUT1 into cytoplasm where it is subjected

to glycolysis generating several substrate intermediates such as phosphoenol pyruvate (PEP). The PEP inhibits endoplasmic reticulum membrane Sarco/ER Ca²⁺-ATPase (SERCA) activity that prevents ER uptake of cytosolic calcium allowing optimal TCR \rightarrow NFAT signaling pathway to proceed normally thereby helping mediate optimal effector functions. Correspondingly, glucose deficiency in tumor microenvironment produces PEP insufficiency promoting SERCA mediated calcium uptake that attenuates TCR signaling and as a result the NFAT activity.^{76,77} Interestingly, in mouse sarcoma tumor model, the systemic analysis of the metabolic changes associated with reactivation of exhausted T cells with CRB inhibitory antibodies PD1, CTLA4, and PD-L1 have shown to restore glucose in tumor microenvironment by restricting glucose uptake in tumor cells and triggering glycolysis and enhanced IFN γ production in antigen-specific T cells.⁷⁵ These data indicate that similar metabolic changes could be taking place in the reactivated antitumor T cells in humans responding to CRB therapy. Confirmatory studies using human samples will be needed to prove this point.

Gene expression signature in exhausted T cells

Several attempts have been made to elucidate the transcription profile of exhausted T cells.^{70,78–80} The T cell exhaustion transcription profile often contains genes that overlapped with the T effector and T cell memory gene expression modules. That essentially could be due to the various subsets of cells present within the T cell exhaustion pool. The composition of heterogeneous subpopulation within T cell exhaustion pool can vary significantly with infections from different strains of viruses or between different lineages of cancers. Moreover, isolating a homogenous population of tumor antigen-specific T cells for obtaining T cell exhaustion specific gene expression profile can be technically challenging and labor intensive. One of the challenges is designing unique MHC-tumor peptide tetramers that TCRs could recognize and bind in sufficient numbers.⁸¹ Until today, very few groups have been successful in isolating tumor antigen-specific T cells in vivo with the exception of melanoma antigens.⁷⁹ Overall, the number of tumor antigen-specific CD8 T cells can be extremely low. They may represent as little as 0.002% of the peripheral T cell population in humans, and approximately 0.1% of TIL ex vivo culture.⁸² Accordingly, several recent studies have performed whole-exome sequencing and RNA-seq analysis on human tumors to identify novel tumor antigens that TCRs could bind in sufficient numbers.^{83–86} These new technologies can prove extremely valuable in identifying unique tumor antigens and in designing MHC-tumor peptide tetramers that eventually could help isolating and analyzing TCR-specific tumor-infiltrating T cells. Moreover, powerful new technologies such as single-cell RNA-seq analysis of tumor-infiltrating T cells has proven powerful resource in revealing a reliable transcriptional profile of homogenous T cell exhaustion pool.^{87–91}

Exhaustion specific transcription factors

Several new transcription factors have recently been identified to be associated with exhaustion in murine chronic viral infections in vivo. Table 2 summarizes the activities of these transcription factors. Some of these transcription factors have

Table 2. Transcription factors involved in regulating T cell exhaustion-specific genes.

| | YY1 | NFAT-AP1 | NFAT1 | BLIMP1 | T-bet | Eomes | IRF4 | BATF | FOXO1 |
|-------------------------------|-----------------|-----------------|-------|--------|-------|-------|------|-----------------|-------|
| <i>PD1</i> | ^h B↑ | ↓ | B↑ | B↓↑ | B↓ | - | B↑ | B↑ | B↑ |
| <i>LAG3</i> | ^h B↑ | ↓ | B | - | - | - | B | B | - |
| <i>TIM3</i> | ^h ↑* | ↓ | B | - | - | - | B | B | - |
| <i>IL2</i> | ^h B↓ | ^h B↑ | B↓ | B↓ | - | - | ↓ | ^h B↓ | - |
| <i>IFNγ</i> | ^h B↓ | B↑ | B↑ | B↓ | ↑ | B↑ | ↓ | ^h ↓ | - |

B indicates binds on consensus transcription factor sites present in genes.

^h denotes in humans.

↑ indicate enhances gene expression.

↓ indicate represses gene transcription.

* Indicate indirect binding.

opposing or overlapping functions in T effector, T cell memory, and T cell exhaustion subsets, which is due to the heterogeneity that exists in these cell types especially in T effector and T cell exhaustion pool. Both T effectors and exhausted T cells contain subsets with varying degrees of differentiation, some present with markers of terminal differentiation and other express markers of T cell memory. In this review, we focus mostly on the transcription factors that have been revealed to regulate the T cell exhaustion fate choices, maturation, metabolism, and memory precursor generation.

IRF4

IRF4 transcription factor was identified in a gene expression analysis to be expressed in the chronic LCMV specific CD8 T exhaustion pool.⁷⁰ The functional role of IRF4 in T cell exhaustion has recently been revealed. Using LCMV chronic infection model, Man et al., showed that IRF4 expression is elevated in exhausted T cells. Furthermore, through in silico ChIP-seq and ChIP assays, they showed that IRF4 binds to immune checkpoint receptor genes, *PD1*, *Lag3*, and *Tim3*.⁴² And, part of the mechanism through which IRF4 exerts its exhaustion specific role was through the disruption of metabolic regulation affecting mitochondrial size and production of reactive oxygen species (ROS). IRF4 disrupted glycolysis and oxidative phosphorylation in exhausted T cells.⁴² In addition, IRF4 forms a transcriptional circuit with NFAT1 and BATF transcription factors. IRF4 and NF-AT1 also form autoregulatory loop with IRF4 promoting expression of NF-AT1. The NF-AT1 in turn binds to *IRF4* promoter.^{92,93} It appears from the insilico ChIP-seq data that IRF4, BATF, and NFAT1 bind together on the *PD1* gene. However, it was not clear whether these composite consensus regulatory sites on the *PD1* were conserved on the human *PD1*. Interestingly, reducing the IRF4 levels attenuates T cells exhaustion, corrected mitochondrial size and ROS production, and promoted differentiation of TCF1+ memory like cell types.⁴² From the data, it is not clear if the T cells in mice infected with chronic viral infections and having lost the IRF4 allele convert to memory like T cells from a reactivating pool of exhausted T cells or represent a separate lineage of cells differentiating from recent effector T cell emigrants. Several lines of evidence support that reactivated exhausted T cells after CRB therapy can generate heterogeneous subset of cells some resembling TCF1+ expressing memory precursors and others the follicular T cells.^{30,42,48,49} Overall, IRF4 is required for the

maintenance of exhaustion phenotype along with the NFAT1 and BATF. These three molecules seem to accumulate with TCR activation. Whether CD28 stimulation (signal 2) was also necessary for their accumulation was not examined. Interestingly, the IRF4 binding site are present on human *PD1*, *Lag3*, and *Tim3* promoters. In my search for transcription factors binding to human *IRF4* promoter using publicly available promoter database <http://epd.vital-it.ch/cgi-bin/>, revealed several IRF4 binding site in ~2kb upstream of TSS on human *PD1*, *Lag3*, and *Tim3* promoter. Whether these sites are functional and important for human T cell exhaustion to chronic infections and in cancer needs elaborate analysis. Furthermore, in CD4 T cells, IRF4 is known to coordinate with AP1, and IRF4-AP1 bind on composite elements on *IL10* gene to promote transcription.⁹⁴ IL10 is one cytokine that increases during exhaustion to chronic LCMV infections.^{95,96} Whether IRF4:AP1 play any role in *IL10* gene transcription during exhaustion remains unknown. It is important to note that intratumoral IL10 released by Tregs into tumor microenvironment contribute to T cell exhaustion. Correspondingly, targeting IL10 or Tregs in combination with checkpoint receptor blockade (CRB) anti-PD1 therapy reverses some aspects of exhaustion to chronic LCMV infection.⁹⁷

T-bet and Eomes

T-bet and Eomes are T-box transcription factors that play a crucial role in effector and memory functions of T cells.^{98,99} The physiologically significant role of T-bet in protective immunity and effector functions was revealed in *T-bet*^{-/-} deficient mice. These mice demonstrated the compromised protection against intracranial LCMV infection.¹⁰⁰ T-bet and its paralogue Eomes appear to have redundant and cooperative functions in effector T cell differentiation. For example, *T-bet*^{-/-} CD8 T cells secrete reduced levels of effector cytokine, IFN γ . Whereas Eomes overexpression rescues IFN γ production in *T-bet*^{-/-} CD8 T cells. Correspondingly, haploinsufficient *Eomes*[±] mice do not produce any defect in IFN γ production that could be due to haploinsufficiency being compensation by the normal T-bet expression.¹⁰¹ The inverse kinetics of T-bet and Eomes expression appear to regulate lineage differentiation of T effector versus T cell memory and T cell exhaustion.^{16,102} The high expression of T-bet and Eomes appears to be important for the effector functions of CD8 T cells in acute infection model.^{99,103} The high T-bet expression in effector T cells during acute infections progressively declines with memory T cell differentiation; however, an inverse kinetics was observed with respect to Eomes.¹⁰⁴ (Figure 2). In chronic LCMV infection exhaustion model, a low T-bet expression is crucial for maintaining exhaustion phenotype because T-bet is revealed to be a repressor of PD1 and was shown to bind directly on *PD1* promoter.¹⁰² Consistent with the murine data, the human chronic HIV antigen-specific exhausted T cells have lower T-bet expression but maintained higher Eomes expression, and these expression kinetics correlated with upregulation of inhibitory immune checkpoint receptor PD1.¹⁰⁵ It remains unclear how reuse of T-bet and Eomes in exhausted T cells in the

same kinetics as in memory T cells contribute to the exhaustion state. One explanation could be that the exhausted T cells like memory T cells remain quiescent with potential for regaining effector activities; therefore, T-bet and Eomes exist in the same kinetics in these two cell types to regulate the quiescence and reactivation programs. The role of Eomes and T-bet to T cell exhaustion in cancers remains unknown; however, similar to chronic infections, in autochthonous melanoma mouse model and in patients with metastatic melanoma expression of Eomes was detected to be upregulated in tumor antigen-specific exhausted T cells.^{43,79}

Blimp1

Blimp1 has been extensively studied for its role in CD8 T effector cells and T cell memory differentiation during acute infections. It emerged from the studies involving acute viral infections that Blimp1 expression increased during T effector cell differentiation and was essential for the effector activities.^{106–108} Blimp1 expression though important for T effector differentiation appears to restrict memory T cell development as the Blimp1 deficient effector T cells showed unrestricted capacity to differentiate into memory precursors.¹⁰⁷ Blimp1 has also emerged as an important player in T cell exhaustion. The gene expression dosage of *Blimp1* appears to be crucial for instructing lineage choices to T effector versus T cell exhaustion. The moderate Blimp1 expression appears to favor CD8 T effector functions. However, in chronic LCMV infections where Blimp1 expression was detected to be elevated promote upregulation of immune checkpoint receptor molecules associated with exhaustion while repressing the genes involved in memory T cell differentiation.¹⁰⁹ Intriguingly, Blimp1 was also reported to repress PD1 expression in acute infection model through a mechanism involving direct transcriptional repression of *PD1* as well as, indirectly, through inhibition of NFAT1, which is a direct transcriptional activator of *PD1*.¹¹⁰ Why Blimp1 does not repress *PD1* and *NFAT1* in exhausted T cells where Blimp1 is detected to be elevated remains unclear. One explanation could be that other transcription factors that activate *PD1* transcription override Blimp1 repressive functions directed at *PD1*. Blimp1 has been proposed to have a role in IL2 shutdown.¹¹¹ That assumption was based on, 1) A Blimp1 binding site on *IL2* promoter 2kb upstream from the transcription start site (TSS), 2) Blimp1 knockout studies in mice.¹¹² However, Blimp1 mediated *IL2* repression in the above studies was not evaluated through promoter mutation analysis, and the site for Blimp1 binding is also not conserved in the human *IL2* promoter. Whether Blimp1 can be a direct mediator of human IL2 failure during T cell exhaustion require further investigations. It is possible that Blimp1 has a role in mouse T cells which is distinct from its role in human CD8 T cell exhaustion (Figure 3a).

NFAT

NFAT is a regulator of T cell activation.^{113,114} *NFAT1* (human ortholog *NFATc2*) is a founding member of the NFAT family which was elucidated to have a prominent role in T cell

activation, and exhaustion to chronic infections (bacterial and viral) and cancer.^{93,115} NFAT1 (NFATc2) including its other members NFAT2 (NFATc1) and NFAT4 (NFATc3) get activated through TCR signaling and triggers biphasic calcium mobilization and binding of cytosolic calcium to calmodulin calcium binding protein. Ca^{2+} /calmodulin activates serine/threonine phosphatase calcineurin, which dephosphorylates cytoplasmic NFAT1 allowing it to translocate to the nucleus to initiate gene transcription program.¹¹⁶ However, for the effector cytokine synthesis such as IL2, NFAT1 cooperates and interacts with AP1 transcription factor that binds to the *NFAT-AP1* composite DNA binding sites on *IL2* promoter.^{117–120} NFAT1 is extensively studied for its role in regulating the gene transcription program associated with anergy and exhaustion.^{80,93,121} In anergy, NFAT1 mediated gene expression is driven primarily by TCR activation in the absence of costimulation that prevents AP1 activation and interaction with NFAT inhibiting in the process the NFAT:AP1 regulated gene transcription program. That includes among others, IL2.¹²² Moreover, anergic T cells in vivo show reduced calcium flux, and as a result inhibited NFAT1 nuclear localization.¹²³

Through gene knockout studies, Martinez et al. showed that NFAT1 and 2 were required for driving the gene transcription program associated with exhaustion. For example, mice deficient in *NFAT1*^{-/-} or *NFAT2*^{-/-} in CD4 T cells when exposed to chronic LCMV infection fail to upregulate *PD1*, *Lag3*, and *Tim3* genes. Furthermore, NFAT1 was shown to directly bind on *PD1*, *Tim3*, *Lag3*, and *IFN γ* genes to upregulate their expression.^{93,124,125} It is clear from these studies that costimulation and NFAT interaction with AP1 could interfere with the exhaustion specific role of NFAT. In the in vivo settings, T cells encountering chronic viral or immunoreactive tumor antigens as also shown recently by the Mognol et al.⁸⁰ may also receive costimulatory signals. It is unclear how the stoichiometry would shift to NFAT monomer activation over NFAT:AP1 dimerization to coordinate exhaustion program when the costimulatory signaling may be unhindered and the calcium flux sustained.¹²⁶ One explanation could be the negative regulation of *AP1* by transcription factor BATF, which has been reported to increase significantly in exhausted CD8 T cells, e.g., in human HIV infections.^{127,128} Future studies utilizing CD28 knockout mutant mice¹²⁹ with intact TCR signaling could very well clear the role of CD28 costimulation and NFAT signaling to initiation and maintenance of T cell exhaustion to immunogenic cancers and chronic viral infections. Nevertheless, inducing NFAT:AP1 interaction might have therapeutic benefits for reversing exhaustion. Promoting CD28 costimulatory signaling pathway should be able to promote AP1: NFAT interactions that in turn could reinvigorate effector T cell program.

BATF

BATF belong to AP1 transcription factor family and is essential for antigen-specific CD8 effector T cell differentiation.¹³⁰ In the acute LCMV infection, expression of BATF increases in effector T cells and its expression also remains high in memory T cells.¹²⁷ The role of BATF in exhaustion has been

reported both in humans and mouse models. The expression of BATF increased significantly in exhausted T cells during chronic HIV and LCMV infection model where it represses *IL2* expression and together with IRF4 regulates *PD1* expression.^{42,127} Yet, major role of BATF appears to be coordinating T effector differentiation and for that its target appeared to be other transcription factors rather than the effector cytokines. BATF directly binds on *T-bet* and *Blimp1* genes to upregulate their expression. Moreover, BATF functionally cooperates with IRF4 and Jun in a cell type specific manner to activate gene transcription program.⁹⁴ BATF forms heterodimer with Jun but unlike Jun/Fos heterodimers, BATF/Jun dimers acts as a negative regulator of AP1 gene transcription and represses gene transcription such as inflammatory cytokine *IFN γ* .^{128,130}

TCF1, TCF7 and FOXO1

Several studies have established the role of transcription factor T cell factor (TCF1) in vivo in T cell exhaustion fate using gene deletion studies.^{30,49} Recently, Chen et al. defined TCF1⁺Ly108⁺PD1⁺ CD8 T cells as precursor population of exhausted T cells using mouse model of LCMV infection.³⁵ TCF1 appears to orchestrate early T cell exhaustion fate differentiation by suppressing KLRG1^{Hi} terminal effector T cells. Interestingly, TCF1+ also maintains T cell exhaustion program by promoting Eomes and cMyb expression. cMyb regulates Bcl2 expression promoting survival of early T exhaustion population.^{34,35} In murine melanoma model and human melanoma patients, the TCF1+ PD1+ population was identified in T cell exhaustion pool that generate proliferative burst after CRB therapy and demonstrate memory like properties.³⁶

The forkhead O transcription factors 1 (FOXO1) and T cell factor 7 (TCF7) play important roles in maintenance of exhaustion and functional T cell memory phenotype. The FOXO1 has been reported to be important for viral antigen-specific CD8 T cell survival, expansion as well as memory T cell identity and differentiation.¹³¹ In murine chronic LCMV infections, FOXO1 transcription factor was shown to activate *PD1* expression in CD8 T cells.¹³² The FOXO1 activity was shown to be controlled through AKT and mTOR signaling. Accordingly, suppression of AKT-mTOR circuit in antigen-specific CD8 T cells was demonstrated to be necessary for enhanced FOXO1 nuclear export and its activity, and for sustaining PD1 expression, therefore, linking the metabolic regulatory signaling pathways to FOXO1 and to T cells exhaustion.¹³² As opposed to continuous activity, FOXO1 loss in CD8 T cells lead to functional anergy in response to latent murine cytomegalovirus (MCMV) infection characterized by decline in the effector cytokine production and reduced proliferation rates.¹³³ Here again, continuous activity of FOXO1 for maintenance of exhaustion and memory phenotype contrasts with its role in anergy pointing to the heterogeneous nature of T cell subsets generated in response to different latent viruses. The FOXO1 regulates expression of *TCF7* in virally infected cells but not in naïve T cells.¹³⁴ In acute infection models, both TCF7 and FOXO1 orchestrate memory differentiation. TCF7 has been shown to

downregulate granzyme-B (GZMB) expression and reduce the KRG1^{hi} effector T cell population. And, its expression is highly elevated in naïve T cells and then downregulate upon pathogen activation but gets reexpressed in the memory precursors.¹³⁴ Likewise, in chronic viral infections, FOXO1 sustains terminal exhaustion phenotype besides maintaining TCF7+ memory like cells. Loss of *FOXO1* in these infections led to the loss of TCF7+ memory like cells.¹³⁵ The TCF7 + CD8 memory precursors in vivo in chronic infections and cancer were recently found to represent two distinct subpopulations of exhausted T cells, the TCF7+ PD1+ and TCF7-PD1+. The TCF7+ PD1+ were found to be with stem cell like properties, and the cells responsible for generating proliferative burst with anti-PD1 immunotherapy mediating tumor control as well as control of chronic viral infections.^{30,36} Whether these two subsets arise from T effector, T exhaustion precursors or from mature pool of exhausted T cells is presently unclear (Figure 4).

Yin Yang 1

The stability of T cell exhaustion involves a unique gene transcription program in which expression of some genes upregulate, i.e., immune checkpoint receptors and others downregulate, i.e., class I cytokines. Perhaps, the transcription factor(s) poised to be an important regulator of exhaustion must have the capacity to regulate both gene activation and repression programs intrinsically, epigenetically, and cooperatively by interacting with other transcription factors or cofactors. Yin Yang 1 (YY1) along with Blimp1 embodies such dual functionalities. YY1 is a ubiquitous multifunctional zinc finger transcription factor that is known to play crucial roles in B cell differentiation, development, and cellular proliferation.^{136–138} Several studies have elucidated the role of YY1 in T cell functions in murine models. For example, CD4-specific knockout of *YY1* has revealed its requirement for Th2 cytokine expression and cell-type specific binding to Th2 cytokine locus.¹³⁹ YY1 is also vital for T effector differentiation to acute LCMV infections in mice. Yu et al., using ATAC-seq and Page Rank analysis revealed chromatin accessibility of YY1 binding motifs to be highly enriched in antigen-specific terminal-effector (TE) cells in mice.¹⁴⁰ However, YY1 has not been recognized to play a significant role in antigen-specific immune responses or in the mechanism of T cell exhaustion and is little expressed in resting normal human T cells. YY1 was recently shown to serve as the master regulator of T cell functional exhaustion in vitro by participating in both activation and repression of a broad array of exhaustion's presently defined elements (Table 2). YY1 was shown to be central for promoting the exhaustion response upon “chronic stimulation” of human T cells through repeat exposure to CD3/CD28 beads or to tumor antigens in vitro. In the repeat exposure system, YY1 was shown uniquely to participate in virtually all defined components of the exhaustion process mediating downregulation of class I and II cytokines, i.e., IL2 and IFN γ , respectively, and upregulation of immune checkpoint receptors (PD1, Tim3, Lag3), with a concomitant decline in functional killing of human antigenic targets. YY1 own transcription and the ensuing

exhaustion process was shown to depend on two signals (Signal 1 + 2) that activates p38MAPK/JNK kinase pathway and transcription factor complex composed of phospho-ATF2/cJun that binds to *YY1* promoter activating *YY1* transcription, *YY1* finally orchestrate functional exhaustion of human T cells.³⁷ *YY1* may meet the standard of “exhaustion-specific” when expressed at high levels in vivo and “representing homogenous cell-type.” The recently published literature on homogenous population of dysfunctional T cells in murine syngeneic tumor model seem to corroborate with the exhaustion specific nature of *YY1*. *YY1* is shown to physically interact with *GATA3* in T cells and is further shown to be required for recruiting *GATA3* on regulatory elements of several Th2 genes.¹³⁹ Singer et al. identified *GATA3* through single-cell RNA-seq analysis of intratumoral CD8+ TILs to be involved in T cell dysfunction. *GATA3* was found to be expressed in highly dysfunction CD8+ TILs in tumor bearing mice.⁹¹ Further in vivo studies would be needed to identify a definitive connection and collaboration between *YY1* and *GATA3* in the initiation, maintenance, and lineage differentiation to a pool of highly dysfunctional exhausted T cells.

Tox

The thymocyte selection-associated high mobility group-box protein (*Tox*) has been revealed to be vital for maintenance of CD8 T cell exhaustion phenotype in chronic LCMV and human HCV viral infections and cancer.¹⁴¹ *Tox* belongs to high mobility group (HMG)-box proteins that can bind to DNA in a manner which is dependent on the structure but independent of specific DNA sequence motif.¹⁴² *Tox* appears to be important for CD4 T cell lineage development program in thymus. CD4 T cell lineage development is disrupted in *Tox*^{-/-} deficient mice without disrupting lineage growth of CD8 T cells.¹⁴³ Moreover, *Tox*^{-/-} CD8 T cells in vitro appear to retain proliferative capacity when exposed to two signal activation as well as cytolytic activity.¹⁴³ *Tox* is revealed to be highly expressed in dysfunctional tumor specific T cells and exhausted T cells in chronic viral infections. Interestingly, utilizing two commonly employed LCMV strains, the Armstrong and Clone 13, *Tox* was shown to be induced during chronic infections but not in acute infections marking *Tox* to be the first exhaustion specific molecule with in vivo relevance to exhaustion. The in vivo experiments demonstrated that CD8 T cell specific deletion of *Tox* down-regulates PD1 expression and upregulated cytokines, however, *Tox* deleted tumor specific dysfunctional “exhausted” CD8 T cells did not regain effector activities.¹⁴⁴ The attenuation of PD1 with *Tox* deficiency or inversely PD1 enhanced expression with *Tox* overexpression does explain a role for *Tox* in maintenance of CD8 T cell exhaustion to chronic infections. However, *Tox* deficient CD8 T cells failure to regain antitumor effector activity also indicate a role which appears not analogous to exhaustion in cancers. Apart from CD8 T cells, CD4 T cells also become exhausted in cancer, chronic viral infections as well as chronic bacterial and parasitic infections. Correspondingly, the instability of *Tox* deficient CD4 T cells suggests that utility of targeting *Tox* for reversing T cell functional exhaustion may be limited.

The transcriptional regulators involved in migration of exhausted CD8 T cells

Apart from the above-mentioned transcription factors, there are several other transcription regulators in particular HIFs, Id2, Id3, that play an important role in T cell exhaustion. The transcriptional regulators Id2 and Id3 belong to inhibitor of DNA binding family and play important roles in differentiation of various lymphocyte subsets such as NK, B and, T cell subsets.¹⁴⁵ Id2 and Id3 during chronic HIV and EBV infections affect migration and differentiation of a subset of antigen-specific exhausted T cells, the CXCR5+ follicular cytotoxic T cells (T_{FC}).⁴⁸ Id2 and Id3 were shown to suppress the expression of *CXCR5* chemokine receptor gene through blocking E protein family transcription factor E2A. Accordingly, CXCR5+ T_{FC} cells deficient in *Id2* and *Id3* genes upon exposure to chronic HIV and EBV infections in humans and mice retained the capacity to migrate to B cell follicles and eliminate the Follicular helper T cells (F_{TH}) HIV and EBV B cell reservoir.⁴⁸ Therefore, Id2 and Id3 by controlling *CXCR5* expression regulate the lineage differentiation of T_{FC} subset of exhausted T cells. Likewise, the VHL-HIF axis plays a crucial role in the control of chronic infections and B16 melanoma tumors in vivo. Under hypoxic conditions of chronic viral infections and malignancy, hypoxia inducible factors (HIFs) and its negative regulator the von Hippel–Lindau tumor suppressor VHL control CTL responses. Under normal oxygen tension (normoxic conditions), the HIFs are constantly degraded in a process that is dependent on VHL tumor suppressor protein complex.¹⁴⁶ The CD8 + T cells deficient in *VHL* gene succumb to chronic LCMV infections due to increased immunopathology triggered by higher expression of effector molecules and cytokines such as granzymes, perforins, TNF α , and IFN γ . The expression of these genes in turn depend on enhanced HIF1 activity. As expected, CD8 T cells deficient in *VHL* gene with higher expression of effector molecules show remarkable control of implanted B16 tumors.¹⁴⁷ Overall, these data demonstrate that enhanced HIF1 activity can maintain effector activity and resist exhaustion despite sustained immune checkpoint receptors expression.

Epigenetic landscape of exhausted CD8 T cells

The epigenetic landscape of exhausted CD8 T cells is distinct from T effector and T cell memory subtypes.^{40,148,149} Even within the exhausted T cell pool, the epigenetic landscape varies between partially versus terminally versus therapeutically reactivated exhausted T cell subtypes.^{150–152} The reason why a distinct epigenetic landscape exists is that a distinctive gene expression patterns exist in T cell exhaustion, T effector and T cell memory subtypes indicating that T cell exhaustion represents a distinct cell type. Even though the lineage growth of T cell exhaustion appears to be derived from reprogramming of T effector population in murine chronic infection model,³⁵ the transcription factors that mediate distinct epigenetic landscape in T cell exhaustion subtypes has begun to be revealed only recently. The epigenetic landscape of T cell exhaustion subsets in vivo has been defined utilizing chronic LCMV infection model and method of choice has been the

chromatic accessibility assays. The high throughput assay known as assay for transposase-accessible chromatin (ATAC) is used to identify regulatory regions in the genome.¹⁴⁹ By motif enrichment analysis of the regulatory elements, the transcription factors involved in the process can be revealed. Using these techniques, several transcription factors have been identified to be involved in epigenetic reprogramming of exhausted T cell subtypes. Several thousand chromatin-accessible regions were identified to be unique in T exhausted compared to T effector and T cell memory subtypes. These open chromatin-accessible regions were present in intronic or intergenic regions containing enhancer elements of target genes with binding activity for transcription factors NFAT2, Nr4a, Nur77, Eomes, Egr2, Sox3, T-bet, retinoic acid receptor (RAR).^{40,148,149} Interestingly, upon PD1 treatment, the epigenetic landscape is partially reprogrammed to support effector activity. The T effector population open chromatin-accessible regions contain target genes with binding activity of transcription factors NF- κ B, IRF1, IRF2, Nur77, Blimp1.⁴⁰ Recently, the high-mobility group protein TOX was revealed to be vital for epigenetic reprogramming of T cell exhaustion to chronic viral infections. Using the ATAC assay, Khan et al., revealed that TOX deletion significantly altered chromatin-accessible regions of genes associated with exhaustion.¹⁵³

The chimeric antigen receptor (CAR) T cell exhaustion

CAR-T cells are chimeric antigen receptor-modified T cells that recognize tumor antigens through chimeric receptor antibody. CAR-T cells have demonstrated remarkable success in eradicating refractory B cell malignancies,^{154–156} but have so far shown limited success against solid tumors. The major reasons for this limited impact among others derives from CAR-T cell exhaustion.¹⁵⁷ Similar to unmodified effector T cells, chimeric antigen receptor (CAR)-modified T cells also undergo functional exhaustion in presence of persistent tumor antigens.^{58,158–161} The detailed molecular mechanisms of CAR-T cell exhaustion are yet unknown. However, the 2nd generation CAR modified T cells incorporating costimulatory molecules have provided some vital clues. The second generation (CAR)-T cells incorporating CD28 costimulatory molecules and receiving persistent antigenic stimulation from tumors grown in the in vitro cultures have been shown to differentiate into dysfunctional state with features consistent with perturbation of dual axes of exhaustion, i.e., failure to sustain cytokine production and upregulation of CR axis especially PD1.^{37,160,162–164} A paper recently published by Zolov et al. demonstrated through a direct comparison between CD123-CD28- ζ -CAR, CD123-41BB- ζ -CAR or CD123- ζ -CAR that CD123 CAR with CD28 costimulatory domain was prone to PD1/PD-L1 mediated exhaustion and failure of antitumor activity. Interestingly, both CD28-CAR and 41BB-CAR enhanced exhaustion specific markers PD1, Lag3, Tim3 (CR axis), however, it was only CD28-CAR that when exposed to antigenic targets showed IL2, IFN γ (Cytokine axis) failure as well as demonstrated reduced proliferative capacity in consistent with the terminal exhaustion phenotype.¹⁶⁴ These observations are clinically significant as

majority of the active clinical trials with CARs listed in NIH clinical research trials database have been utilizing second generation CARs incorporating CD28 costimulatory molecule. <https://clinicaltrials.gov/ct2/results?cond=&term=CAR&cntry=&state=&city=&dist=>. Furthermore, the second generation CARs incorporating CD28 costimulatory domain targeting neuroblastoma, ovarian cancer, and metastatic renal carcinoma have reported partial clinical responses in the clinical trials.^{157–159,165,166} It remains unclear if persistent signal 2 contributed to the reported partial clinical responses with second generation CARs versus other factors such as restricted tumor access and immunosuppressive tumor micro-environment. It is likely that the mechanisms driving CAR-T cell exhaustion are different from the mechanisms contributing to exhaustion of antigen-specific unmodified T cells.

T cell exhaustion specific signaling pathways in cancer and chronic viral infections

Not much is known about the signaling pathways driving T cell exhaustion in cancer or chronic infections. For example, there is limited understanding if signaling pathways driving exhaustion to chronic infections and cancers are alike or different. Or, if any synergy exists in the signaling pathways between murine models and humans. Therefore, knowledge is limited to construct a comprehensive signaling pathway map specific to exhausted T cells. Nevertheless, based on known activities of transcription factors in T cell exhaustion and the knowledge of their signaling pathways in T cell functions can help constructing exhaustion specific signaling pathway maps. The two important evidences exist that point to activities of two distinct pathways in exhaustion: 1) TCR mediated NFAT signaling pathway, which has emerged as a common link to exhaustion in cancer and chronic viral infections in vivo, 2) The in vitro two-signal model of human T cell exhaustion which is centered around YY1 and is relevant to CAR-T cell exhaustion.

TCR \rightarrow NFAT1 signaling pathway

Significant amount of data provide strong evidences linking TCR signaling with NFAT1, IRF4, and BATF activation to T cell exhaustion. All three are induced in response to TCR signaling to murine chronic infections and are essential components of maintaining exhaustion specific transcriptional profile. The exact membrane proximal TCR events associated during persistent antigenic activation in vivo is unknown (Figure 5a). Data exist, however, that suggested the occurrence of TCR responsive calcium flux and calcineurin activation in T cells experiencing chronic infections, thus, indicating an involvement of NFAT activation.⁹³ NFAT1 has emerged as a crucial player of TCR responsive exhaustion specific gene transcription program both in cancer and chronic infections. Therefore, a signaling model can be designed based on constitutive NFAT1 nuclear activity driven by constitutive TCR proximal signaling complex, calcium influx and calcineurin phosphatase activity (Figure 5a). The nuclear NFAT1 directly binds on *PD1* promoter and activates its transcription. NFAT1 also contributes to *PD1* transcription

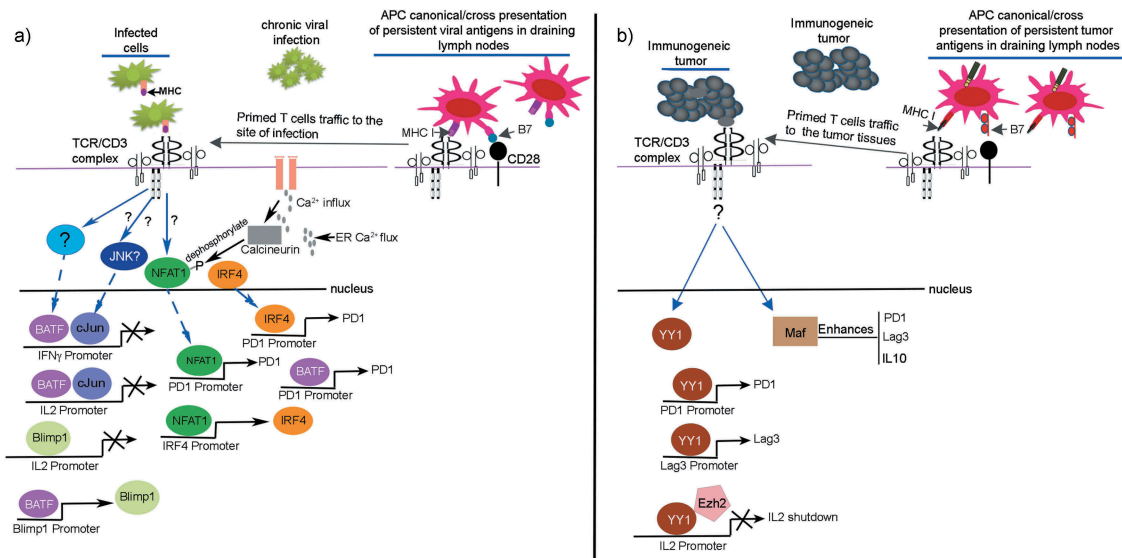


Figure 5. T cell exhaustion specific signaling pathways. (a) TCR → NFAT1 → exhaustion, which is relevant to chronic viral infections in vivo. The antigen-specific T cells receive activation from APCs in draining lymph nodes followed by their reactivity against infected cells. (b) TCR → YY1 → exhaustion of human T cells, which is relevant to immunogenic cancers. The tumor antigen-specific T cells in vivo receive activation from APCs in draining lymph nodes followed by their reactivity with immunogenic tumors, e.g., melanoma. The YY1 along with c-Maf are highly expressed in T cells reacting with immunogenic melanoma, however, the upstream signaling pathways regulating their transcription during development of T cell exhaustion are unknown. The YY1 is shown to upregulate CR axis and suppresses IL2 production in cooperation with Ezh2.³⁷ Maf enhances PD1, Lag3, and IL10.

indirectly through IRF4. IRF4 binds on *PD1* promoter enhancing its transcription. Another component of TCR exhaustion pathway is the BATF and Jun that form heterodimer and repress effector cytokine expression. In addition, BATF also potentially binds on *PD1* promoter to promote its transcription. Taken together, NFAT1, and BATF in response to persistent TCR activation and calcium mobilization accumulate in the nucleus promoting a cumulative effect on *PD1* transcription by binding on its promoter (Figure 5a). However, the scheme of signaling pathway presented in Figure 5a is not without a caveat. As data exist that shows in response to chronic LCMV and HIV infections, NFAT1 paralog NFAT2 translocation to the nucleus was inhibited in the setting of persistent TCR activation and calcium flux producing profound defect on IFN γ production.¹²⁶ Additional studies will help clear the apparent dichotomy that exist between NFAT1 and NFAT2 in exhaustion.

TCR → YY1 → exhaustion

The signaling pathways active in exhausted T cells in human cancers is unknown. YY1 was revealed to be highly elevated in melanoma-infiltrating tumors.³⁷ The upstream signaling pathway regulating YY1 expression in vivo in immunogenic tumors is not known. However, the in vitro human T cell exhaustion model that uses CD3/CD28 beads to repeatedly stimulate T cells have revealed activation of MAPK/JNK signaling pathway leading to heterodimerization of phospho-cJun and phospho-ATF2 that bind to the *YY1* promoter to initiate its transcription.^{37,167} YY1 binds directly to *PD1* and *Lag3* genes in vitro to upregulate their transcription. Moreover, YY1 binds directly on *IL2* promoter and recruits Ezh2 that catalyzes H3K27me3 marks repressing *IL2* transcription (Figure 5b). Extensive RNA-seq data exist that

shows enrichment of the elements of cJun/ATF signaling pathway in T cell exhaustion in vivo cancer models and chronic viral infections providing relevance to MAPK/JNK signaling pathway in exhaustion.^{93,141,153} Apart from YY1, the c-MAF transcription factor was also revealed to be highly elevated in melanoma antigen-specific T cells in mouse model and melanoma draining lymph node in humans.^{31,43} Maf enhances expression of CR axis as well as immune-suppressive cytokine IL10.⁴³ However, the signaling pathway regulating Maf expression in T cell exhaustion is also unknown.

Conclusion

As evident in this review, knowledge is still evolving about the transcriptional regulation and developmental fates of T cell exhaustion both in human cancers and chronic viral infections. Exhaustion is revealed to be a time sensitive process that occurs via perturbation of two axes of exhaustion, i.e., failure of cytokine axis and the upregulation of checkpoint receptor axis that include among others the PD1. The PD1 recruits Src homology 2 (SH2) domain-containing tyrosine phosphatase SHP2 to dephosphorylates CD3 ζ chain¹⁶⁸ as well as phospho-CD28¹⁶⁹ leading to the inhibition of both TCR and CD28 costimulatory signaling. Finally, once the T cells are fully exhausted, the pressure to maintain exhaustion associated genetic and epigenetic program remains high as the process itself is reversible proportional to the pool of chronically infected cells or tumor volume. With the shifting dynamics of antigen pool, the exhaustion specific genes expression may be altered to reflect the change.

In chronic viral infections, the development of T cell exhaustion appears to be a linear differentiation process. If the rise of T cell exhaustion in immunogenic cancers also

follows a linear differentiation model will require further investigation. The lineage tracing techniques may help define molecularly the stages of exhaustion. Taken together, T cell exhaustion is dynamic, transcriptionally, and metabolically active and energy-consuming state in which transcription factors may often alter their expression kinetics to reflect the state of differentiation of T cell exhaustion pool. The T cell exhaustion pool responding to the therapeutic interventions could provide vital clues regarding the pattern of genomic and epigenetic changes associated with the suppression of T effector functions. Moreover, immunotherapy induced reactivation of T cell exhaustion pool can also help to identify the origin of reinvigorated population whether these being pre-exhausted CD8 population or recent emigrants to antigen rich environment.

BOX

T_{Naïve}: Naïve T cells are recent thymic emigrants, self-antigen tolerant and having undergone TCR rearrangements. Typically, after their development from thymus and subsequent positive and negative selection, T cells circulate through blood and secondary lymphoid organs as naïve T cells and have not yet responded to antigens.

T_{Effector}: CD8 T Effector cells arise during acute viral infections. For T effector differentiation, naïve T cells must react with professional antigen presenting cells that present viral antigens to CD8 TCR through MHC Class I and coengages costimulatory, adhesion, and cytokine receptor molecules. T effector are antigen specific and have huge potential to proliferate and can synthesize and secrete effector molecules such as granules containing granzymes and perforins, cytokines and chemokines. Effector T cells effectively perform antigen target killings. After the pathogenic target is elimination, effector T cells contract leaving behind a small percentage of long-lived memory precursors.^{170,171}

T_{Memory}: T effector cells responding to pathogenic targets differentiate into terminal T effector population and generate memory precursor cells.^{172,173} The generation of T cell memory precursors are regulated through epigenetic mechanisms involving changes in DNA methylation patterns orchestrated by methyltransferase Dnmt3a¹⁷³ and histone methylation notably histone H3 lysine 9 catalyzed by histone methyltransferase Suv39h1.¹⁷⁴ Memory precursor differentiate into central memory (T_{CM}) and effector memory T cells (T_{EM}). T_{CM} reside and travel between lymphoid organs whereas T_{EM} reside and travel to tertiary tissues, e.g., mucosal surfaces, lungs, liver, skin, intestine, etc.¹⁷⁵ Both T_{CM} and T_{EM} retain effector functions including lytic activity upon antigenic rechallenge.^{17-19,176}

T_{Exhaustion}: T cell exhaustion is quasi-terminally differentiated state, presented with altered metabolic, and epigenetic state that develops in response to chronic infections and immune reactive cancers.^{8,40,148,149,177} Exhaustion is initiated with persistent delivery of TCR signaling and maintained by the checkpoint PD1/PDL1 signaling, as well as by immune suppressive cellular, molecular and metabolic intermediators, DNA and histone methylation. The activity of transcription factors such as YY1, IRF4, NFAT, Maf, Blimp1, Tox have been shown to be vital for the exhaustion phenotype. The inhibitory PD1/PDL1 signaling in exhausted T cells may be

a response to control the proliferation of persistently activated antigen-specific T cells helping to avoid autoimmune responses and promoting peripheral tolerance.^{178,179} Therefore, exhausted T cells even though consistently expressing activation markers have lost the capacity to proliferate and have poor effector functions. One of the distinctive characteristics of exhausted T cells is that they can regain effector functions especially their killing functions against antigenic targets with both immune checkpoint receptor blockade inhibitors as well as with non-checkpoint therapeutic interventions such as cytokines.

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No potential conflicts of interest were disclosed.

Availability of data and material

I agree to share all the data and content of this review.

Author contributions

M.Y.B. conceived, planned, and wrote the review article.

Consent for publication

I consent to publication.

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