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Review article

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Epigenetic regulation of T cell exhaustion in cancer

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Abstract

Current T cell-based immunotherapy strategies, including immune checkpoint blockade (ICB) and chimeric antigen receptor (CAR) T cells, have revolutionized cancer care. However, many patients with cancer who are treated with these approaches fail to respond or do not achieve durable protection against disease relapse, highlighting the need for further optimization of such strategies. The advent of cancer immunotherapy has ushered in an era of research centred on immune oncology with a specific focus on defining T cell-intrinsic mechanisms that delineate therapeutic responders and non-responders. Among the major barriers limiting immunotherapy efficacy, T cell exhaustion — which is characterized by repression of the effector functions and proliferative potential of T cells — has emerged as a common mechanism among various cancers. Here, we review transcriptional and epigenetic mechanisms that control T cell exhaustion. We discuss how T cell subset-specific gene regulatory programmes limit immunotherapy success and theorize on the development of next-generation strategies for increasing the clinical breadth, efficacy and durability of T cell immunotherapy.

Sections

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Introduction

The past decade has witnessed the translation of fundamental principles of T cell biology into cancer therapies such as immune checkpoint blockade (ICB), adoptive cell therapy (ACT) and cancer vaccination for the treatment of relapsed or refractory haematological malignancies and solid tumours[1]. Immune-oncology efforts over the past few decades have demonstrated that T cells survey for and recognize tumours as foreign cells that need to be eliminated, but the antitumour effector response can be prematurely terminated. Discovery of the inhibitory signals contributing to the premature termination of the effector T cell response eventually led to the development and US Food and Drug Administration (FDA) approval of ipilimumab in 2011 as the first ICB therapy targeting cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) for the treatment of metastatic melanoma, and shortly after, inhibitors for programmed cell death protein 1 (PD-1) were developed and approved for patients with metastatic melanoma[2,3]. Such ICB therapies have now received FDA approval for the treatment of various tumours, including non-small-cell lung cancer, urothelial carcinoma and renal cell carcinoma[4].

Juxtaposed to strategies that leverage an existing endogenous antitumour T cell response, chimeric antigen receptor (CAR) T cell therapy has been developed on the basis of the rationale that tumour specificity can be engineered into functional T cells. Following the approval of CD19-directed CAR T cells for the treatment of B cell acute lymphoblastic leukaemia (B-ALL), B cell maturation antigen (BCMA)-targeted CAR T cell therapy received FDA approval in 2019 for treatment of individuals with multiple myeloma[5], facilitating the development of further T cell engineering strategies for targeting cancer and autoimmune diseases.

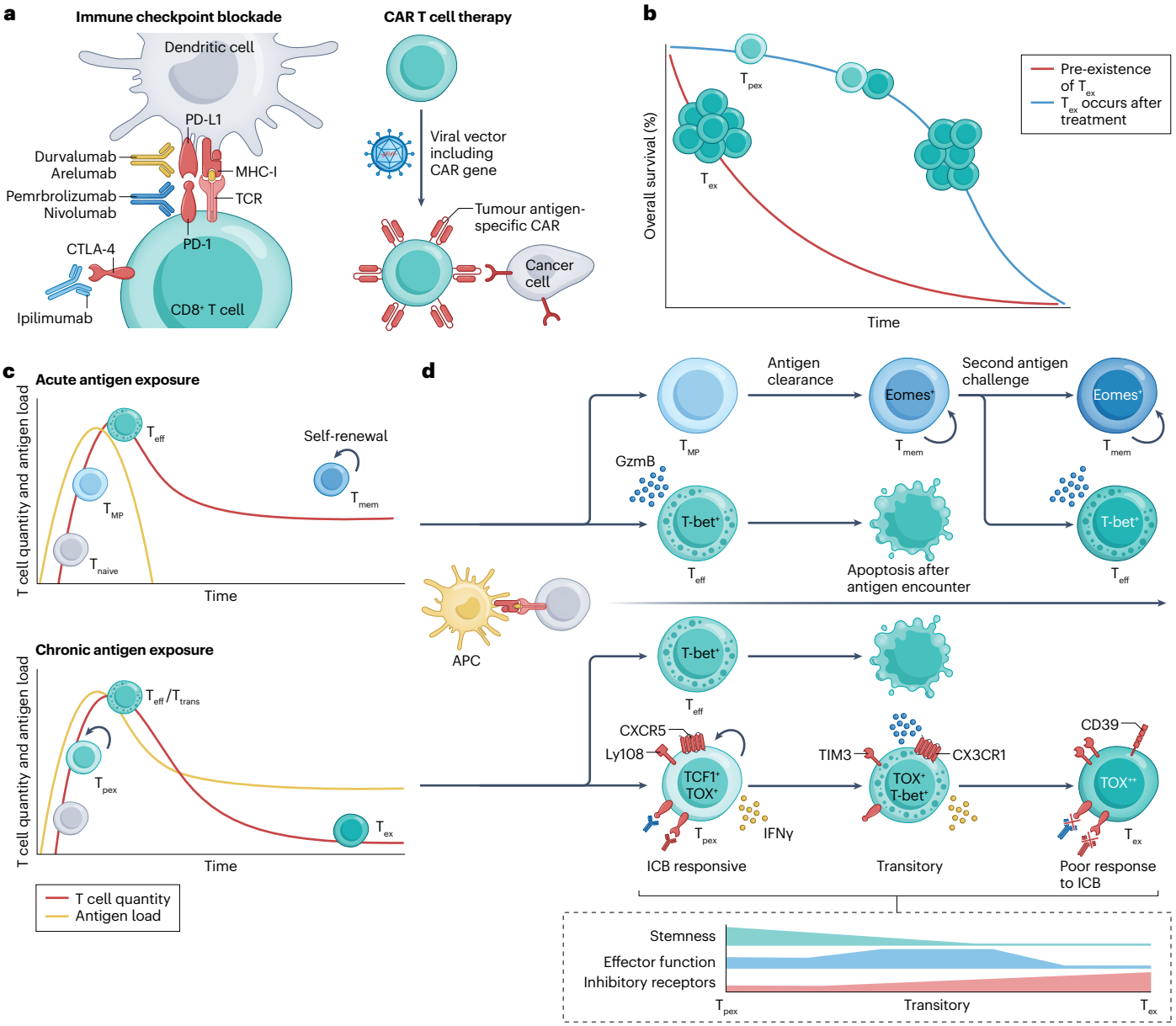
Although the above-described development of ICB and CAR T therapy for cancer treatment highlights the curative potential of T cell-mediated immunotherapy, a substantial number of patients with cancer have not responded to either strategy (Fig. 1a), necessitating improvement of lasting treatment efficacy. To enhance current immunotherapy approaches, mechanisms limiting the breadth and durability of antitumour T cells are being investigated through the lens of T cell differentiation.

The total repertoire of $CD8^+$ T cells available for immunotherapy in any individual is in part dictated by the adaptations that each cell has undergone starting from their naive state. When a naive T cell encounters its cognate antigen, it undergoes a wide range of developmental changes during effector differentiation that are tailored to the strength and duration of the antigenic stimulus (Fig. 1b). Investigating these adaptations in the context of acute antigen resolution versus persistence has revealed striking developmental divergences among the responding T cells[6,7]. Under optimal conditions, a subset of T cells that acutely encounter their cognate antigen retain the potential to differentiate into a long-lived memory population, termed memory T cells, that can persist in the absence of antigen. This ultimately provides immunological protection to the host by maintaining a population of T cells poised to mount a rapid recall response after re-encountering the antigen[8] (Fig. 1c). However, if the source of antigen persists, the $CD8^+$ T cells will continue to adapt to the chronic stimulation and differentiate into a state that limits the cell's proliferative capacity and ability to recall effector cytokines, termed T cell exhaustion[9–11] (Fig. 1b,c). Exhausted $CD8^+$ T cells have been found in various mouse and human settings that involve the chronic presence of antigen, including viral infections and cancer[12–14]. This terminally differentiated state is thought to limit the efficacy to the above-described T cell therapies[9,12,15–18]. Importantly, recent studies have revealed that T cells destined to become exhausted, but not yet terminally differentiated, can retain hallmarks of a multipotent cellular state[19,20] (Fig. 1c). Such progenitor exhausted T (T_{pex}) cells are capable of self-renewal and also retain the capacity to transiently differentiate into functional effector-like transitory T cells[21–23]. A distinct differentiation pathway has also been reported, leading to a terminal effector T cell subset expressing natural killer (NK) cell-related markers coupled with effector functions discussed in further detail elsewhere[22,24,25]. Documentation of these

Fig. 1 | T cell exhaustion limits clinical response to immunotherapy. **a**, Immune checkpoint blockade (ICB) transiently enhances T cell function within the tumour microenvironment by blocking inhibitory receptors such as programmed cell death protein 1 (PD-1) or cytotoxic T lymphocyte-associated protein 4 (CTLA-4) on T cells. Chimeric antigen receptor (CAR) T cell therapy genetically modifies T cells to possess a CAR that recognizes tumour-specific antigens, enabling the T cells to specifically attack cancer cells in patients. Although T cell-based immunotherapy clinical results have demonstrated an improvement in overall survival for several patient populations compared to conventional therapies, the development of T cell exhaustion driven by both intrinsic and extrinsic mechanisms during cancer development limits the durability of clinical response and remains a major barrier to long-term T cell responses. **b**, This conceptual curve describes how the timing of T cell exhaustion could influence the survival outcomes for patients with cancer. On the basis of current understandings from clinical and preclinical studies[54,55,59,60], if T cell exhaustion occurs before treatment with ICB therapy or the generation of CAR T cells, the overall survival will decrease. However, if exhaustion is acquired among the therapeutic population of T cells after treatment with ICB therapy or the generation of CAR T cells, the overall survival will be extended. This acquisition of T cell exhaustion relative to the time of intervention necessitates two general approaches for improving immunotherapy outcomes: 'reversal' of pre-existing programmes and 'prevention' of post-therapy programmes during extended T cell activation. **c**, This schematic depicts T cell expansion and differentiation in settings of acute versus chronic antigen exposure. Under chronic antigen stimulation conditions, such as in the tumour microenvironment, T cells progressively lose effector functions and acquire differentiated exhaustion states, resulting in limited long-term antitumour effect. This figure highlights how chronic antigen exposure leads to a hierarchical differentiation pathway characterized by the loss of proliferative potential and function compared to acute antigen exposure environment. **d**, Phenotypic and functional hallmarks demarcate distinct developmental stages along memory and exhaustion T cell differentiation trajectories during an immune response to an acute versus chronic antigen. During acute antigen exposure, naive T (T_{naive}) cells differentiate into either effector T (T_{eff}) cells or memory precursor T (T_{MP}) cells. T_{eff} cells have the capacity to directly kill and eliminate pathogens or infected cells through production of cytolytic molecules, including Granzyme B (GzmB). After antigen clearance, T_{eff} cells die and T_{MP} cells differentiate into memory T (T_{mem}) cells. T_{mem} cells undergo self-renewal and survey the host for their cognate antigen. Antigenic re-encounter promotes heightened differentiation of T_{mem} cells into either T_{eff} or T_{mem} cells. During chronic antigen exposure, T_{naive} cells can differentiate into either T_{eff} cells or precursors of exhausted T cell (progenitor exhausted T (T_{pex}) cells). T_{pex} cells, which express the transcription factor TCF1, can self-renew like T_{mem} cells and exhibit hyper-responsiveness to ICB. After ICB or further differentiation, T_{pex} cells develop into a transitory T (T_{trans}) cell subset that has effector functions that facilitate disease control. Continuous antigenic stimulation of T_{pex} cells promotes their development into exhausted (T_{ex}) cells characterized by a loss of effector function, increased inhibitory receptor expression, and retained expression of the transcription factor TOX. APC, antigen-presenting cell; TCR, T cell receptor.

discrete stages of the adaptive T cell response has lent new insight into the therapeutically efficacious populations of T cells that enable effector functions during chronic antigen exposure and long-term immunity in settings of tumour remission. Although the concept of 'reversing' T cell exhaustion has often been used in describing the clinical response, it is now clear that the efficacy of such therapies does not arise from reversing the developmental state of individual T cells, but rather engaging T cells that are already in a less-differentiated state[26,27]. To achieve true reversal of terminal exhaustion, it is probable that the epigenetic programmes discussed further below will need to be therapeutically erased to revert the T cell back into a more plastic stage of differentiation.

In this Review, we discuss mechanisms that contribute to failed antitumour T cell responses and specifically address the role of T cell exhaustion (Fig. 1). We contextualize the T cell differentiation process described above in settings of cancer immunotherapy and discuss how such T cell fate commitment processes may delineate therapeutic responders from non-responders (Fig. 1a). Next, we dissect epigenetic mechanisms involved in T cell fate commitment and elaborate on specific epigenetic modifications utilized in preserving cell type-specific gene expressions that establish therapeutically unresponsive T cells. Lastly, we discuss engineering efforts that address the distinct hurdles of having T cell exhaustion exist before therapeutic intervention versus exhaustion that arises during or after therapy (Fig. 1a). With a focus on the future of immunotherapy, we conclude with examples wherein the biology of T cell exhaustion has been leveraged for development of next-generation strategies that further deliver on the promise of immunotherapy. Although this Review focuses on the epigenetic mechanisms governing T cell fate commitment, there are other important regulatory mechanisms for T cell development, such as metabolic reprogramming or transcription factor-mediated regulations. These aspects have been comprehensively covered in



other recent reviews, including discussion on the interplay between metabolite and epigenetic regulations[28,29], as well as broader regulatory networks influencing T cell differentiation[30].

Discovery and implications of T cell exhaustion

The suppressive tumour microenvironment can be filled with various immune cells that actively limit T cell effector functions, raising the question of whether the dysfunction of tumour-associated T cells could simply be explained by these T cell-extrinsic mechanisms, or whether the effector T cells themselves have indeed lost their functionality owing to an intrinsic exhaustion. To address this mechanistic question, investigators leveraged the molecular hallmarks of T cell exhaustion, historically derived from the study of chronic viral infection, towards their study of tumour progression and immunity. The term 'T cell exhaustion' was first coined while describing a virus-specific $CD8^+$ T cell population that lost the ability to recall effector cytokines in the setting of chronic lymphocytic choriomeningitis virus (LCMV) infection in mice[13,31,32]. This led to the conclusion that, rather than simply dying, T cells could continue to adapt to the chronic source of antigen by dampening the effector response (Fig. 1c). Specifically, antigen-specific exhausted $CD8^+$ T cells underwent a duration-associated hierarchical reduction in their effector cytokine production, with a loss of interleukin 2 (IL-2), then tumour necrosis factor (TNF) and, lastly, interferon γ (IFN-γ)[9,12,33,34]. Extending this mouse-based observation to human conditions, it was further confirmed that chronic viral infections, such as infection with HIV or hepatitis C virus (HCV)[15–17], result in exhaustion of anti-viral T cells, establishing this T cell-intrinsic mechanism for suppression of the effector response as a generalizable adaptation towards a chronic source of antigen. However, many fundamental questions remained about the developmental origin and kinetics of T cell exhaustion and whether the same molecular mechanisms contributed to the decline in tumour-specific T cell effector functions.

To broadly identify changes associated with chronic T cell stimulation, transcriptional profiling studies were initially performed using a virus-specific T cell population that underwent effector and memory differentiation under conditions of acute and chronic infection. These formative studies have revealed changes in gene expression among exhausted T cells, including downregulation of glycolysis and the citric acid cycle, arrest of cell cycle progression at the G0 or G1 phase, and altered transcriptional regulation of transcription factors[7,35]. From these initial gene expression profiling studies, it was observed that T cells experiencing prolonged antigen exposure also maintained elevated expression of inhibitory receptors such as PD-1, T cell immunoglobulin and mucin domain-containing protein 3 (TIM-3), T cell immunoreceptor with Ig and ITIM domains (TIGIT), and lymphocyte activation gene 3 (LAG-3)[36,37]. Investigation into the role inhibitory receptors have in the attenuation of the effector functions and proliferative capacity of T cells has revealed that they blunt T cell receptor (TCR) signalling and indeed limit the effector response of the T cells[7,14,36,38–41]. Given their causal role in limiting T cell effector function, it was tempting to use the expression of these molecules as a 'hallmark' of T cell exhaustion. However, because acutely stimulated T cells also express several of these inhibitory receptors during their transient activation, the field realized that 'exhausted' T cells could not be readily delineated from acutely activated T cells based solely on the expression of these molecules[7]. Therefore, additional phenotypic features and a deeper understanding of the molecular drivers of T cell exhaustion were needed. Insights into the molecular mechanism of exhaustion came from studies that showed that exhausted T cells maintained an imprinted dysfunctional state even when they were removed from their source of antigen[42,43]. Notably, removing exhausted T cells from antigen exposure resulted in a downregulation of the inhibitory receptors, emphasizing the limited ability for these receptors to be used as the definition of exhaustion[42,43]. Although these T cells exhibited downregulated inhibitory receptors, they maintained a heightened ability to re-express higher PD-1 when exposed to their cognate antigen, suggesting that exhausted T cells are fate-committed after prolonged TCR stimulation[42,43]. These collective studies have led to a general reliance on gene expression profiles that go beyond simply looking at phenotypic surface markers as a tool to determine if T cells have undergone exhaustion (Box 1).

Concurrently, tumour immunologists began to apply these molecular definitions to their study of tumour-infiltrating $CD8^+$ T cells. Initially, using cells isolated from patients with melanoma, melanoma-infiltrating $CD8^+$ T cells expressing PD-1, LAG3 and TIM-3 exhibited oligoclonal expansion and were enriched for clonotypes specifically reactive to autologous tumour cells[44]. Subsequent studies have further demonstrated that circulating PD-1^+ T cells in gastrointestinal cancers were also enriched for neoantigen-specific T cells[45]. Further investigation using single-cell RNA sequencing has revealed that more than one-third of the tumour-infiltrating $CD8^+$ T cells possessed an exhaustion-related gene signature[46]. Similarly, analysis of tumour-infiltrating $CD8^+$ T cells from patients with melanoma has revealed that the T cells within the tumour exhibited a robust exhaustion phenotype, marked by reduced cytokine production and increased expression of inhibitory receptors such as PD-1, CTLA-4 and TIM-3. Collectively, enrichment of exhaustion-associated hallmarks among $CD8^+$ tumour-infiltrating lymphocytes (TILs) suggested that $CD8^+$ T cells responding to tumour antigens underwent differentiation programmes that paralleled those observed with T cells responding to chronic viral infections[12].

To explore the developmental overlap between tumour versus viral-induced T cell exhaustion, longitudinal studies of tumour-specific T cells were performed using mouse tumour models. For instance, using a mouse liver cancer model, it was revealed that naive tumour-specific T cells rapidly become dysfunctional after exposure to their cognate antigen, acquiring features similar to T cells found in late-stage tumours, including increased inhibitory receptor expression and reduced cytokine production. Importantly, these dysfunctional properties coincided with the tumour-specific T cells acquiring exhaustion gene expression programmes during the early formation of the tumour[47]. Another study has demonstrated that tumour-specific T cells undergo a stepwise process of epigenetic reprogramming that defines their dysfunctional exhausted state within the tumour microenvironment. Early dysfunctional T cells contained accessible chromatin at effector gene loci such as *Ifng*, allowing potential functional rescue. However, these T cells gradually acquired a more repressive chromatin landscape associated with irreversible functional exhaustion[48]. Notably, investigation by assay for transposase-accessible chromatin using sequencing (ATACseq) has identified a distinct region in the chromatin near the *Pdcd1* locus that was specifically accessible in exhausted tumour-specific and virus-specific T cells, relative to functional memory and effector T cells generated in response to an acute viral infection[26,48]. These studies have established that substantial transcriptional and epigenetic features associated with canonical exhaustion are coupled to the T cell dysfunction that occurs during the early formation of a tumour[49]. Moreover, these findings reinforced the idea that exhaustion can help to restrain and limit hyper-immune responses. Additionally, the terminally differentiated state of tumour-reactive

Box 1 | Metrics for defining T cell exhaustion

T cell exhaustion represents a major barrier limiting the efficacy of many anticancer cellular therapies. Efforts to prevent or revert T cell exhaustion require analytical approaches for measuring this developmental state. Here, we provide phenotypic, functional, transcriptional and epigenetic metrics that can be used to delineate T cell exhaustion from other T cell differentiation states.

1. Phenotype
Chronically stimulated T cells express multiple inhibitory receptors, such as programmed cell death protein 1 (PD-1), lymphocyte activation gene 3 (LAG-3), T cell immunoglobulin and mucin domain-containing protein 3 (TIM-3), cytotoxic T lymphocyte-associated protein 4 (CTLA-4), and T cell immunoreceptor with Ig and ITIM domains (TIGIT). However, this phenotype can sometimes overlap with recently activated effector T cells that are in the process of responding to an acute antigenic challenge[7,37]. Inhibitory receptor cell surface expression can be used as an indication of T cell exhaustion but should be used in conjunction with other metrics if feasible.

2. Function
T cells experiencing sustained antigenic challenge progressively lose the ability to proliferate in response to their cognate antigen, as well as lose the ability to express the effector cytokines interleukin 2 (IL-2), tumour necrosis factor (TNF) and interferon γ (IFN-γ) and undergo degranulation[9,12,13,152,153]. The loss of effector cytokine production occurs progressively, with some cells retaining residual protective effector functions[49,154,155]. Functional assessment of a T cell's loss of effector potential is a direct measurement of exhaustion.

3. Transcription
Increased inhibitory receptor transcript production (associated with cell surface protein expression, as mentioned in point 1) is part of a broader collection of gene expression programmes that make up a unique molecular signature for exhausted T cells[7]. Gene expression profiles established from bona fide mouse and human exhausted T cells can be used as a comparator for gene set enrichment analyses (GSEA)[21,55,56,156], to assess experimentally or clinically derived T cell populations for features of exhaustion.

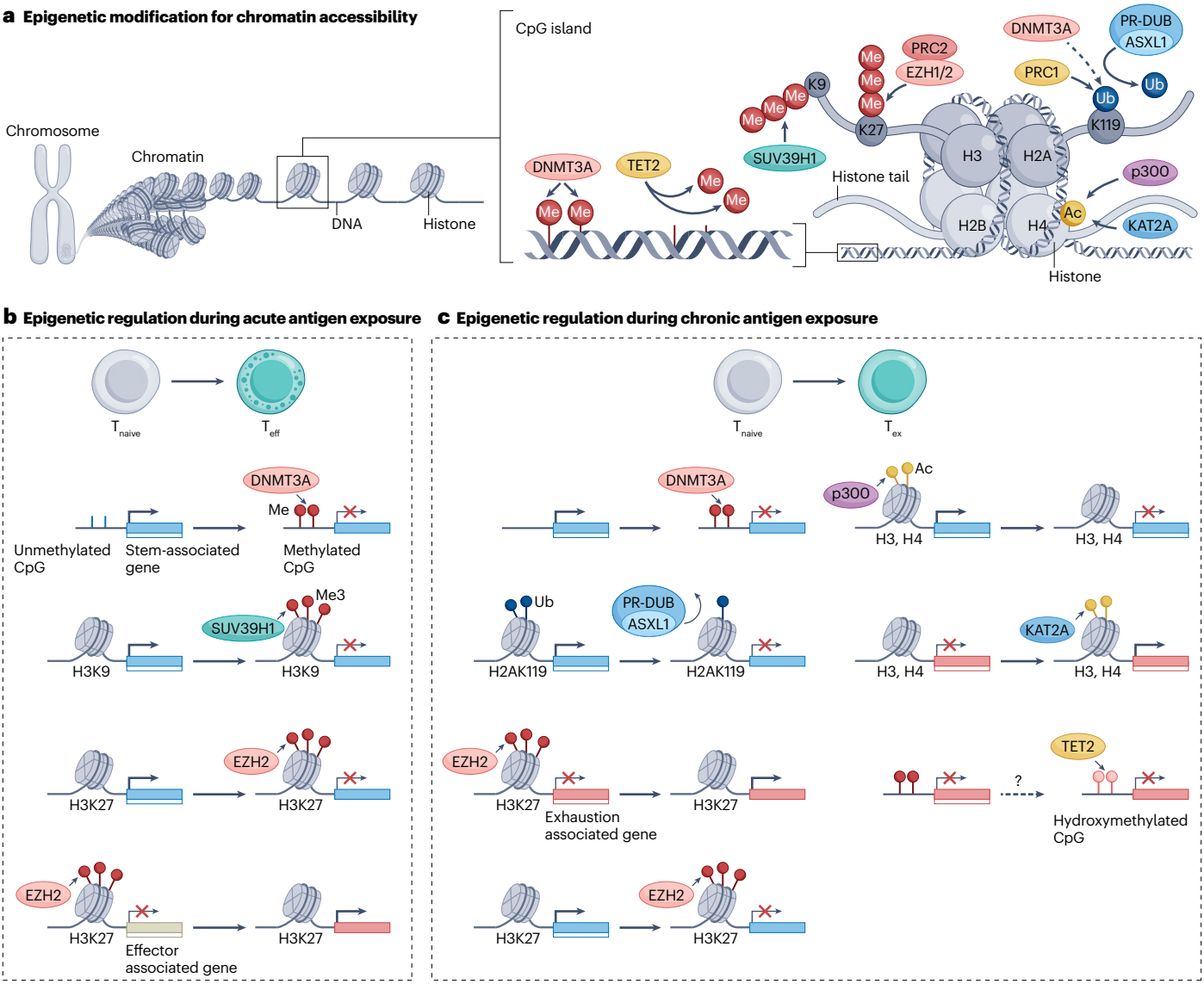
4. Epigenetic landscape
Terminally exhausted T cells can retain a memory for their suppressed effector potential in the absence of continued antigenic stimulation. The preserved memory for terminal exhaustion has been linked to discrete epigenetic modifications that can be maintained for long periods of time and during cell division[27,86]. Epigenetic modifications that are transmissible during cell division and associated with T cell exhaustion include DNA methylation[92]. Similar to gene expression profiles described in point 3, epigenetic profiles established from bona fide mouse and human exhausted T cells can be used as a comparator for gene set enrichment analyses[26,27,48,55,92,156,157]. Notably, DNA methylation profiles have been used to develop a phenotype-agnostic prediction tool that provides a normalized quantitative assessment of the developmental potential of a T cell. As additional epigenetic modifications are incorporated into the T cell multipotency index prediction tool, finer resolution of different T cell exhaustion states may be quantifiable in the future[124].

T cells provided context for the limitations of T cell-based immunotherapies and provided a rationale for investigating the events that lead to terminal T cell exhaustion.

Origin and maintenance of TIL exhaustion

Longitudinal characterization of antigen-specific T cells responding to chronic viral infections, including LCMV infection in mice[7,37] and cross-sectional investigation of antigen-specific T cells during HIV or HCV infection in humans[15–17], has revealed that T cells accumulated various inhibitory receptors on their cell surface with extended duration of the infection (Fig. 2). In particular, there was a notable distinction between T cells that expressed just PD-1 versus T cells that co-expressed PD-1 and TIM-3 (ref. 39); the latter were also enriched with functional and transcriptional hallmarks of the terminally exhausted state. This heterogeneity raised the possibility of a lineage relationship between the different phenotypic subsets. Investigation into this has revealed that T cells destined to become exhausted retained hallmarks of a multipotent state at the early stages of a chronic infection[19,20]. Notably, this subset of cells, now described as T_{pex} cells, possessed a gene signature similar to the less-differentiated $CD4^+$ follicular helper T (T_{fh}) cells, as well as the ability to establish a pool of long-lived memory T cells[50]. The phenotypic and transcriptional similarity between the two subsets led to the investigation of T_{pex} cells as the reservoir of cells that give rise to functional transitory T cells during chronic antigen exposure[19,20] (Fig. 1c). To test this hypothesis, adoptive transfer experiments into chronically infected mice were performed using a subset of antigen-specific T cells enriched for C-X-C chemokine ligand receptor 5 $(CXCR5)^+$ expression. This revealed that the $CXCR5^+$ T cell subset retained its proliferative capacity in a chronic antigen environment, enabling the cells to continue to undergo robust proliferation after PD-1 blockade, whereas the $CXCR5^-$ exhausted T cells exhibited limited proliferation in response to PD-1 blockade[21,22]. Importantly, this T cell subset was reported to retain expression of the stem-associated transcription factor T cell factor 1 (TCF1), which was subsequently identified as a transcription factor controlling the generation and maintenance of T_{pex} cells[20,51]. This further demonstrated that the T_{pex} population of T cells served as the cellular origin of exhaustion (Fig. 1).

Work over the past decade has revealed that the presence of a stem-like or T_{pex} cell subset is a critical determinant for the success of T cell-based immunotherapy. T_{pex} cells replenish the pool of functional effector T cells, which in turn can differentiate into terminally exhausted cells in the tumour microenvironment[23,30,52]. In mouse tumour systems, the tumour niche can be made up of a heterogeneous $CD8^+$ T cell pool that includes polyfunctional T_{pex} cell subpopulations that can respond to anti-PD-1 therapy by giving rise to effector T cells that combat tumour growth[49,53] (Fig. 1b,c). The synergy between T_{pex} cells and ICB was compromised in the absence of TCF1, suggesting that expression of TCF1 was required for maintenance of T_{pex} cells and efficient ICB immunotherapy[54]. Consistent with the critical role of T cell stemness in cancer, high-dimensional single-cell RNA sequencing



analysis of CD8$^+$ T cells from human lung cancer or melanoma samples has revealed that the stem-like T cells consistently exhibited gene networks associated with better clinical outcome[55,56]. Also, CD8$^+$ T cells within kidney tumours consist of distinct populations of stem-like and terminally differentiated T cells[23]. Importantly, stem-like CD8$^+$ T cells within kidney tumours reside in antigen-presenting cell niches and sustain antitumour immunity by differentiating into effector T cells, maintaining their presence and microenvironment for enhanced antitumour immunity[23,57]. In addition, it was reported that CD8$^+$ T cells isolated from patients with human papillomavirus (HPV)-positive head and neck cancer contained gene expression profiles that segregated them into three transcriptionally distinct subsets, including *Tcf7*-expressing PD-1$^+$ T_{pex} cell populations, transitory populations with effector features, and populations exhibiting enriched terminally differentiated gene signatures. Isolation and in vitro culture of the HPV-specific TCF1$^+$ T_{pex} cell populations demonstrated that they can proliferate and differentiate into more effector-like cells after stimulation with an HPV peptide, whereas the more terminally differentiated exhausted T cells did not exhibit an effector response[58]. High-dimensional analysis of human ACT products from patients with melanoma revealed that the CD39$^-$ T_{pex} cell-like subset was coupled to tumour regression, whereas enrichment of the terminally differentiated exhausted CD39$^+$ T cell subset was found to be associated with poor clinical response[59]. In addition to the higher proportion of T_{pex} cells among TILs, several studies have suggested that the anatomical distribution of T_{pex} cells in lymphoid tissue and tertiary lymphoid structures serves as a self-renewing reservoir. This results in the capacity to replenish effector populations in the tumour microenvironment and, thereby, enhances the efficacy of immunotherapy[60–64]. Collectively, this work has made it evident that a large portion of T_{pex} cells among CD8$^+$ T cells is crucial for efficient and successful T cell-based immunotherapy, as this supplies effector T cells to the tumour microenvironment.

Immunological memory for tumour antigens

ICB and CAR T cell therapy can establish tumour remission in patients that lasts years and even decades after treatment[65]. As an example,

Fig. 2 | Epigenetic imprinting of CD8$^+$ T cell exhaustion during tumour immunotherapy. a, Nucleosomal chromatin forms by DNA wrapping around a histone octamer. Outlined here are modifications to nucleosomal DNA in T cells that impact their differentiation by epigenetic regulators. This includes DNA methyltransferase 3A (DNMT3A) methylation of CpG dinucleotides, TET2-mediated hydroxylation of methylated CpG sites, SUV39H1-induced tri-methylation at lysine 9 (K9) of histone H3, tri-methylation at K27 by the polycomb repressive complex 2 (PRC2) complex in concert with EZH1 or EZH2, ubiquitination at K119 of histone H2A (H2AK119Ub) by the PRC1 complex, and ubiquitin removal at K119 of histone H2A by the polycomb repressive complex PR-DUB. In stem cells, it was reported that DNMT3A can bind H2AK119Ub[110] (dashed arrow). Several studies have reported that DNMT3A-mediated DNA methylation[92] or additional sex combs-like 1 (ASXL1)-mediated H2AK119Ub modifications[96] have a causal role in the development of T cell exhaustion. **b**, During acute antigen exposure, DNMT3A methylates stem-associated loci and provides an epigenetic programme to limit stem-associated gene transcription, skewing differentiation towards an effector fate. SUV39H1 induces histone 3 lysine 9 trimethylation (H3K9me3) at stem-associated loci, resulting in inhibition of stem-associated gene transcription and promoting effector T (T_{eff}) cell differentiation. EZH2 was reported as having two different mechanisms. EZH2-mediated H3K27me3 at stem-associated loci inhibits gene transcription and promotes effector T cell differentiation. Additionally, EZH2 can establish H3K27me3 at effector-associated loci, resulting in inhibition of gene transcription and promoting memory T cell differentiation. **c**, During chronic antigen exposure, DNMT3A can methylate stem-associated loci, resulting in inhibition of stem-associated gene transcription and promoting exhausted T (T_{ex}) cell differentiation. ASXL1 can eliminate H2AK119Ub as a part of the PR-DUB complex at stem-associated gene locus, resulting in inhibition of stem-associated gene transcription and promoting exhausted T cell differentiation. Similar to acute antigen exposure, EZH2 regulates T cell differentiation through two distinct mechanisms during chronic antigen exposure. EZH2 can generate H3K27me3 at exhaustion-associated loci, resulting in inhibition of exhaustion-associated gene transcription and promoting stem-like T cell differentiation. In addition, EZH2-mediated H3K27me3 at stem-associated gene loci inhibits the stem-associated gene transcription and promotes exhausted T cell differentiation. p300 establishes histone acetylation at stem-associated loci, activating transcription and supporting maintenance of progenitor exhausted T (T_{pex}) cells. Lysine acetyltransferase 2A (KAT2A) establishes histone acetylation at exhaustion-associated loci, activating gene transcription and promoting T cell exhaustion. TET2 may be involved in T cell exhaustion through modification of methylated DNA into hydroxymethylated DNA. Each red 'x' (in panel **b**) indicates inhibition of gene transcription.

durable remission among ICB-treated patients with melanoma has persisted in some cases for more than a decade, and this tumour control was coupled to the maintenance of tumour-specific memory T cells[66]. Such durable remission is not unique to melanoma as some patients with lung cancer have experienced extended tumour control after therapy, which was coupled to the generation and maintenance of tumour-specific CD8$^+$ T cells in circulation for over 3 years after therapy[67]. These clinical observations provide compelling evidence that immunological memory can be established after therapy-induced tumour control and presumed clearance of the cognate antigen of T cells. This antigen-free stage of an immune response raises questions regarding the cellular origin of the memory T cells protecting against remission and, specifically, whether or not stem-like T_{pex} cells, which function in the presence of persisting antigen, also serve to establish a self-renewing antigen-independent population of T cells.

Defining T cell memory for human tumour antigens is often challenged by the limited ability to define the epitope specificity of T cells, which, if known, would allow classical assessment of the functional responses of T cells to antigenic restimulation, such as cytokine production and proliferative capacity, long after tumour clearance. In lieu of an ability to assess tumour-specific memory T cell functions, investigators often turn to surrogate phenotypic markers such as CD39, CD127 or CD62L[68,69]. Although widely used, these phenotypic markers often fail to capture the history of a T cell and its future potential. Thus, expression of memory-associated markers alone should be used judiciously, and when possible, antigen-specific T cell recall responses should be assessed to determine whether a T cell is truly imprinted with a heightened effector response[70]. Initial investigation into the lineage relationship of memory T cells and T_{pex} cells using preclinical tumour models has revealed that a subset of tumour-specific CD8$^+$ T cells in tumour-draining lymph nodes was not functionally exhausted and displayed traditional memory T cell features, including memory-associated epigenetic programmes[68,71]. These studies further demonstrated that the memory-like tumour-specific T cells gave rise to the T_{pex} cell subset that subsequently further differentiated into terminally exhausted T cells within the tumour microenvironment. A similar memory-like T cell subset was also detected in hepatic lymph nodes from patients with liver cancer, suggesting that a memory-like subset of T cells could indeed give rise to the therapeutically efficacious T_{pex} cells and, thus, may indeed have a causal role in durable disease remission. However, more recent studies now suggest that there are important distinctions between memory-like T cells and T_{pex} cells. Single-cell RNA sequencing of samples from mouse models of non-small-cell lung cancer has revealed that tumour-specific T cells exhibited clonal expansion; however, they also exhibited limited expression of genes regarding conventional effector or exhausted T cells. Additionally, this T cell differentiation programme was established at early time points in the lymph node[72]. It has also been reported that 'inefficient' priming of T cells against tumours leads to a T cell phenotype that is similar to peripheral tolerant CD8$^+$ T cells. However, the differentiation trajectory of these cells appears to be distinct from exhausted, memory and effector T cell subsets. These tolerant T cells diverged early and progressively from effector populations, adopting a transcriptionally and epigenetically distinct state, separated from both effector and exhausted T cell populations, within 60 h of antigen encounter[73]. These data suggest that insufficient tumour-specific T cell priming in tumour-draining lymph nodes could limit the response of CD8$^+$ T cells to ICB therapy.

More recently, studies investigating the origin of T_{pex} cells have found that they can be generated early during an acute infection and develop in parallel with conventional memory T cells, subsequently persisting long after antigen clearance[74,75]. Compared to conventional memory populations, T_{pex} cells generated during the early phase of an acute infection exhibited higher expression of TOX, a transcription factor critical for maintaining exhausted T cells[76,77]. These cells also had elevated levels of PD-1 and acquired a chromatin landscape similar to those T cells generated in response to a chronic infection. These studies have demonstrated that T_{pex} cells can be generated early during an immune response, and they can persist in both chronic and antigen-free settings. This information also raises the question of whether or not terminally exhausted T cells are capable of developing into functional memory T cells in an antigen-free environment. Several recent studies have provided insight into this important question. When T cells with an exhaustion phenotype are taken from mice

infected with LCMV clone 13 (CL13) and adoptively transferred into mice immunized with the acute strain of LCMV, a subset of the adoptively transferred T cells recovered both phenotypic and transcriptional features of memory T cells, including CD127 and TCF1 expression. However, despite exhibiting some phenotypic features of memory T cells, the cells remained functionally compromised in terms of reduced expansion and cytotoxicity relative to memory T cells generated in an acutely infected mouse[78]. Other studies examining human T cells that persisted long after resolution of an HCV infection have revealed that T cells exhibited phenotypic changes resembling memory-like T cells that have increased CD127 and TCF1 memory markers. However, the HCV-specific T cells exhibited limited effector cytokine production, indicating that an exhaustion-associated repression of effector functions was imprinted. Despite the successful restoration of HCV by antiviral treatment, HCV-specific T cells retain exhaustion-associated chromatin accessibility at enhancer elements near key genes such as *TOX* and *HIF1A*[79,80]. This specific example provides important insight into the durability of exhaustion-associated programmes in humans and highlights a contradiction between phenotype and function that serves as a cautionary note for studies that rely primarily on phenotypic definitions for classifying stages of memory T cell differentiation (Box 1). This highlights the role of exhaustion-associated epigenetic imprinting in inhibiting T cell reinvigoration potential in cancer.

Further linking long-lived tumour immunity with the maintenance of a stem-like T cell subset, recent engineering efforts have attempted to recapitulate T cell-mediated durable remission by disrupting genes known to restrict T cell multipotency in the tumour microenvironment[81–83]. Importantly, these gene-disruption studies (both engineered disruptions and naturally occurring disruptions) established that a long-lived quiescent pool of memory T cells could be established through genetic modification of T cells. It was recently reported that disruption of ten-eleven translocation 2 (TET2) in CD19 CAR T cells results in the preservation of memory potential[84]. Consistent with this, other groups have reported that TET2 disruption in $CD8^+$ T cells responding to a chronic viral infection preserves the ability of T cells to establish a long-lived pool of memory-like T cells that maintain their effector potential without uncontrolled effector differentiation[82,85]. Similarly, in tumour-bearing mice treated with DNA methyltransferase 3A (DNMT3A)-knockout CD19 CAR T cells, the therapy led to a long-lived pool of memory T cells that repressed their effector response after tumour clearance. When the tumour-free mice were rechallenged with $CD19^+$ tumour cells, the DNMT3A-knockout memory CAR T cells exhibited heightened tumour control compared to treatment with the wild-type (WT) CAR T cells[83]. These engineering efforts broadly document the feasibility of modifying T cells so that they maintain their memory potential without compromising the effector response needed to control tumours and maintain protection against disease relapse. Thus, persistent antitumour immunity can be rationally engineered by modifying the mechanisms that reinforce T cell differentiation.

Epigenetic checkpoints limiting tumour immunotherapy

Although the term exhaustion may evoke imagery of marathon runners at the end of a race, this analogy could give the impression that our exhausted T cells just need a little rest before their next challenge. Unfortunately, however, terminally exhausted T cells in an antigen-free environment will not revert key hallmarks of the exhausted state[42,86]. These original fate commitment observations described in the prior sections served as an impetus to define mechanisms that reinforce this differentiation state. Epigenetic modifications can serve as a mechanism to reinforce cell fate decisions among a dividing population of cells by propagating instructions for tissue-specific and cell-specific gene expression programmes to their cellular progeny[87] (Fig. 2). It is now well-appreciated that epigenetic mechanisms have a critical role in reinforcing the transcriptional changes associated with various $CD8^+$ T cell differentiation states and have thus emerged as promising molecular determinants that can be modulated to restore and/or augment long-lived T cell immunity against tumours. Therefore, substantial emphasis has been placed on resolving the specific epigenetic machinery and developmental kinetics for their involvement in T cell exhaustion. In this section, we review efforts to define the epigenetic readers, writers and erasures of chromatin modifications that regulate $CD8^+$ T cell fate commitment.

DNA methylation and $CD8^+$ T cell subset specification

Cytosine methylation was one of the first-described epigenetic modifications found in vivo[88], and characterization of it revealed that it was largely enriched at CpG sites among mammalian DNA. Moreover, the palindromic nature of the CpG substrate led to the idea that it could be a heritable feature in the genome of dividing cells[89,90] (Fig. 2a). It is now known that methylation of the CpG dinucleotide can result in recruitment of transcriptionally repressive methyl-binding domain proteins that block access of transcriptional machinery to gene regulatory promoters and enhancer elements[91]. CpG methylation is a key epigenetic mechanism that stably represses gene expression through the recruitment of transcriptionally repressive methyl-binding domain proteins[88,91]. The first assessment of exhaustion-associated epigenetic events in T cells was the analysis of DNA methylation changes that occur at the PD-1 (*Pdcd1* and *PDCD1*) locus in mouse and human $CD8^+$ T cells, respectively, that are responding to chronic viral infections[43,86]. Using the LCMV model of acute versus chronic infection, it was observed that the PD-1 locus underwent transient DNA demethylation in acutely infected animals; however, this demethylated state was reinforced during a chronic infection. Moreover, the demethylated state persisted even when the virus was undetectable and PD-1 cell surface protein expression was notably reduced (an indication that the T cells were no longer seeing their cognate antigen)[43,86]. To further assess the impact of DNA methylation on T cell exhaustion, a conditional knockout (cKO) for DNMT3A was utilized among activated $CD8^+$ T cells in an LCMV mouse model system. Notably, the DNMT3A cKO virus-specific $CD8^+$ T cells retained their effector functions despite chronic exposure to their cognate antigen. The retained effector potential among the cKO T cells was coupled to a lack of de novo DNA methylation at effector and stem-associated loci, including *Tcf7* and *Ifng* (Fig. 2b). Moreover, these cells retained the capacity to mount a proliferative burst during PD-L1 blockade treatment of the chronically infected mice compared to WT antigen-specific T cells. These studies have definitively established de novo epigenetic programmes as a causal mechanism for reinforcing T cell exhaustion[92]. Moreover, documentation of this epigenetic mechanism further supported the notion that T cell exhaustion is an authentic cell fate.

To more broadly assess the stability of exhaustion-associated chromatin alterations, several groups performed genome-wide chromatin accessibility profiling studies of chronically stimulated T cells in mouse and human viral systems[26,27]. Using ATACseq to profile various $CD8^+$ T cell subsets, it was observed that the exhausted T cell subsets have distinct epigenetic signatures, such as exhibiting

increased chromatin accessibility in the *Pdcd1* locus compared with effector or memory T cells. In addition, PD-1 blockade fails to recover the memory-associated recall capacity of T cells, and more broadly, fails to convert the exhausted T cell epigenetic landscape into effector or memory-associated chromatin states[26,27,48,93,94]. This epigenetic fingerprint was also detected in long-lived human memory T cells retaining an epigenetic imprint of their effector history, and it was coupled to the poised effector response of a cell after antigen rechallenge[95]. Collectively, these studies have confirmed that the fate commitment step of exhaustion could be delineated using discrete epigenetic modifications that programme for cell subset-specific chromatin profiles, providing the field with a refined molecular definition and further defining specific epigenetic regulators as potential therapeutic targets for preventing or reversing T cell exhaustion (Box 1).

Moreover, studies examining T cell dysfunction in the tumour microenvironment have incorporated these molecular features of T cell exhaustion into the analysis of T cell antitumour responses. Methylated cytosine can be demethylated through the addition of a hydroxyl group, catalysed by methyl cytosine dioxygenase TET2, which can facilitate site-specific DNA demethylation (Fig. 2a,b). Disruption of TET2 in $CD8^+$ T cells by using either conditional knockout mice having $CD8^+$ T cell-specific *Tet2* or CRISPR–Cas9-mediated *Tet2* deletion resulted in a memory-like phenotype among acutely infected mice and a stem-like phenotype in chronic viral infection settings, respectively[82,85,96] (Fig. 2b). Additional investigation of TET2-mediated regulation of T cell terminal differentiation using mouse tumour models has demonstrated that *Tet2*-knockout $CD8^+$ T cells retained robust antitumour effector functions and an ability to sustain the overall pool of effector T cells, which collectively contributed to heightened tumour regression[82]. Together with data from viral infection models, these results suggest that disruption of *Tet2* in $CD8^+$ T cells may preserve functional plasticity and prevent terminal exhaustion in both chronic infection and cancer settings. Although disruption of TET2 in $CD8^+$ T cells limits the overall DNA demethylation that occurs to enable an effector response[85,96], whether this directly prevents hydroxylation of these specific DNA methylation programmes remains to be determined.

Histone modifications and $CD8^+$ T cell subset specification

As another layer of epigenetic regulation, histone modifications have been broadly associated with cell fate-associated chromatin landscapes[97] (Fig. 2a) and therefore have recently been investigated for their role in establishing exhaustion-associated chromatin changes. Deletion of bromodomain-containing protein 4 (BRD4), a histone acetyltransferase facilitating acetylation of histone H3, in $CD8^+$ T cells responding to a mouse model of melanoma lead to a reduction of terminal effector T cells. Using a small-molecule inhibitor of bromodomain and extra-terminal domain (BET), the binding site of BRD4, resulted in an increase of $TCF1^+$ T_{pex} cells within the tumour microenvironment, ultimately contributing to enhanced tumour regression[98]. Recently, it was observed that T_{pex} cells retain a high level of histone acetylation at stem-associated loci, mediated through p300 (histone acetyltransferase)–acetyl-CoA synthetase 2 (ACSS2) pathways. It was further demonstrated that as the T_{pex} cells develop into the exhausted T cell subset, histone acetylation at these specific stem-associated loci is decreased[99]. Notably, the modification of the histone acetylation programme was specific to the stem-associated loci as the level of histone acetylation at exhaustion-associated loci remained unchanged. Ultimately, preventing histone acetylation through overexpression of ACSS2 resulted in better antitumour functions (Fig. 2b).

Beyond the more commonly described histone acetylation, many other histone post-translational modifications have been reported to impact antitumour T cell transcriptional programmes, including histone methylation. On the basis of a report of higher histone 3 lysine 9 trimethylation (H3K9me3) deposition at effector-associated gene loci in $CD8^+$ T cells from the colon tumour microenvironment[100], small molecule-mediated inhibition of H3K9me3 in $CD8^+$ T cells was shown to enhance expression of $CD8^+$ effector T cell molecules in a mouse model of colorectal cancer, resulting in colon cancer regression[100]. Similar results were also reported when SUV39H1, a histone methyltransferase that facilitates H3K9me3 (Fig. 2a), was disrupted in mouse and human $CD8^+$ T cells[101] (Fig. 2b). In the mouse B16 melanoma model, SUV39H1-disrupted $CD8^+$ T cells retained hyper-responsiveness to ICB therapy, which was coupled to chromatin accessibility at common effector-associated loci[101]. Extending these findings into human studies, it was shown that SUV39H1-knockout human CAR T cells exhibited enhanced antitumour properties in patients with leukaemia and prostate cancer, and they were able to persist for an extended time after tumour control[102]. Profiling of exhausted $CD8^+$ T cells in a mouse model of melanoma revealed increased bivalent chromatin, defined as the presence of both transcriptionally permissive (H3K4me3) and repressive (H3K27me3) histone modifications[103]. Further investigation has revealed that hypoxia, a common feature of solid tumours, drives this bivalent chromatin state by impairing histone demethylation. Overexpression of the hypoxia-insensitive histone demethylase KDM6B, which demethylates H3K27me[104], in $CD8^+$ T cells rescued the effector cytokine response and resulted in reduced tumour growth[103]. During acute LCMV infection, disruption of *Ezh2*, a component of the polycomb repressive complex 2 (PRC2) that catalyses H3K27me3 (ref. 105) (Fig. 2a), in $CD8^+$ T cells resulted in their impaired expansion. Additionally, deposition of H3K27me3 at stem-associated loci lead to the development of terminally differentiated effector T cells[106]. Notably, when *Ezh2* was knocked out in memory $CD8^+$ T cells in mice acutely infected with LCMV, it impaired recall responses after antigenic rechallenge[106]. In mouse model of melanoma, transferring *Ezh2*-knockout tumour antigen-specific $CD8^+$ T cells resulted in poor antitumour functions with a lower frequency of memory-like T cells[107] (Fig. 2b). This finding suggested that *Ezh2*-mediated epigenetic regulation is essential for maintaining memory-like features in tumour-reactive T cells, important for sustained antitumour response. In the absence of this epigenetic regulation, $CD8^+$ T cells may more easily acquire a terminally exhausted phenotype, limiting therapeutic potential. Genome-wide profiling of *Ezh2* binding sites in the various T cell subsets revealed that *Ezh2* bound to the loci of transcriptional regulators, including *Id3*, *Id2* and *Prdm1*. Mutating *Ezh2* to specifically disrupt its negative regulatory functions resulted in *Id3* activation and established a higher frequency of memory T cells with improved tumour control, suggesting that *Ezh2* could limit the formation of memory-like T cells by promoting the differentiation of T cells towards an exhausted state.

Leveraging recent insights into the role of *Ezh2* in T cell differentiation, it has been reported that halting CAR T signalling using a drug-regulatable degron system to transiently suppress CAR expression or by dasatinib treatment to inhibit CAR signalling, limited the development of exhaustion and improved antitumour function[108]. Such 'rested' CAR T cells indeed exhibited reduced exhaustion markers with a shift to a memory-like phenotype and improved cytokine function. Using H3K27me3 chromatin immunoprecipitation sequencing (ChIP-seq), it was demonstrated that rested CAR T cells maintained H3K27me3 levels at exhaustion-associated gene loci, resulting in

inhibition of differentiation into fully exhausted T cells. Additionally, during CAR T cell resting state, EZH2 activity was required for deposition of H3K27me3 at exhaustion-associated gene loci, reprogramming the CAR T cells to have enhanced antitumour functions. When EZH2 was pharmacologically inhibited using tazemetostat during the rest period, tumour killing and cytokine production of T cells were impaired, suggesting that EZH2-dependent chromatin remodelling is required to maintain function of CAR T cells during their manufacturing[108]. By contrast, it was reported that transient inhibition of EZH2 can preserve T cell stemness and improve the functional quality of T cells for adoptive T cell therapy[109]. In this work, short-term treatment with tazemetostat during in vitro T cell culture delayed T cell exhaustion and enhanced polyfunctionality and persistence without curtailing the proliferative capacity of T cells. More specifically, tazemetostat treatment reduced H3K27me3 levels at promoters of genes such as *Tcf7*, consistent with the stem-like transcriptional state of the T cell. In a mouse model of melanoma, tazemetostat-treated T cells showed enhanced antitumour efficacy, especially when combined with anti-PD-1 therapy[109]. These data collectively demonstrate that disruption of EZH2 in $CD8^+$ T cells produces distinct effects dependent on the specific microenvironment (that is, acute viral infection versus tumour microenvironment) and the differentiation state of the T cell. Although genetic deletion of *Ezh2* in naive T cells led to curtailed maintenance of memory-like characteristics in acute viral infection[106] or a mouse model of melanoma[107], its transient inhibition after activation could promote T cell stemness and enhance adoptive T cell therapeutic efficacy[109]. This highlights the need for further investigation using inducible systems to comprehensively elucidate the role of *Ezh2* at distinct stages of T cell differentiation in both infection and tumour models. Furthermore, these studies suggest that EZH2-induced H3K27me3 controls the T cell fate, which raises the possibility of disrupting or overexpressing EZH2 as a therapeutic strategy for establishing long-lived antitumour T cells. Additionally, the EZH2 inhibitor tazemetostat is already approved for epithelioid sarcoma and under clinical investigation in various solid tumours, including prostate, breast and ovarian cancers. However, whether this treatment modulates T cell differentiation in patients remains unclear. To better understand the impact of EZH2 inhibition as an immunomodulatory target, further studies will need to focus on T cell fate-commitment and therapeutic durability in the tumour microenvironment.

Investigation of DNA methylation and histone modifications are often siloed, and thus, discussion of their biology can give the impression that they operate as parallel events controlling discrete cell fate mechanisms. However, recent investigation of a novel polycomb repressive complex has linked these two mechanisms through the deposition of ubiquitin at lysine 119 histone 2A (H2AK119Ub) and the binding of DNMT3A to this histone mark[110,111] (Fig. 2a,b). Notably, H2AK119Ub is one of the most abundant histone modifications in mammalian cells[111], yet the function of H2AK119Ub in terms of gene regulation remains debated. A recent study has reported that H2AK119Ub can either promote gene transcription by H1-mediated chromatin condensation or repress gene expression by inhibiting polycomb repressive complex 1 (PRC1) in mouse embryonic stem cells[112]. Removal of H2AK119Ub is controlled by the polycomb repressive complex PR-DUB, and a core component of this complex is the additional sex combs-like 1 protein (ASXL1)[111]. Notably, ASXL1 is among the three dominant epigenetic regulators, the other two being DNMT3A and TET2, commonly mutated in haematopoietic stem cells (HSCs) providing a survival advantage and leading to the age-associated clinical manifestation of clonal haematopoiesis[113]. Given that HSCs and T cells share a core transcriptional programme[114], and that disruption of DNMT3A and TET2 in T cells preserves a stem-like state[82,92], a recent series of experiments have assessed the broader role of clonal haematopoiesis-associated regulators in controlling T cell fate commitment through the PR-DUB complex. Using the LCMV chronic infection model of T cell exhaustion, it was found that disruption of each of the clonal haematopoiesis-associated epigenetic regulators among the virus-specific T cells preserved their ICB-responsive state and established long-lived T_{pex} cells. Importantly, because chronic LCMV infection does not kill the mice, the durability of these mutated T cells could be assessed for the life of the animal, and therefore, the mutated T cells were found to be quantitatively maintained for over 1 year in the chronically infected mice. Moreover, when these year-old cells were adoptively transferred into naive animals and rechallenged with the chronic strain of LCMV, they mounted a substantial proliferative response and again entered a homeostatic state that sustained their quantity. These multi-year studies have documented the durable nature of these genetically engineered T_{pex} cells.

Deeper investigation into the mechanism linking the PR-DUB complex to T cell longevity has demonstrated that H2AK119Ub patterns among stem-like T cells were preserved when ASXL1 was disrupted in mouse and human T cells experiencing conditions that would normally lead to terminal T cell differentiation. Moreover, ASXL1-disruption in $CD8^+$ T cells was coupled to preservation of H2AK119Ub at specific regions of chromatin that remained accessible in T_{pex} cells, and maintenance of the H2AK119Ub was coupled to T_{pex} cell self-renewal in chronically LCMV infected mice[96] (Fig. 2b). Together, these investigations document a differentiation process that is collectively controlled by coordinated DNA methylation and histone modifications. Moreover, they provide a framework for therapeutic epigenetic reprogramming of T cells to prevent or reverse exhaustion and increase the durability of T cell-based immunotherapy.

Chromatin remodellers and CD8 T cell subset specification

Chromatin accessibility for transcription factor binding is controlled by a network of chromatin remodelling factors, including the switch/sucrose non-fermentable (SWI/SNF) family of regulators[115,116]. The SWI/SNF chromatin remodelling complex consists of three subfamilies, canonical BAF (cBAF), polybromo-associated BAF (PBAF), and non-canonical BAF (ncBAF)[116]. Disruption of ARID1A, a subunit of the cBAF complex, in T cells enhanced their proliferative capacity and reduced expression of exhaustion markers by regulating chromatin regions associated with T cell exhaustion. *Arid1a* deletion in primary $CD8^+$ T cells enhanced tumour control, in both mouse and human models[117]. Another study that performed an in vivo CRISPR screen in a mouse model of chronic viral infection has identified PBAF as a key regulator of CD8 T cell exhaustion. Disruption of PBAF, particularly deletion of its subunit *Arid2*, led to enhanced proliferation and survival of $CX3CR1^+$ T cell effector populations and enhanced antitumour immunity and improved responses to anti-PD-L1 in a mouse model of melanoma[118]. It was suggested that components of the cBAF complex, such as ARID1A and SMARCD2, promote effector differentiation while inhibiting the generation of memory populations. Mechanistically, cBAF and MYC are asymmetrically distributed during the first cell division of activated $CD8^+$ T cells, shaping the eventual fate towards effector T cells. cBAF physically interacts with MYC, co-binding to chromatin regions that activate effector-associated genes, enhancing the cytotoxic programming. Importantly, transient inhibition of cBAF using a small molecule (BD98) before CAR T cell manufacturing

led to a stem-like phenotype of CAR T cells and enhanced their persistence in mouse models of osteosarcoma and glioblastoma, resulting in improved tumour control[119]. In human $CD8^+$ T cells stimulated acutely or chronically in vitro, genetic deletion of ARID1A or SMARCD4 reduced expression of exhaustion markers such as TIM3 and PD-1 and promoted a memory-like phenotype with increased cell numbers[120]. Notably, when OT-1 $CD8^+$ T cells with *Smarcd4* knockout were transferred into B16-OVA-inoculated mice, these T cells exhibited an enhanced antitumour effect that was coupled to decreased T cell exhaustion and increased memory-like T cell populations. Although disruption of cBAF components promote enrichment of T cells with a stem-like phenotype at the early stages of an immune response in tumour and viral infection models, the durability of these stem-like properties remains unknown.

Engineering T cell stemness for cell therapy

Although T cell exhaustion continues to be a major roadblock during tumour immunotherapy, recent discoveries into the molecular mechanisms governing T cell exhaustion have enabled efforts that restrict T cell stemness[108,121,122]. In addition, given that T_{pex} cells are responsible for generating a therapeutic effector response during ICB therapy[52], investigators have begun to develop approaches that modify the specific regulators controlling the durability of this T cell subset.

Leveraging the critical role that epigenetic regulators have in reinforcing T cell exhaustion, several studies have targeted these regulators with the goal of engineering T cells to retain their stem-like properties (Fig. 3). One of the initial reports of therapeutic success arising from genetic modification of an epigenetic regulator was described in a patient with chronic lymphocytic leukaemia (CLL) treated with CD19 CAR T cells[84]. Assessing the CAR T cell response in the patient revealed that disease control was coupled to the expansion of CAR T cells arising from a single clone with the *TET2* gene disrupted by the integration of the CAR vector construct. These *TET2*-deficient CAR T cells exhibited an altered epigenetic profile that was enriched for a central memory phenotype[84]. Notably, this patient exhibited sustained B cell aplasia for years after treatment, indicating that the CD19 CAR T cells maintained a capacity to kill antigen-positive cells for a long period of time. Building on the clinical success observed from the unintended *TET2* disruption, it was further demonstrated that rationally designed disruption of TET2 in human CD19 or prostate-specific membrane antigen (PSMA) CAR T cells indeed enhanced T cell-mediated tumour regression in leukaemia and prostate cancer models, respectively[123].

Similar to the disruption of *TET2*, deletion of DNMT3A in either first-generation or second-generation human CAR T cells has been reported to enhance T cell persistence and effector potential during chronic tumour antigen exposure[83]. Longitudinal DNA methylation profiling of wild-type and knockout CAR T cells further defined the epigenetic signature and timing for human T cell exhaustion. Naive or stem-like memory T cells normally acquire DNMT3A-mediated methylation during differentiation into terminally differentiated cells. Notably, *DNMT3A*-knockout CAR T cells remained unmethylated at stem-associated genes, consistent with their functional persistence. This work has also demonstrated that epigenetic signatures could be predictive of the relative developmental potential of the CAR T cells in preclinical and clinical settings[83,124] (Fig. 3). Collectively, these studies demonstrate that DNA methylation can be a target to enhance therapeutic durability and a biomarker for predicting therapeutic potential of T cell-based strategies.

As part of an effort to define engineering targets to generate an ICB-responsive T_{pex} cell subset, one recent study has assessed mutations associated with the survival of patients with relapsed and refractory acute myeloid lymphoma (AML) after treatment with the hypomethylating agent azacitidine and an anti-PD-1, nivolumab. This revealed that patients with *ASXL1* mutations experienced improved survival[125], which was corroborated by a clinical trial, in patients with myelodysplastic syndrome (MDS) who were treated with a hypomethylating agent (guadecitabine) and an anti-PD-L1. Although typically thought of as a myeloid-associated disease, the patients with MDS underwent extensive genetic profiling of their myeloid and lymphoid compartments. Notably, several patients with *ASXL1* mutations in both the lymphoid and myeloid compartments achieved complete remission, whereas the patients with *ASXL1* mutations restricted to the myeloid compartment did not respond to combination therapy of guadecitabine and anti-PD-L1 (ref. 126). Although these observations were made in a limited number of patients, the remarkable difference in clinical outcome suggested that *ASXL1* mutations in T cells may have a pivotal role in maintaining an ICB-responsive population of cells. These clinical results prompted experimental investigation into the role of *Asxl1* in T cell function. Preclinical studies using *Asxl1*-deficient $CD8^+$ T cells have revealed that adoptive transfer of the edited T cells into various mouse tumour models resulted in enhanced antitumour activity. Moreover, the antitumour properties of *Asxl1*-deficient $CD8^+$ T cells synergized with anti-PD-L1 therapy, suggesting that targeting *ASXL1* could be a promising strategy to enhance the therapeutic efficacy of adoptive cell therapies, including CAR T cell therapy in combination with ICB[96].

In addition to disrupting the clonal haematopoiesis-associated epigenetic regulators, other groups have focused on additional targets, such as SUV39H1 and FOXO1. For instance, genetic disruption of *SUV39H1* in human CAR T cells exhibited early expansion and long-term persistence resulting in an enhanced antitumour effect in leukaemia and prostate cancer models[102]. Furthermore, overexpression of FOXO1 in T cells generated a stem-like phenotype, including increased chromatin accessibility at *FOXO1*-binding motifs. CD19 CAR T cells that overexpressed *FOXO1*, *JUN* and *FOXP1* exhibited enhanced function and persistence with augmented antitumour effects[127–130] (Fig. 3). These studies provide further rationale for manipulating core epigenetic regulators to enhance durable responses in T cell-based immunotherapies.

Although the above engineering efforts highlight a bottom–up approach to engineering T cells with 'stemness' programmes, strategies that leverage the signalling events that drive stable epigenetic programmes as a 'top–down' engineering approach are also being explored. Signal three cytokines have long been known to serve as a critical determinant of T cell fate decisions[131], and both preclinical and clinical studies have highlighted the requirement for cytokine signalling in effective antitumour responses[132]. Notably, several examples of cytokine-driven therapies have specifically focused on enhancing T_{pex} cell generation and maintenance (Fig. 3). Combination treatment with IL-2 and PD-L1 blockade exhibited a substantial synergistic effect in driving increased differentiation of functional antigen-specific T cells through an alternative differentiation path from T_{pex} cells[133]. In addition, an engineered IL-2 partial agonist, activating IL-2 pathway through binding IL-2 receptor β (IL-2Rβ) in the absence of IL-2Rα binding, promoted the expansion of T cells without driving terminal differentiation. This treatment preserved TCF1 expression among the T cells, maintaining a stem cell-like state and robust antitumour activity in mouse models of melanoma and acute lymphoblastic leukaemia[134]. In addition, CAR T cells preconditioned with either IL-7 or IL-15, memory-associated

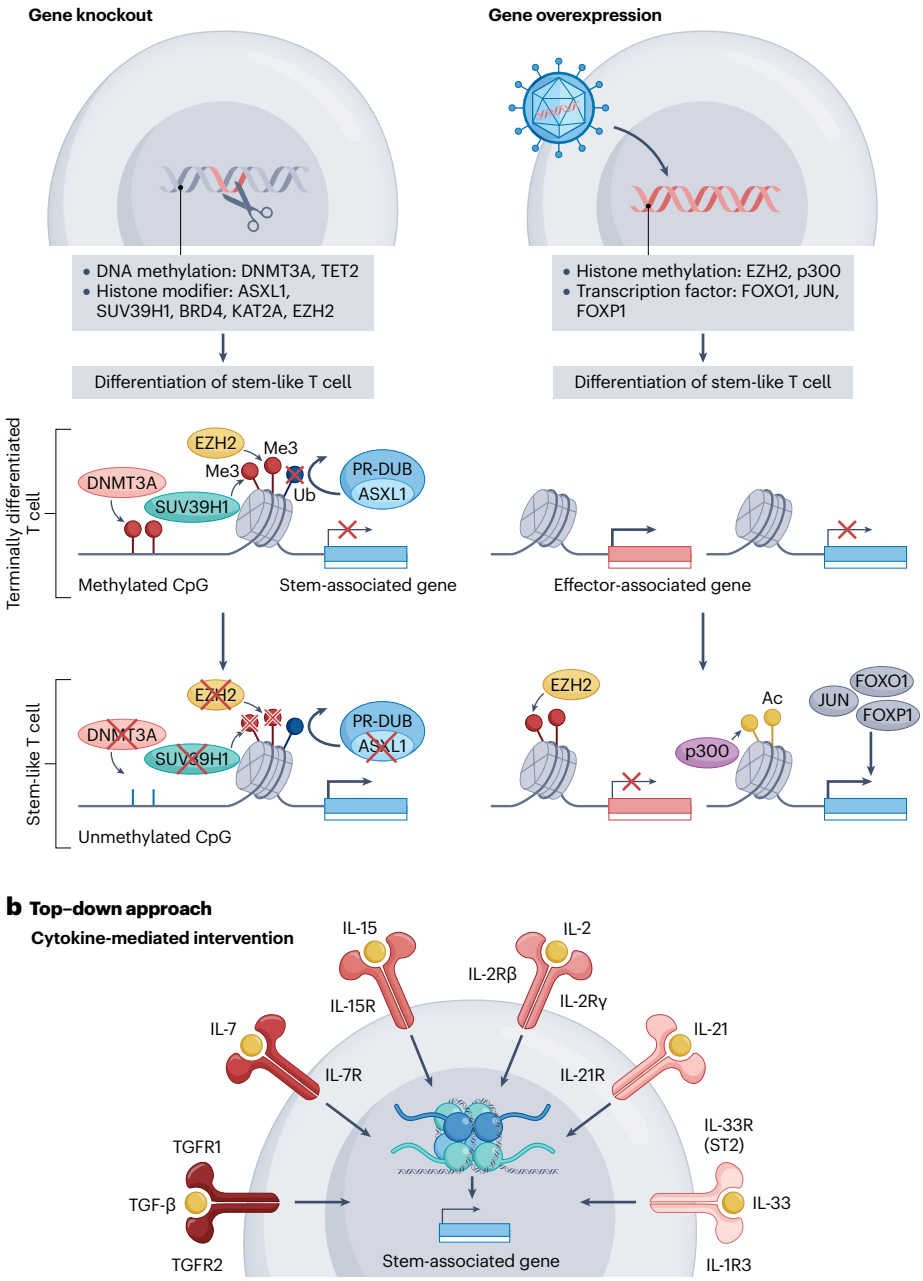


Fig. 3 | Two data-driven engineering approaches for improving T cell stemness. a, T cell stemness may be improved through direct genetic manipulation of various immune checkpoints by disrupting (left) or overexpressing (right) epigenetic regulators or transcription factors. Disruption of genes such as those encoding DNA methyl transferase 3A (DNMT3A) or TET2, which are involved in DNA methylation, could promote and maintain the development of stem-like T cells. Overexpressing genes such as those encoding EZH2, p300, FOXO1, JUN or FOXP1 could support maintenance of progenitor exhausted T (T_{pex}) cells. **b**, T cell stemness may be improved by extracellular modulation of the T cell through cytokine signalling. Cytokines such as interleukin 2 (IL-2), IL-7 or IL-33 could function through their respective receptors to modulate intracellular signalling pathway to enhance T cell stemness. Cytokine-based therapy for patients with cancer or the pre-treatment of T cells before transferring them into patients with cancer may promote the generation of T_{pex} cells.

cytokines, exhibited augmented antitumour effects by maintaining proliferation and effector functions compared with CAR T cells cultured with IL-2 alone in human lymphoma and a mouse breast cancer model[135–137]. Another study has revealed that CAR T cells preconditioned with IL-7 and IL-15 showed increased ICB responsiveness that was coupled to a preservation of stem-like T cell populations[138]. CD19 CAR T cells cultured with IL-21 in vitro exhibited a more stem-like phenotype and enhanced antitumour effects against $CD19^+$ B cell malignancy[139]. In addition, IL-21 treatment could induce the generation of stem-like $CD8^+$ T cells from naive precursors, and the transfer of these cells into a humanized melanoma mouse model resulted in a superior antitumour response[140]. When patients with metastatic melanoma that was refractory to both anti-CTLA-4 and adoptive T cell therapy were treated with IL-21-primed $CD8^+$ T cells and anti-CTLA-4, they showed durable complete remission and durable and functional persistence of transferred $CD8^+$ T cells[141].

Further top–down engineering strategies for T cells include modulation of metabolic pathways or unconventional cytokines to enhance T cell function for antitumour immunity. For example, transforming growth factor β (TGF-β) regulates the metabolic and functional fate of $CD8^+$ T cells by limiting mTORC1 activity, which in turn preserves mitochondrial fitness and promotes the maintenance of

Glossary

Adoptive cell therapy
(ACT). A type of immunotherapy in which T cells are isolated, expanded or genetically modified ex vivo and then re-infused into patients.

Chimeric antigen receptor (CAR) T cell
Genetically engineered T cells expressing synthetic receptors that specifically bind to an antigen of interest.

Chromatin immunoprecipitation sequencing
(ChIP-seq). A genomic technique used to analyse genome-wide protein–DNA interactions.

Conditional knockout
(cKO). A genetic technique in which gene deletion is restricted to specific cell types or developmental stage.

Follicular helper T (T_{fh}) cells
A $CD4^+$ subset of T cells that aid in orchestrating B cell responses within secondary lymphoid organs.

Oligoclonal expansion
The proliferation of a limited number of T cell clones in response to specific stimulus.

Signal three cytokines
Inflammatory cytokines important for T cell activation along with T cell receptor stimulation (Signal1) and co-stimulation (Signal2).

Tertiary lymphoid structures
Organized immune cell clusters that form in non-lymphoid tissue, resembling secondary lymphoid organs and supporting local immune responses.

T_{pex} cell populations[142]. Transient inhibition of mTORC1 by rapamycin during early T cell differentiation resulted in an increase of T_{pex} cells with improved mitochondrial fitness, suggesting the temporal modulation of the TGF-β and mTORC1 axis could be a promising strategy to improve T_{pex} cell generation[142]. Another example of a top–down engineering strategy is exemplified by work showing that CAR T cells engineered to produce IL-10 exhibit enhanced metabolic fitness, resistance to exhaustion, and an overall improvement in antitumour activity, particularly against solid tumours and metastases[143]. Unlike conventional CAR T cells, IL-10-expressing CAR T cells maintain mitochondrial integrity along with increased oxidative phosphorylation, resulting in the increased expression of effector cytokines and enhanced polyfunctionality. As an unconventional cytokine, it was demonstrated that IL-33 serves as a critical factor regulating T cell differentiation. When T cells are engineered with a modified IL-2 variant binding to the IL-2Rβγ receptor and IL-33, they did not show an exhaustion phenotype, but exhibited a unique effector state showing superior function[144]. Notably, this unique T cell state displayed enhanced antitumour effects independent of either lymphodepletion or external cytokine support, which is commonly used in adoptive cell therapies. These findings highlighted the potential of engineered $CD8^+$ T cells to overcome an exhausted state and reduce the need for preconditioning in solid tumour immunotherapy. Importantly, this unique effector state was not found in T cells responding to natural infections or checkpoint blockade, or in conventional CD19 CAR T cells from the peripheral blood of treated patients, suggesting that this is a completely new engineered state[144]. Another research group highlighted the role of IL-33 as a critical cytokine promoting the expansion of stem-like T cells[145]. Disruption of ST-2, the receptor for IL-33, in $CD8^+$ T cells led to a decrease in the proportion of $TCF1^+$ T cells and shifted them towards terminal effector differentiation, resulting in reduced proliferation and an impaired anti-viral response.

In addition to engineering T cells with a stem-like phenotype, reprogramming terminally exhausted T cells has demonstrated potential in enhancing tumour control in mouse models. For example, in vitro treatment of $CD8^+$ T cells with a modified IL-10-Fc fusion protein directly boosts the mitochondrial oxidative phosphorylation in exhausted T cells, resulting in improved proliferative and cytotoxic T cell responses in a mouse model of melanoma. Combining IL-10-Fc with adoptive cell therapy or anti-PD-1 ICB successfully led to tumour regression of established mouse melanoma and colon adenocarcinoma models and led to long-lasting immune memory[146]. Moreover, T cells experience severe hypoxia within the tumour microenvironment[147]. In mouse tumour models, it was suggested that continuous antigen stimulation and hypoxia led to T cell exhaustion through rapid mitochondrial dysfunction marked by high levels of reactive oxygen species[148]. In mouse models of melanoma wherein the tumour is either genetically engineered to lack a critical component of mitochondrial complex 1, *Ndufs4*, or the cells are treated with a low dose of an inhibitor against vascular endothelial growth factor receptor (VEGFR), axitinib, it was observed that the reduced intratumoural oxygen consumption and hypoxia resulted in improved T cell function and responsiveness to ICB[148]. This finding suggested that modulating the hypoxic tumour microenvironment can improve T cell metabolic fitness and responsiveness to immunotherapy. By using metabolic reprogramming strategies, future T cell-base immunotherapies could be improved, particularly in solid tumours wherein T cell dysfunction remains a major problem.

Conclusions and future perspectives

Although T cell exhaustion remains a considerable barrier limiting the efficacy of cancer immunotherapy, the past decade has greatly advanced our understanding of the molecular mechanisms that reinforce the developmental states of T cells and their cellular origins. As we look to the future of cancer immunotherapy, we must address several critical open questions that still hinder the durability of this approach. First, what are the key underlying molecular mechanisms governing the generation and persistence of stem-like T cells within the tumour microenvironment? Second, is it possible to revert the differentiation state of terminally exhausted T cells into a functional state? Last, can combination therapy that includes epigenetic-targeted approaches engage exhausted T cells in the tumour microenvironment? Identifying naturally occurring and long-lived T cell subsets that have the ability to resist exhaustion could address the question above and uncover pivotal pathways to enhance therapeutically durable stem-like T cell populations in the tumour microenvironment[149]. As an example, self-reactive T cells can persist for decades, and this pathology is associated with the persistence of a stem-like phenotype among the beta-cell-specific T cells[150,151]. Therefore, studying how stem-like T cells are generated and maintained in the context of autoimmune diseases and leveraging these studies for T cell-based cancer immunotherapy could also be beneficial.

These examples of T cell functional durability highlight opportunities to define novel exhaustion-avoidance strategies that may be engineered or selected for in the development of next-generation cancer immunotherapies. Therefore, future efforts to resolve the underlying mechanisms governing the development of therapeutically relevant T cell subsets, such as exhausted or stem-like T cells, and identifying T cell populations that naturally resist exhaustion mechanisms will be critical. These advances will provide a foundation for refining adoptive T cell therapies and ICB to achieve long-lived tumour immunity.

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