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Transcriptional rewiring in CD8$^+$ T cells: implications for CAR-T cell therapy against solid tumours

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T cells engineered to express chimeric-antigen receptors (CAR-T cells) can effectively control relapsed and refractory haematological malignancies in the clinic. However, the successes of CAR-T cell therapy have not been recapitulated in solid tumours due to a range of barriers such as immunosuppression, poor infiltration, and tumour heterogeneity. Numerous strategies are being developed to overcome these barriers, which include improving culture conditions and manufacturing protocols, implementing novel CAR designs, and novel approaches to engineering the T cell phenotype. In this review, we describe the various emerging strategies to improve CAR T cell therapy for solid tumours. We specifically focus on new strategies to modulate cell function and fate that have precipitated from the growing knowledge of transcriptional circuits driving T cell differentiation, with the ultimate goal of driving more productive anti-tumour T cell immunity. Evidence shows that enrichment of particular phenotypic subsets of T cells in the initial cell product correlates to improved therapeutic responses and clinical outcomes. Furthermore, T cell exhaustion and poor persistence are major factors limiting therapeutic efficacy. The latest preclinical work shows that targeting specific master regulators and transcription factors can overcome these key barriers, resulting in superior T cell therapeutic products. This can be achieved by targeting key transcriptional circuits promoting memory-like phenotypes or sustaining key effector functions within the hostile tumour microenvironment. Additional discussion points include emerging considerations for the field such as (i) targeting permutations of transcription factors, (ii) transient expression systems, (iii) tissue specificity, and (iv) expanding this strategy beyond CAR-T cell therapy and cancer.

KEYWORDS

immunotherapy, adoptive cell therapy (ACT), transcription factor, cancer, CAR-T cell, CD8$^+$ T cell, solid tumour

1 Introduction

A critical arm of cancer immunity involves $CD8^+$ T cell activity. $CD8^+$ T cells are cytotoxic lymphocytes that eliminate infected and malignant cells based on the recognition of non-self or altered-self antigens presented by class I major histocompatibility complex (MHC) molecules on the cell surface. Recent decades of basic, fundamental research have seen rapid growth in our understanding of T cell biology, from the stringent selection criteria enforced during T cell development to their striking phenotypic plasticity post activation. This provided the foundations for driving important discovery research down the translational pipeline, leading to a critical new pillar of treatment known as cancer immunotherapy. This treatment modality has drawn immense scientific and clinical attention due to its ability to drastically improve outcomes for aggressive cancers through two primary streams: (*i*) immune checkpoint blockade (ICB) and (*ii*) adoptive cell therapy (ACT) (1). ICB has achieved impressive clinical outcomes for patients with advanced solid tumours, and include CTLA-4 inhibitors (e.g. ipilimumab), PD-1/PD-L1 inhibitors (e.g. nivolumab, pembrolizumab and atezolimumab) and LAG-3 inhibitors (e.g. relatlimab) (2). ACT protocols employ either tumour-infiltrating lymphocytes (TILs) grown out from tumours in the presence of interleukin-2, or chimeric antigen receptor (CAR)-T cells, which are genetically engineered T cells expressing a synthetic receptor that redirects cytotoxicity towards target antigens. TIL therapy consists of an autologous T cell product that has shown efficacy in a few solid tumours (3). CAR-T cells have demonstrated remarkable remissions achieved for patients with various haematological cancers (4). Very recently, a TCR transgenic T cell product was approved by the FDA for the rare but aggressive synovial subtype of sarcoma (5). However, despite these clinical successes, many patients present with immunotherapy-resistant disease, often due to failures associated with the $CD8^+$ T cell response. Thus, defining the molecular drivers and mechanisms underpinning highly effective $CD8^+$ T cell immunity remains an important goal in the field for improving therapeutic outcomes.

The activated $CD8^+$ T cell compartment is remarkably diverse. In acute infections, $CD8^+$ T cells adopt a continuum of functional states, from short-lived effector cells that efficiently eliminate infected cells, to quiescent memory populations that provide long-lived immunity for the host (6). More recently, it has been shown that persistent antigen exposure, a condition frequently observed with chronic infection or cancer, generates an additional spectrum of 'exhausted' phenotypes, varying in functional capacities and stem-like features (7). The formation of these different phenotypes are directed by an array of extracellular signals, such as the strength of antigen signalling, the cytokine milieu, cell-to-cell communication, and the availability of metabolites and nutrients. Transcription factors play a crucial role in establishing these heterogeneous cellular phenotypes, and as such, play a central role in shaping $CD8^+$ T cell activity and maintenance. Importantly, the balance of these different phenotypes dictates the quality of $CD8^+$ T cell responses, including the efficacy of therapeutic products targeting this immune compartment.

Transcription factors are proteins that bind to key regulatory elements in the DNA to regulate gene expression and steer the developmental trajectory of cells. Transcriptional networks guide the developmental program of cells over our lifetime, regulating the differentiation of embryonic stem cells into the numerous unique cell types found throughout the body. A striking demonstration of the control that transcription factors have on cellular fate is the ability of forced expression of the OKSM/Yamanaka factors (four transcription factors: Oct3/4, Sox2, Klf4, and c-Myc) to reprogram adult human and mouse fibroblasts back into pluripotent stem cells (8, 9). Due to the advent of novel genetic engineering platforms, bioinformatics approaches, and high throughput screening tools, we are on an exciting precipice of unravelling the numerous transcriptional networks underpinning effective $CD8^+$ T cell. It has become evident that the transcription factors regulating these networks serve as actionable molecular targets that can be leveraged to enhance the performance of cellular therapies. Thus, there is significant interest in identifying and modulating the expression of transcription factors in $CD8^+$ T cells to improve ACT protocols, with a particular desire to extend its clinical application into the realm of treatment-resistant solid tumours. This review aims to provide an up-to-date view of transcription factors that have been targeted in preclinical T cell therapy protocols, and the outcomes of this strategy. We discuss novel approaches to deciphering and rewiring these transcriptional circuits, such as using revolutionary next-generation sequencing technologies and emerging genetic engineering platforms and their applications to cancer immunotherapy. Finally, in light of this cutting-edge research, we speculate on the future directions of this field.

2 T cell development and differentiation

A T cell's journey begins with the migration of common lymphoid progenitors from the bone-marrow to the thymus. It is here that thymocytes are programmed and selected to develop into naive $CD4^+$ or $CD8^+$ T cells, steered towards their fate by lineage-determining transcription factors (10, 11). Random recombination of T cell receptor (TCR) genes generates an extraordinarily diverse repertoire of antigen-specificities, which is pruned by selective processes to calibrate T cell responses towards 'altered self'. Once selection is complete, successful naive T cells undergo a small burst of proliferation, followed by each naïve clone emerging from the thymus to seed the periphery. These naive T cells are destined to circulate throughout the secondary lymphoid compartment, traversing the host's intricate immune surveillance network in search of their cognate antigen. During this stage they are transcriptionally and epigenetically maintained in a state of quiescence and homeostasis (12). Whilst many naive T cells never undergo activation, those that encounter their cognate antigen on an antigen presenting cell (APC) undergo extensive transcriptional, epigenetic, and metabolic rewiring to initiate rapid clonal

expansion. T cell activation requires three signals: antigen recognition provides the first signal, costimulation provides the second signal, and cytokine support provides the third signal. This results in the generation of a highly specific army of T cells directed against infected or malignant cells.

The formation of these different phenotypes are directed by an array of extracellular signals, such as the strength of antigen signalling, the cytokine milieu, cell-to-cell communication, and the availability of metabolites and nutrients. Transcription factors play a crucial role in establishing these heterogeneous cellular phenotypes, and as such, play a central role in shaping $CD8^+$ T cell activity and maintenance. Importantly, the balance of these different phenotypes dictates the quality of $CD8^+$ T cell responses, including the efficacy of therapeutic products targeting this immune compartment.

3 CAR-T cell therapy in solid tumours

ACT is an established, powerful branch of immunotherapy that broadly refers to *ex vivo* expansion and modulation of autologous T lymphocytes that are re-infused into patients as a 'living drug' to directly target cancer cells. Whilst cancer vaccines and ICB rely on the stimulation and reinvigoration of natural immunity, ACT bypasses the need to promote clonal expansion *in vivo* by expanding lymphocyte numbers in the laboratory (13). CAR-T cells are one form of ACT where T cells are engineered to express synthetic receptors constructed by fusing a surface antigen binding domain to T cell receptor and co-receptor signalling domains, redirecting T cell specificity towards target surface antigens. To date, the impact of CAR-T cell therapies has expanded across a range of haematologic malignancies, primarily targeting CD19 and CD20 on B cell-derived cancers, and B cell maturation antigen (BCMA) on multiple myeloma (Table 1).

Although CAR-T cell therapy has undoubtedly saved many lives, clinical responses to CAR-T cell therapy are varied, with some patients experiencing no responses or relapses post treatment. Possible reasons for this include poor quality of the initial T cells, poor expansion of CAR-T cells, and poor persistence of the cells over time. Furthermore, although CAR-T cell therapy has improved therapeutic outcomes for subsets of patients with haematological cancers, there remains a clear unmet clinical need for better

TABLE 1 Approval of CAR-T cell therapies for various haematological malignancies across the globe, their design (target antigen and costimulatory domain) and key clinical trials investigating the clinical efficacy for respective clinical indications.

Approved CAR-T cell therapies and related trials										
Generic Name and trade name(s)	CAR Design	Clinical Indication	Region Approved				Pivotal Clinical Trials			
			US	EU	JP	AU	Trial Name	Primary endpoint	Median follow-up/cut-off (months)	Outcome
Tisagenleucel Kymriah, CTL019, CART-19	Anti-CD19, 4-1BB	R/R B cell ALL	•	•	•	•	**ELIANA** (244)	ORR	3	81%
		R/R FL	•	•			**ELARA** (245)	CRR	16.59	69.1%
		R/R DLBCL	•	•	•	•	**JULIET** (246)	BORR	14.0	52%
Axicabtagene ciloleucel Yescarta, Axi-cel, KTE-C19	Anti-CD19, 4-1BB	R/R LBCL	•	•	•	•	**ZUMA-7** (247)	EFS	24.9	8.3 months
		R/R FL	•	•			**ZUMA-5** (248)	ORR	17.5	92%
Brexucabtagene autoleucel Tescartus, KTE-X19	Anti-CD19, CD28	R/R B cell ALL	•	•			**ZUMA-3** (249)	CRR	16.4	71%
		R/R MCL	•	•		•	**ZUMA-2** (250)	ORR	12.3	93%
Lisocabtagene maraleucel Breyanzi, JCAR017, Liso-cel	Anti-CD19, 4-1BB	R/R LBCL	•	•	•		**TRANSCEND NHL 001** (251)	ORR	18.8	73%
		R/R PMBCL	•	•	•					
		R/R FL	•	•	•					
Idecabtagene vileucel Abecma, Ide-cel	Anti-BCMA, 4-1BB	R/R MM	•	•	•		**KarMMa** (252)	ORR	13.3	73%
Ciltacabtagene autoleucel Carvykti, Cilta-cel	Anti-BCMA, 4-1BB	R/R MM	•	•	•		**CARTITUDE-1** (253)	ORR	12.4	91%

R/R, relapsed or refractory; ALL, acute lymphoblastic leukaemia; FL, follicular lymphoma; DLBCL, diffuse large B cell lymphoma; LBCL, large B cell lymphoma; MCL, mantle cell lymphoma, PMBCL, primary mediastinal large B cell lymphoma; MM, multiple myeloma; US, United States of America; EU, Europe; JP, Japan; AU, Australia; ORR, objective response rate; CRR, complete response rate; BORR, best overall response rate; EFS, event free survival.

treatments for patients with advanced solid tumours (14). In contrast to haematological 'liquid' tumours, solid tumours are composed of highly dynamic and complex ecosystems commonly referred to as the tumour microenvironment (TME). Clinical efficacy is hindered by several challenges intrinsically present within the TME, including tumour heterogeneity, dysregulated trafficking and infiltration, T cell exhaustion and immunosuppression (15). Heterogeneous antigen expression too often leads to incomplete eradication of tumours by promoting the outgrowth of antigen-negative clones or loss-of antigen variants. Approaches to overcome this include the development of CAR-T cells targeting multiple antigens, such as the 'quad' CAR-T cells in the Brainchild-04 Phase I clinical trial (16), or boosting epitope spreading by enhancing DC cross presentation and recruitment of endogenous T cell responses using cytokines such as FLT3L (17) and IL-12 (18). CAR-T cells must also effectively home to the tumour site and successfully infiltrate the tumour. Features of the TME such as abnormal vasculature, obstructive extracellular matrix, and dysregulated chemokine signalling inhibit effective trafficking and infiltration of CAR-T cells. To enhance trafficking and penetrance, studies have investigated genetically engineered expression of chemokine receptors (19), local and/or regional cell delivery (20), and administration of anti-VEGF drugs to stabilise aberrant vasculature (21). Furthermore, prolonged periods of antigen exposure within the TME drives T cells towards exhaustion. T cell function is further dampened by expression of inhibitory molecules on cancer cells, and additional microenvironmental factors such as hypoxia, limited metabolites, and co-opted immunosuppressive cells. Different approaches such as dominant negative receptors (22), knock-out of inhibitory receptors (23), and modified manufacturing protocols with the inclusion of different cytokines or molecules (24, 25) have been studied to enhance resistance to exhaustion and immunosuppression. Providing cytokine support can additionally overcome poor persistence of T cells, a key barrier for therapeutic efficacy in both haematological and solid tumours. Indeed, studies in mice have demonstrated that the survival and therapeutic efficacy of anti-HER2 CAR-T cells is dramatically improved in mice transgenic for interleukin (IL)-2 (26, 27). With the numerous challenges of effectively targeting solid tumours with CAR-T cell therapy, it is beneficial to develop novel strategies that can augment multiple aspects of the CAR-T cell. One such strategy is modulating the expression of transcription factors, master regulators of cell function and fate, to enhance the CAR-T cell phenotype and address multiple barriers to therapeutic efficacy.

4 Using transcription factors to rewire CAR-T cells

Transcriptional networks drive strikingly diverse changes in the phenotype and function of a T cell over its lifetime (Figure 1). Transcription factors are typically classified as repressors or activators of gene expression, although certain proteins are capable of either activation or repression in different contexts. Modulation of gene expression is mediated by binding at promoters, found near transcriptional start sites, or at enhancers, distal regulatory regions (28). Whilst some transcription factors can operate as single functional units, such as those with a helix-turn-helix (HTH) structure, others must dimerise, such as basic leucine zipper (bZIP) transcription factors e.g. the activating protein 1 (AP-1) family (29, 30). Furthermore, these dimers can bind to additional cofactors to mediate activation or repression at composite element sites. For instance, heterodimers of two AP-1 family members, BATF and JUNB, can bind to interferon regulatory factors (IRF) to modulate transcription at AP-1-IRF composite elements (AICEs) (31). Post-translational modifications play an important role in the regulation of transcription factor activity, with different modifications driving specific outcomes depending on the target protein (32). For example, phosphorylation of nuclear factor of activated T cells (NFAT) sequesters the protein in a resting, cytosolic state, whereas phosphorylation of certain IRFs or signal transducer and activator of transcription proteins (STATs) activates these transcription factors and is required for nuclear translocation (33, 34). Ultimately, the endogenous control of a cell's transcriptional activity is mediated by the integration of upstream signalling to coordinate appropriate changes in gene expression induced by various stimuli. Accordingly, T cell differentiation is orchestrated by networks of transcription factors that integrate a range of stimuli, such as antigenic stimulation, cellular crosstalk, and environmental cues to allow crucial fate decisions to unfold. Transcriptional networks drive strikingly diverse changes in the phenotype and function of a T cell over its lifetime (Figure 1).

In naïve T cells, a maintenance transcriptional program promotes a state of quiescence during its patrol for cognate antigen. This involves the epigenetic and transcriptional silencing of effector-related genes by important transcription factors such as TCF1, BCL2 and LEF1, and maintenance of regular naïve migratory patterns such as suppression of inflammatory chemokine receptors by KLF2 (12, 35–37). Once a T cell receives the three requisite signals of activation, this program is swiftly downregulated, allowing for the transcriptional framework governing effector differentiation and clonal expansion to assume control. TCR-responsive transcription factors such as NFAT and IRF4, and transcription factors induced by co-stimulation, such as c-Jun and NF-κB, integrate to orchestrate the necessary molecular rewiring of the cell (38–41). These transcriptional circuits are further tuned by cytokine support, such as IL-2, IL-7, IL-12, IL-15 and type I IFN signalling (42, 43). With all activating signals delivered to the T cell, these transcriptional networks induce robust proliferation, metabolic rewiring, effector differentiation and the trafficking to sites of pathogenic or malignant insult (44). In contrast, where incomplete signals are delivered, a tolerogenic program is initiated to restrict the T cell response and limit harm to self (41).

In addition to the generation of short-lived effector cells (SLECs), which are purged from the system once the antigenic threat is neutralised, early activated T cells also give rise to a small population of effector cells that are destined to survive contraction and form memory (45, 46). These are known as memory precursor effector cells (MPECs). Their fate is determined by sustained expression of transcription factors driving longevity and stemness, and lower levels of effector-related transcription factors such as

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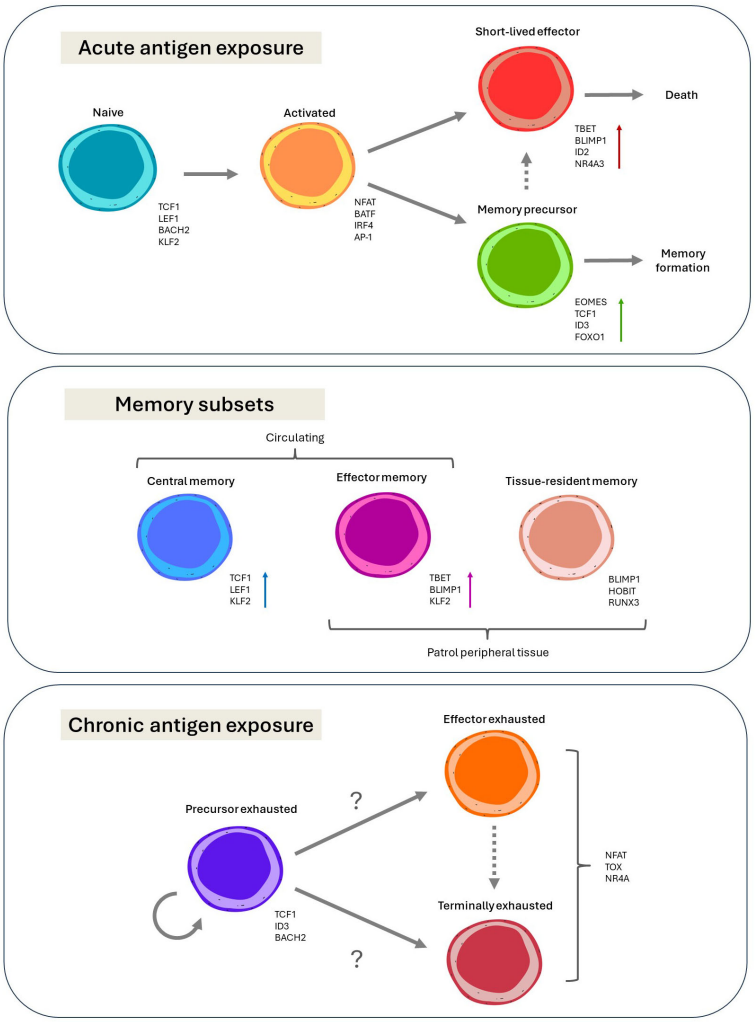

FIGURE 1
Current paradigms on the diversity and differentiation of CD8$^+$ T cells, and the transcription factors associated with the various phenotypic states, with arrows indicating differentiation trajectories.

IRF4 and BLIMP1 (47, 48). Whilst some of the transcription factors associated with longevity are shared with the naive transcriptional program, such as TCF1, others are uniquely induced after activation, such as EOMES (49–51).

Several transcriptional networks run in parallel during the formation of memory, producing several subsets with unique differentiation states, migratory patterns, and functional capacities to provide comprehensive long-term protection. These include two subsets of circulating populations – central memory T (T_{CM}) and effector memory T (T_{EM}) cells – and one subset that stably occupies peripheral tissues - tissue resident memory T (T_{RM}) cells (52–54). T_{CM} cells adopt similar migratory patterns to that of naive T cells, utilising shared transcription factors such as KLF2 to regulate its course (55). In contrast, low level activity of the effector T cell transcriptional program likely maintains T_{EM} cells in a more terminally differentiated state than T_{CM} cells, and permits trafficking through both blood and peripheral organs (56, 57). Furthermore, a distinct tissue-residency program involving RUNX3, BLIMP1 and HOBIT, governs the T_{RM} compartment, which provides local surveillance at previous sites of antigenic insult (58–60).

In certain disease settings, such as cancer or chronic infection, the epigenetic and transcriptional landscapes of T cells are gradually remodelled to produce a unique phenotypic state known as exhaustion (61). T cell exhaustion is driven by complex mechanisms including chronic antigen exposure, excess of inflammatory signals, and suppressive signals (e.g. cytokines, cell-to-cell signalling) (62). The transcription factor TOX has been identified as an important contributor to the epigenetic 'scarring' (63). Over the past few years, our understanding of exhausted T (T_{EX}) cells has expanded to appreciate that heterogeneity exists within this T_{EX} population, and that transcription factors likely play a fundamental role in directing these divergent fates. Transcription factors driving quiescence and self-renewal, once again including T cell factor-1 (TCF1), ID3, and BACH2, maintain a progenitor exhausted T (T_{PEX}) cell pool (64). These T_{PEX} cells can be a source of sustained anti-tumour activity through the generation of cytotoxic progeny. The definitions surrounding T_{EX} subsets are

constantly evolving. However, the field has broadly converged on the existence of at least two distinct fates: (i) effector exhausted cells, thought to provide potent cytotoxic effects; and (ii) terminally differentiated exhausted T cells, which have poor cytotoxicity and are deficient in cytokine production (65–67). Furthermore, a hostile TME alters nutrient and oxygen availability, ultimately affecting the transcriptional program of infiltrating T cells (68, 69). For instance, lower oxygen levels within the TME drives the activity of hypoxia inducible factors, which are thought to allow for cellular adaptations to the challenging environment. In this setting, distinct transcription factors calibrate and fine tune the T cell phenotype in response to chronic exposure to antigen and other factors within the TME.

Transcription factors and their downstream effects thus endow $CD8^+$ T cells with an array of phenotypic states. Importantly, certain fates are correlated with better clinical outcomes for patients receiving ACT. For example, stemness – a central feature of naive and central memory $CD8^+$ T cells – has been highlighted as a characteristic that correlates with improved CAR-T cell persistence and durable remissions (57, 70). Targeting transcription factors that promote these favourable phenotypes is thus a promising strategy to enhance ACT protocols; this strategy has the potential to attenuate exhaustion-related transcriptional circuits, enhance stemness and/or memory-like potential, and promote tumour accumulation, thus overcoming the various challenges faced when targeting solid tumours. One barrier that is not obviously overcome with transcription factor modulation is tumour heterogeneity. Transcriptional rewiring of T cells into hybrid natural killer (NK)-like phenotypes can overcome MHC-restriction, but does not expand the repertoire of cancer antigens targeted by T cells (71). However, as novel insights into transcription networks involved during successful immunity are unravelled as a consequence of DC-T cell crosstalk, transcription factor targets enhancing important phenomena such as epitope spreading will no doubt assist with overcoming this key obstacle. However, identification of transcription factors that drive effective T cell immunity against cancer remains a significant challenge due to the complexity of its spatiotemporal regulatory patterns during T cell differentiation. Nevertheless, recent years have seen immense advances in molecular and bioinformatics platforms that have been instrumental in garnering mechanistic insight at unprecedented rates.

5 Unbiased strategies for prioritising novel transcription factor targets

In silico analyses using powerful bioinformatics approaches have accelerated drug discovery beyond the capabilities of traditional 'reductionist' (biased) approaches. As such, next generation sequencing technology that captures epigenetic and transcriptomic information (at bulk and single cell resolution) are now at the forefront of cancer immunotherapy research to unlock the heterogeneity of the anti-tumour T cell response. This process typically involves leveraging pre-clinical and clinical models, particularly those with divergent outcomes, and dissecting these models with next generation sequencing to prioritise candidates for downstream experimental validation. Indeed, conventional analysis of transcripts in TILs from human melanoma and non-small cell lung cancer revealed TOX as a critical driver of intratumoral T cell exhaustion which was confirmed by flow cytometry and siRNA knock-out experiments (72). Integrative analysis evaluating ATAC- and RNA-seq data highlighted KLF4 as a novel transcriptional regulator that reinvigorated exhausted $CD8^+$ T cells in MC38 murine tumours (67). A similar approach was adopted by Chen et al., reporting the role of NR4A transcription factors in driving CAR-T cell function in humanised animal models for B cell leukaemia (73). New technology involving the simultaneous single cell profiling of chromatin states and RNA in CAR-T cells has also unveiled FOXP1 and KLF2 as reciprocal regulators of stemness and effector function respectively (74). In addition to the more conventional analyses involving these technologies, a suite of *in silico* prediction tools have been specifically developed to garner further mechanistic insight from omics data to identify novel transcriptional regulators. These tools utilise various computational approaches, each with distinct strengths and limitations: 1) Gene regulatory network (GRN) inference methods like ARACNe (75), GENIE3 (76) and WGCNA (77) reveal complex regulatory interactions from transcriptomic data but are computationally intensive and can generate noisy GRNs. 2) Motif enrichment analysis tools, such as HOMER (78), predict TF binding sites through DNA motif analysis, though they may oversimplify predictions and don't capture secondary or indirect targets. 3) Transcription factor activity scoring approaches, like DoRothEA (79) and VIPER (80) estimate TF activity based on gene expression but is limited in scope due to their reliance on *a priori* information. 4) More advanced tools leverage multi-omic information generated by an array of single cell technologies capturing transcriptomic and epigenetic information. The SCENIC algorithm can predict TF activity from transcriptomic data derived from individual cells (81), but data sparsity inherent to scRNA-seq technology can limit the resolution of transcription factor-target gene predictions. Integrative multi-omic approaches such as MARINa (82) and CellOracle (83) combine gene expression and TF binding data from scRNA-seq and ChIP-seq respectively to generate more robust predictions models. In the context of cancer and T cell biology, the use of tools such as SCENIC and GENIE3, which are computational frameworks that reverse engineer's gene regulatory networks from transcriptomics data (81), has been instrumental in identifying novel transcriptional networks underlying TIL activity in various human cancer atlas datasets (84, 85), as well as long-term CAR T cell persistence (86). It is important to note that these computational frameworks serve merely as predictive tools, and rigorous laboratory validation is necessary. When combined with *in vitro* high throughput screening tools involving the use of the CRISPR/Cas9 gene editing system, this represents the current state-of-the-art in the field that efficiently and systematically interrogates regulators of T cell fate in cancer. This has been exemplified in numerous studies utilising CRISPR screening that delineate the importance of specific transcription factors programs in tumour-specific $CD8^+$ T cell (87, 88) and Treg (89) populations.

6 Current tools for the modulation of gene expression

A suite of strategies can be used to modulation the expression of transcription factors, such as the use of cytokines (90, 91), chemical drugs (92, 93), immunomodulators (94, 95), and different nutrient levels (96, 97). In contrast to these approaches, which require the identification of biologically active molecules and compounds, genetic engineering platforms are capable of highly targeted manipulation of any known gene (Figure 2). The fundamental advantage of genetic engineering strategies is that the transcriptome of the cell becomes largely customisable for the purposes of basic and therapeutic research. The manipulation of transcription factors in CAR T cells can be broadly categorised into approaches aimed at decreasing or enhancing their expression.

Decreasing the expression of transcription factors that give rise to inferior phenotypes can improve CAR-T cell efficacy by diverting cellular products from poor phenotypic subsets and/or promoting effective populations. Several tools exist for the ablation or reduction of gene expression in living systems. The CRISPR-Cas9 system permanently inactivates targeted genes from the cellular genome by introducing loss-of-function mutations (98, 99). CRISPR-Cas9 is favoured for its simplicity, efficiency and accuracy. Novel iterations of CRISPR technology, particularly CRISPR interference (CRISPRi) can also achieve transient repression of target gene transcription (100, 101). The use of small interfering RNAs (siRNA) or short hairpin RNAs (shRNA) can also transiently repress or knockdown (KD) gene expression, although the incorporation of their sequences into viral vectors can establish stable downregulation of target genes (102–105). The Cre/lox system is a sophisticated tool for genetic deletion in mice, that is widely used for its ability to efficiently ablate genes *in vivo*, at designated times, and in designated cellular subsets, by the inducible expression of Cre recombinase under different promoters (106, 107). For example, floxed genes can be deleted in thymocytes at the double positive stage by expression of Cre under the CD4 promoter, in developing or mature T cells by expression of Cre under the proximal or distal Lck promoters, and in activated $CD8^+$ T cells by expression of Cre under the Granzyme B promoter (108–110). Whilst the use of CRISPR tools and siRNA or shRNA-based approaches can be feasibly adopted into an ACT protocol, the Cre/lox system can be utilised only in murine models. However, this system has been incredibly valuable for developing fundamental understanding of many genes and continues to be a powerful platform for genetic studies.

In contrast, increasing the expression of transcription factors that drive superior phenotypes can improve CAR T cell efficacy by enriching effective cellular subsets. A frequently used tool is retroviral viral transduction to achieve constitutive overexpression of genes of interest. Benefits of retroviral overexpression include stable and efficient levels of expression (111). Stable expression of genes of interest can also be achieved by HDR-CRISPR approaches to express genes under chosen promoters (112). Although this is a technically challenging approach, it circumvents issues relating to random integration into the genome such as the introduction of dangerous mutations. Ectopic gene expression can additionally be achieved by transfection with mRNA or DNA, although this increased expression is transient in nature (113, 114). Amplification of the wildtype gene can also be achieved through CRISPR activation (CRISPRa) (100, 115). Although there are many novel tools for the introduction or amplification of target genes, retroviral overexpression is most widely used in preclinical studies.

To date, numerous transcription factors have been modulated in $CD8^+$ T cells and shown to improve anti-tumour efficacy by conferring the adoptive cellular product with a range of functional or phenotypic advantages (Table 2).

7 Current outcomes in modulating transcription factor expression in $CD8^+$ T cells

7.1 NFAT

The NFAT transcription factor family is comprised of 5 members, four of which are regulated by the Ca^{2+}-calcineurin signalling axis (NFATc1-c4), whilst NFAT5 responds to hyperosmotic stress (116–118). Calcium-responsive NFAT

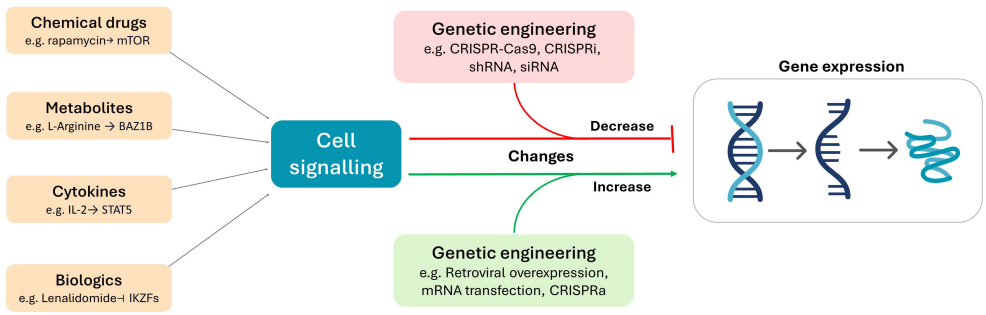

FIGURE 2
The use of metabolites, chemical drugs, cytokines, and biologics can be used to deliver cellular signals that alter gene expression. In contrast, genetic engineering platforms directly target gene expression by altering or influencing the cell at the genomic (e.g. CRISPR-Cas9, retroviral overexpression) or transcriptomic (e.g. CRISPRi, CRISPRa, shRNA, siRNA, mRNA transfection) level.

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TABLE 2 Mechanisms of improved CD8$^+$ T cell-driven anti-tumour immunity due to the engineered increased (↑) or decrease (↓) in transcription factor activity.

Family	Protein	Change	Method	Mechanisms of improved anti-tumour response
NFAT	NFAT5	↓	-/- CD4-Cre (120)	Limits exhaustion
Tox	TOX	↓	+/- CD4-Cre (63, 124)	Limits exhaustion
	TOX + TOX2	↓	shRNA (TOX), genetic deficiency (TOX2) (125)	Limits exhaustion
NR4A	NR4A1	↓	-/- CD4-Cre (135)	Limits exhaustion
	NR4A3	↓	Genetic deficiency (131), CRISPR-Cas9 (141)	Limits exhaustion
	NR4A1, 2, + 3	↓	-/- Transduced-Cre (NR4A1, NR4A2), genetic deficiency (NR4A3) (73)	Limits exhaustion
PRDM	BLIMP1	↓	CRISPR-Cas9 (141, 142), CRISPRi (143)	Limits exhaustion, improves persistence
JUN	c-Jun	↑	Retroviral overexpression (146, 147)	Limits exhaustion
TCF/LEF	TCF1	↑	Retroviral overexpression (153)	Limits exhaustion, promotes stemness
ID	ID3	↑	Retroviral overexpression (155)	Promotes stemness
MYB	c-Myb	↑	Retroviral overexpression (160)	Promotes stemness
FOXO	FOXO1	↑	Retroviral and lentiviral overexpression (164, 165)	Promotes stemness and memory phenotypes
RUNX	RUNX3	↑	Retroviral overexpression (171–173)	Promotes effector function and accumulation
HIF	HIF-1α + HIF-2α	↑	-/- dLck-Cre or ER-Cre deletion of VHL (177, 178)	Promotes effector function and accumulation
	HIF-2α	↑	Retroviral overexpression (180)	Promotes effector function
ETS	ETS1	↓	CRISPR-Cas9 (87)	Promotes T_{EX} differentiation and function
	FLI1	↓	CRISPR-Cas9 (190)	Promotes effector function
BATF	BATF3	↑	Retroviral overexpression (184)	Promotes memory phenotypes
	BATF	↑	Retroviral overexpression (198)	Limits exhaustion
		↓	shRNA (195), siRNA (196), CRISPR-Cas9 (197)	Promotes memory phenotypes
IRF	IRF4	↑	Retroviral overexpression (201)	Enhanced effector function
		↓	shRNA (195, 203)	Promotes memory phenotype *(in vitro only)*
BCL11	BCL11B	↓	CRISPR-Cas9 (208)	Overcomes MHC restriction

CRISPR interference (CRISPRi), knockout of two alleles (-/-), knockout of one allele for haploinsufficiency (+/-), Cre expressed under different conditions, including the CD4 promoter (CD4-Cre), retroviral transduction (Transduced-Cre), tamoxifen-induced (ER-Cre), short hairpin RNA (shRNA).

isoforms play a fundamental role in initiating early transcriptional responses following TCR signalling (38). NFAT, when combined with other prominent transcriptional regulators such as AP-1 confers a range of effects on T cell phenotype. For instance, AP-1/NFAT cooperation elicits downstream IL-2 and cytokine production (119), whilst NFAT activity alone drives exhaustion-like programs in CD8$^+$ T cells (120). Indeed, deletion of NFAT5 in a CD4 promoter-driven Cre-Lox recombination system improves tumour control by tumour-specific T cells in a mouse model of melanoma (121). Using a same recombination system, Heim et al. demonstrated that the deletion of NFATc1 resulted in ablated memory T cell formation and impaired effector differentiation, highlighting distinct biological activities of NFAT isoforms in driving fate (122).

7.2 TOX

The TOX family of transcriptional regulators comprises four members: TOX1 (also referred to as TOX), TOX2, TOX3 and TOX4 (123). In 2019, numerous studies identified and underscored a role for TOX in establishing exhausted T cell populations in viral and cancer studies (63, 124–128). Evidence suggests that the TOX pathway is activated downstream of NFAT during TCR-stimulation, and cooperates with NR4A to establish exhaustion (63, 125). In light of this central role in mediating exhaustion, several studies investigated the effects of knocking out TOX to limit $CD8^+$ T cell exhaustion in cancer. Two studies showed that haploinsufficiency of TOX improves *in vivo* anti-tumour efficacy of $CD8^+$ T cells in murine models of melanoma and hepatocellular carcinoma (63, 124). Furthermore, KO of TOX2 in addition to shRNA downregulation of TOX in CAR-T cells (*Tox* DKO T cells) further enhanced tumour control (125). Multiomic interrogation of haploinsufficient $Tox^{-/+}$ and *Tox* DKO T cells typically revealed an effector-like phenotype with reduced features of exhaustion. In contrast, Scott et al. found that T cells ablated of TOX retained features of dysfunction, such as poorer cytokine production, and failed to persist *in vivo*, possibly due to activation-induced cell death (126). This may reflect context-specific differences between studies, as well as complexities of transcriptional circuits governing exhaustion. Furthermore, the unique functional role of TOX2 remains unclear. On one hand, knockout of TOX2 synergises with TOX deficiency to prevent exhaustion, whilst another study suggests that TOX2 may regulate memory formation (125, 129). As such, we are yet to decipher the precise nature of TOX– and TOX2–driven transcriptional networks, however evidence suggests that targeting this axis is indeed a promising strategy to attenuate exhaustion.

7.3 NR4A

The NR4A family of transcription factors – NR4A1 (*Nur77*), NR4A2 (*Nurr1*), and NR4A3 (*Nor1*) – are orphan nuclear receptors that operate within several major transcriptional circuits, including induction by NFAT signalling, cooperation with TOX, and negative regulation of Jun and Fos (125, 130). As such, they are involved in multiple aspects of $CD8^+$ T cell biology, including the negative selection of autoreactive thymocytes, SLEC formation, establishing peripheral tolerance, and promoting exhaustion (73, 131–134). Thus, NR4As appear to negatively regulate the survival and function of developing and activated $CD8^+$ T cell subsets to prevent overactive or inappropriate immune responses. In mice bearing B16-OVA-CD19 melanoma tumours, CD19-targeting CAR-T cells lacking all three NR4As display significantly enhanced control of tumour growth compared to WT CAR-T cells, and were characterised by distinctly less exhausted, more effector-like phenotype capable of increased cytokine production (73). Ablation of individual NR4As, such as NR4A1 and NR4A3 have also been shown to enhance *in vivo* ACT efficacy against lymphoma and melanoma, respectively. In $Nr4a1^{-/-}$ OT-I cells formed a two to three-fold larger TIL population, reduced expression of exhaustion markers, and greater expression of Bcl-2, IFN-γ, TNF and CD107a (135). $Nr4a3^{-/-}$ OT-Is are similarly more polyfunctional, but adopt a memory-like phenotype, likely due to the role of NR4A3 in SLEC formation (131). Taken together, these results suggest that targeting the NR4As may generate more effective CAR T cell products.

7.4 BLIMP1

B lymphocyte-induced maturation protein-1 (BLIMP1), encoded by the *PRDM1* gene, plays an important role in both B and T cell effector differentiation (136, 137). Upon $CD8^+$ T cell activation, BLIMP1 is upregulated in effector cells, particularly SLECs, and controls key effector-related transcriptional events and functions, including the suppression of memory-related genes, induction of cytolytic molecules, and trafficking to tissues (56, 137, 138). In addition, BLIMP1 drives exhaustion in both cancer and viral infection, enforcing a battery of inhibitory genes and repressing stemness, and limiting effector function when expressed at high levels (139, 140). The regulation of Blimp1 expression has thus been investigated as a strategy to enhance CAR-T cell therapy. Two studies have shown that CRIPSR/Cas9 KO of *PRDM1* in CAR-T cells results in improved persistence and overall enhanced tumour control in preclinical models of haematological and solid cancers including leukaemia, melanoma and prostate cancer (141, 142). Both studies showed that knockout of BLIMP1 epigenetically rewired T cells, particularly increasing accessibility at the promoters of stemness and memory-related transcription factors. Interestingly, Jung et al. found that *PRDM1*-ablation also increased accessibility at a set of genes for exhaustion-related transcriptional regulators, suggesting the activation of compensatory exhaustion programs in the absence of BLIMP1 (141). This was circumvented by additional KO of NR4A3, resulting in a further improvements of tumour control. However, both studies also noted that BLIMP1 deficiency impaired cytolytic activity. Strategies to knockdown rather than completely ablate BLIMP1 expression may therefore preserve effector function. Indeed, CRISPRi downregulation of BLIMP1 in human T cells maintains cytotoxicity whilst limiting exhaustion and enriching the central memory subset (143). Overall, targeting the Blimp1 axis may be an effective strategy to combat exhaustion and enhance the persistence of CAR-T cells, although more refined approaches to maximise cytotoxicity whilst suppressing exhaustion may be optimal to best leverage this transcriptional network.

7.5 c-Jun

The transcription factor c-Jun is paramount for $CD8^+$ T cell activation. c-Jun is induced by costimulation, and cooperates with TCR-induced NFAT to activate the production of IL2, stimulating clonal expansion (144, 145). Thus, c-Jun plays a fundamental role in mediating activation, ensuring the expansion of robust effector populations. Two studies have shown that constitutive expression of c-Jun protects T cells from exhaustion and potently restored polyfunctional cytokine production and improved cellular persistence. This ultimately enhanced *in vivo* control of a range of human cancers, including leukaemia, osteosarcoma, and

hepatocellular carcinoma, by CAR-T or TCR-transgenic T cells (146, 147). Evaluation of a panel mutants deficient in transcriptional activation or chromatin binding ability revealed that the functional advantages conferred by c-Jun were at least partly mediated by displacement of AP-1 – IRF complexes from the chromatin, and not direct transcriptional effects (146). Overall, the overexpression of c-Jun reshapes the transcriptional landscape of $CD8^+$ T cells towards superior phenotypes, and is a promising target to enhance CAR-T cell performance.

7.6 TCF1

TCF1 is a transcription factor that is highly critical in establishing T cell phenotypes that relate to self-renewal, memory, and quiescence (35, 50). TCF1 is highly expressed in naïve T cells, and although silenced over the course of T cell activation it is important for the generation of MPECs in acute infections, and subsequent formation of memory T cell populations (148–150). TCF1 is also a key marker and transcriptional regulator of the T_{PEX} niche, and represses exhaustion-related genes such as Blimp1, IRF4, and NFAT (151, 152). Indeed, ectopic expression or overexpression of TCF1 in tumour-specific T cells has been shown to enhance the formation of the T_{PEX} population, enhance polyfunctionality, reduce expression of inhibitory receptors, and thus improve *in vivo* control of murine melanoma (153). TCF1-overexpressing T cells additionally possess heightened sensitivity to checkpoint blockade. Thus, overexpression of TCF1 can enrich T_{PEX} subsets that give rise to sustained anti-tumour responses.

7.7 ID3

Inhibitor of DNA Binding 3 (ID3) regulates properties of stemness and memory in $CD8^+$ T cells. Higher levels ID3 expression in early activated $CD8^+$ T cells demarcates memory precursors from short-lived effectors, which instead express increased levels of ID2 (138, 154). Furthermore, ID3 expression delineates T_{PEX} populations, and is downregulated in terminally differentiated subsets (151, 155). Thus far, one study has demonstrated that enforcing an ID3-driven transcriptional program can confer resistance to exhaustion in $CD8^+$ T cells. $CD8^+$ T cells overexpressing ID3 displayed enhanced polyfunctional cytokine secretion, increased cytotoxicity, and increased intratumoral accumulation, ultimately achieving enhanced control of liver tumours in a murine ACT model. Conversely, knockdown of ID3 dampened anti-tumour control and hampered T cell function (155). Therefore, overexpression of ID3 may be a viable strategy to limit exhaustion in CAR-T cells.

7.8 c-Myb

Recent studies have revealed that c-Myb, a member of the MYB transcription factor family, plays an important role in regulating properties of stemness and memory in activated $CD8^+$ T cells, building on its previously defined role in thymocyte development (156–159). A stem-like $CD62L^+$ subset of T_{PEX} cells that mediated effective responses to checkpoint blockade was not only enriched for c-Myb expression, but dependent on c-Myb for its formation (156). Furthermore, memory formation after vaccinia virus infection was significantly impaired when c-Myb was deleted in mature $CD8^+$ T cells (160). Evidence suggests that c-Myb potentiates $CD8^+$ T cell longevity and survival by driving the expression of anti-apoptotic molecules, driving the expression of TCF1, and repression of ZEB2, a transcription factor that promotes effector differentiation (157, 160). Gautam et al. found that overexpression of c-Myb successfully enforces properties of stemness, which, importantly, generates more effective anti-tumour responses. c-Myb overexpression improved the metabolic fitness and polyfunctionality of $CD8^+$ T cells, generated greater numbers of stem-like populations, and preserved CD62L expression after repetitive stimulation, a process that typically drives terminal effector differentiation. Adoptively transferred c-Myb overexpressing $CD8^+$ T cells provided curative anti-tumour immunity in mice bearing melanoma tumours. Furthermore, c-Myb overexpressing cells formed effective memory populations that protected hosts against the development of tumours upon a secondary melanoma challenge (160). As such, ectopic expression of c-Myb is an attractive approach to enhance stem-like phenotypes that promote persistence and superior anti-tumour responses in $CD8^+$ T cell ACT products.

7.9 FOXO1

FOXO1 is an important transcriptional regulator of stemness in both naive and antigen-experienced $CD8^+$ T cells and is crucial for the establishment of memory and long-term survival (161–163). Two recent studies found that direct overexpression of FOXO1 in CAR-T cells improves anti-tumour efficacy (164, 165). Phenotypically, human CAR-T cells overexpressing FOXO1 were enriched for markers of memory, and decreased levels of exhaustion. FOXO1-overexpressing CAR-T cells additionally demonstrated increased expansion, and greater polyfunctionality. Metabolically, FOXO1-overexpressing CAR-T cells demonstrated oxidative phosphorylation than control cells, indicating superior cellular fitness. Interestingly, both studies found that using a constitutively active version of FOXO1 that was insensitive to nuclear export resulted in blunted production of cytokines by the CAR-T cells. In addition, use of an EF1α promoter which drove higher expression levels of wildtype FOXO1 resulted in a stronger memory-like phenotype as compared to expression driven by other promoters (165). These findings elegantly demonstrate that balanced and fine-tuned expression of FOXO1 maximized the functional and phenotypic advantages conferred to CAR-T cells. Finally, when evaluated in an *in vivo* setting, FOXO1 overexpression improved CAR-T cell anti-tumour control in a range of preclinical solid tumour models, including murine breast carcinoma and colon adenocarcinoma, and human ovarian cancer and osteosarcoma. Importantly, these studies provided direct comparisons between FOXO1 and TCF1-overexpressing CAR-T

cells, demonstrating FOXO1 reduced exhaustion, improved expansion, and enhanced *in vivo* outcomes in a model of leukaemia (164, 165)vghy. Numerous studies have now identified that overexpression of various master regulators of memory and stemness can indeed improve the therapeutic efficacy of CAR-T cell therapy in several preclinical models, and experiments to identify the most effective of these transcription factors are of utmost importance.

7.10 RUNX3

RUNX3 governs multiple aspects of $CD8^+$ T cell fate decisions, from commitment to the $CD8^+$ lineage during thymopoiesis, to governing transcriptional networks post-activation (166–168). RUNX33 is critical for formation of functional memory T cells, underpinning widespread chromatin remodelling upon TCR stimulation including increased accessibility at effector-related genes such as *Irf4*, *Prdm1*, *Id2*, *Eomes*, and *Il2ra* (167). Recently, RUNX3 has additionally been highlighted for its key role in establishing T_{RM} subsets in $CD8^+$ T cells, driving tissue residency in a range of tissues (58, 169, 170). Exploiting RUNX3-driven transcriptional networks is thus an attractive strategy to enhance CAR-T cell therapy, as accumulation within the tumour bed is imperative for $CD8^+$ T cell-mediated tumour control. In a model using murine P14 T cells targeting GP_{33}-expressing B16 melanoma, overexpression of RUNX3 drove greater abundance of TILs, granzyme B expression, and tissue-residency gene modules to ultimately delay tumour growth and prolong survival (171, 172). RUNX3-overexpression synergises with inhibition of protein kinase B (Akt) to promote additional T_{CM} differentiation in CAR-T cells, which produces robust and improved anti-tumour responses in pancreatic ductal adenocarcinoma patient-derived xenograft tumour models (173). However, these results conflict with a study using a different human CAR-T cell model, which found that RUNX3-overexpression in anti-mesothelin CAR-T cells neither promoted a tissue-residency phenotype, nor improved control of mesothelioma *in vivo* (174). Thus, RUNX3 is a promising target that may engineer CAR-T cells with favourable characteristics such as tissue residency features and improved persistence to achieve superior therapeutic outcomes, although evidence is unclear as to whether these outcomes are consistent across multiple models and systems.

7.11 HIF-1α and HIF-2α

Hypoxia inducible factors 1 and 2 alpha (HIF-1α and HIF2α) are dimeric transcription factors that mediate homeostatic responses to low oxygen levels, such as those found within the tumour microenvironment (175, 176). In addition to governing homeostatic transcriptional circuitry, HIFs support $CD8^+$ effector cell differentiation and tissue resident fates (177–179). Two studies demonstrated that deletion of the von Hippel-Lindau tumour suppressor gene (VHL), a negative regulator of HIF, to promote HIF activity improves the anti-tumour immunity of $CD8^+$ T cells against primary and metastatic melanoma, and colorectal cancer in murine models. Unrestrained HIF activity enhanced effector functions, particularly cytotoxicity and cytokine production, along with increased transcription of inhibitory molecules and increased protein expression of costimulatory markers (177, 178). VHL deletion could also generate T_{RM}-like TILs with superior accumulation and survival within tumours. Therefore, genetic engineering platforms to reduce or ablate VHL in CAR-T cells, such the use of CRISPR knockout or interference tools, may improve anti-tumour efficacy by enforcing HIF-driven transcriptional circuits. Veliça et al. showed that ectopic overexpression of HIF-2α, particularly a factor inhibiting HIF (FIH)-insensitive mutant, was shown to more effectively enhance their anti-tumour efficacy (180). These $CD8^+$ T cells with enhanced cytotoxicity, increased expression of costimulatory molecules, and greatest *in vivo* efficacy against melanoma and leukaemia. This suggests unknown complexities in this transcriptional circuit, including the differences in HIF-1α vs HIF-2α driven-networks, and the nuances of different regulatory systems. Overall, the same cellular machinery used to adapt to hypoxia can drive improved effector phenotypes that benefit CAR-T cells for tumour elimination.

7.12 BATF3

The basic leucine zipper transcriptional factor ATF-like (BATF) family of transcription factors includes BATF1, BATF2 and BATF3 (181). Unlike BATF1 (discussed below), $CD8^+$ T cell-intrinsic BATF3 is non-essential for mounting the primary response to infection, however it is important for the establishment of memory $CD8^+$ T cell populations to ensure effective recall responses, provides protection against apoptosis, and maintains cellular fitness (182, 183). Overexpression of BATF3 promotes $CD8^+$ T cell survival and enforces a battery of genes associated with the establishment and maintenance of memory phenotypes as well as $CD8^+$ T cell function (182). In a pipeline for identifying transcription factors regulating memory phenotypes in human $CD8^+$ T cells, BATF3 was identified as a positive regulator of memory-like phenotypes and cellular fitness, amongst other more characterised proteins such as MYB and FOXO1. Overexpression of BATF3 suppressed CAR-T cell exhaustion and enhanced *in vivo* tumour control in an orthotopic breast cancer model (184). However, a recent report showed that unrestrained BATF3 due to loss of TET2 drove clonal expansion of CAR-T cells (185). Given concerns about the safety of engineered cellular products, particularly with respect to the development of secondary cancers derived from the infused cells, it is important to approach potent drivers of proliferation with caution. To overcome such issues, transient expression systems, as discussed later in this review, may be useful tools to create safer, more controlled CAR-T cells.

7.13 ETS1

ETS1 is an important enforcer of quiescence, driving other stem-like transcription factors such as TCF1 and BCL6, whilst

repressing effector molecules and regulators such as CD25 and BLIMP1 (186, 187). Although transiently downregulated during activation, ETS1 deficiency compromises the survival and proliferation of murine T cells during activation (188). In activated T cells, ETS1 is important for the maintenance of IL-7 receptor, which regulates survival and homeostasis in memory $CD8^+$ T cells (189). Recently, Zhou et al. utilised a single-cell CRISPR screening platform to unravel transcriptional regulators of diverse $CD8^+$ TIL fates, particularly those that governed transitions between different T_{PEX} and T_{EX} states. ETS1 was identified as a key repressor of differentiation from T_{PEX} into T_{EX}, and CRISPR-Cas9 KO of ETS1 in activated $CD8^+$ T cells improve anti-tumour efficacy in a suite of murine melanoma models, alongside increased cytokine production and cytotoxicity (87). Whilst many strategies revolve around enforcing memory or stem-like populations, this approach highlights the importance and advantages of permitting transition from quiescent phenotypes into differentiated subsets with effector capacities.

7.14 FLI1

FLI1 is another member of the ETS transcription factor family, however with a largely unexplored role in $CD8^+$ T cell biology. In a CRISPR screening platform, FLI1 was identified as a negative regulator of effector subsets. Consequently, KO of FLI1 generated robust effector $CD8^+$ T cell populations that displayed enhanced control of tumours and various pathogens, whilst limiting T_{PEX} numbers. Furthermore, FLI1 epigenetically restricted accessibility at ETS: RUNX binding sites, which were then exposed upon FLI1 ablation. This epigenetic remodelling due to KO of FLI1 synergised with overexpression of RUNX3 to further enforce effector subsets (190). Thus, CRISPR screens have successfully identified two members of the ETS transcription factor family as actionable molecular targets to promote effective $CD8^+$ T cell responses (87, 190). Manipulation of these transcriptional networks may enhance the performance of CAR T cells via the bolstering of effector activity.

7.15 BATF

BATF1, typically referred to as BATF, is critical for the commitment of $CD8^+$ T cells to the effector lineage as well as the proliferative burst following activation (191–193). The activity of BATF can involve complexing with partner transcription factors, most notably IRF4, as well as other AP-1 family members such as c-Jun, to promote the effector transcriptional program (193). In addition to its role in the effector T cell response, BATF expression has been correlated with $CD8^+$ T cell exhaustion, however a causal relationship has not been determined (194, 195). Interestingly, studies have shown both up- and down-regulation of BATF in $CD8^+$ T cells can improve anti-tumour immunity. Silencing or knockdown of BATF promoted memory-like phenotypes capable of superior anti-tumour immunity in a range of ACT models, including patient-derived pancreatic carcinomas (195–197). On the contrary, a recent paper by Seo et al. demonstrated that constitutive overexpression of BATF attenuated T cell exhaustion and drove superior anti-tumour control by OT.Is and CAR-T cells in preclinical mouse melanoma models. However, instead of promoting stem-like features, overexpression of BATF reinforced effector-like phenotypes (198). Notably, BATF has been shown to drive the differentiation of effector-like CX_3CR1^+ cells in chronic LCMV infection (199). This suggests a potential mechanism by which BATF overexpression in anti-tumour $CD8^+$ T cells generates robust responses. Thus, because BATF is both a driver of effector differentiation and a suppressor of memory, its transcriptional circuits may be activated to generate more effector-like $CD8^+$ T cells, or repressed to generate more memory-like $CD8^+$ T cells, ultimately improving anti-tumour efficacy.

7.16 IRF4

IRF4 is a TCR-responsive transcription factor that plays a dual role in $CD8^+$ T cell effector and exhaustion programs (39, 200). IRF4 is necessary for the differentiation of fully functional effector $CD8^+$ T cells, maximal proliferation, cytokine production, cytotoxicity, and thus critical for effective responses to bacterial, viral, and malignant threats (39, 47, 193). The role of IRF4 in $CD8^+$ T cell exhaustion is nuanced, with several studies point towards an exhaustion-inducing function of IRF4, whilst others argue that it is instead crucial for sustained cytotoxic activities (195, 200–202). In an *in vitro* model of artificially induced CAR-T cell exhaustion, IRF4 was upregulated in terminally exhausted $CD8^+$ T cells, and shRNA knockdown of IRF4 reduced inhibitory receptor expression, and enriched the T_{CM}-like population (195, 203). In contrast, another study showed that depletion of IRF4 using the DTR system compromised tumour control of by endogenous $CD8^+$ T cells and impaired the efficacy of adoptively transferred T cells (202). Thus, it remains unclear how to optimally leverage the activity of IRF4 in anti-tumour T cells. Interrogation of its downstream targets may reveal distinct circuits for driving effector responses over exhaustion. As novel genetic engineering platforms emerge alongside increased understanding of these circuits, it may be possible to more finely tune this axis to maximise anti-tumour responses.

7.17 BCL11B

BCL11B is a transcription factor that protects the T cell identity during development, particularly safeguarding from the NK lineage (204, 205). Interestingly, transcriptional networks that orchestrate immune cell lineages can be manipulate to induce transdifferentiation between different immune cell types (206). Indeed, downregulation or loss of BCL11B in T cells generates induced-T cells with NK-like features (ITNKs) that acquire an NK-like genetic signature and express functional NK receptors. This has been demonstrated in several models and at various stages of T cell differentiation, including engineered deletion using Cre/lox systems at different stages of

thymopoiesis (71), and an expanded BCL11B^{low} T cell subset in human cytomegalovirus-seropositive individuals (207). Importantly, ITNKs generated by CRISPR-Cas9 deletion of BCL11B in peripheral blood-derived human T cells demonstrate robust anti-tumour activities against leukemic cell lines *in vitro* and *in vivo* (208). Deletion of BCL11B additionally enables killing of MHC-low or MHC-negative cancer cells. Furthermore, *in vivo* anti-tumour activity against leukemic and hepatocellular carcinoma of ITNKs was enhanced when equipped with a CAR targeting CD19 or glypican-3, respectively (208). Thus, reprogramming of T cells with a hybrid phenotype that leverages both innate and adaptive lymphocyte qualities is a novel strategy to enhance the anti-tumour activity of CAR-T cells.

8 Advancements into clinical trials

Preclinical screening pipelines and novel technologies have successfully identified a number of candidate transcription factors that can be modulated to confer CAR-T cells with improved efficacy against solid tumours. To date, two of these discussed targets have advanced into preliminary phase I clinical trials. RUNX3-overexpressing CAR-T cells have been assessed for their safety profile in the treatment of metastatic hepatocellular carcinomas, demonstrating manageable toxicities thus far (209). The safety and tolerability of ITNKs, generated by BCL11B deletion, are being investigated for the treatment of a range of MHC-low or MHC-negative advanced solid tumours, and preliminary results from nine patients have been reported (208, 210). Thus far, clinical benefit (either disease stabilisation or partial remission in one case) has been observed in two thirds of treated patients, with no toxicities experienced (208). As such, transcription factor-modulated T cell products are successfully progressing into human trials, demonstrating the feasibility of generating such products. Comparisons with non-transcription factor-modulated products will be highly interesting to define the therapeutic benefit provided by rewiring these T cell phenotypes.

9 Future considerations

9.1 Targeting transcription factor combinations to improve ACT

Transcription factors in cells operate in a highly cooperative and interconnected fashion, with different binding partners or co-factors influencing the biological effects exerted. An effective strategy of rewiring anti-tumour immunity may therefore require modulating multiple transcription factors rather than a single target in isolation. Targeting combinations of transcription factors may additionally address redundancies in the biological circuitry of the cell. For example, the BLIMP1/NR4A3 dual KO (141), TOX/TOX2 dual KO (125), or NR4A triple KO T cells (73) all outperformed single KO counterparts in their respective studies. Another potential approach may be the disruption of specific transcriptional complexes. Mognol et al. used a FRET-based high-throughput screen to identify a compound that effectively disrupted NFAT: AP-1 complexes at target DNA binding sites (211). It would be interesting if this compound could redirect T cells from an activated phenotype towards tolerance or dysfunction. This could be therapeutically relevant for immunopathology caused by excessive T cell-mediated activity, such as in autoimmune diseases like multiple sclerosis or type 1 diabetes (212, 213). Alternatively, to enhance CD8$^+$ T cell responses for elimination of malignant cells, compounds that, for example, stabilised NFAT: AP1 interactions, or targeted other important transcriptional complexes, could be a promising approach for fine-tuning the molecular circuitry in T cells.

9.2 Transient TF expression

Genetic engineering platforms continue to advance at a rapid pace, providing cellular therapies with increasingly sophisticated tools. New methods may be capable of achieving more tailored transcriptional programming to better maximise CD8$^+$ T cell responses. As these methods emerge, we may find that more simplistic means of modulating gene expression such as overexpression or CRISPR KOs lack the ability to capitalise on complex biological circuits that dynamically regulate the transcriptome. For example, although promoting memory-like phenotypes is a promising approach of improving the persistence and overall functionality of T cells, transcription factors that establish memory phenotypes can work to dampen effector functions (214, 215). Could constitutive activation of memory transcriptional programs oppose full recovery of CD8$^+$ T cell effector function upon antigenic stimulation? Alternatively, attenuating T cell exhaustion often relies on downregulation of transcription factors such as BATF, NFAT and BLIMP1 which typically have a dual role in promoting effective effector responses and/or activation (137, 141, 191, 195). Could constant repression or total ablation of these transcription factors be partially limiting effector responses? Furthermore, complete ablation or constitutive overexpression of transcription factors with tumour-suppressor functions (e.g. BLIMP1 (216)) or oncogenic potential (e.g. BATF3 (185, 217)) may be dangerous in modifying a cellular product. A more refined approach to enhancing T cell anti-tumour immunity may instead be temporal, context-dependent modulation of transcription factors as and when they are needed.

Druggable systems exist, such as the Tet-On/Off system, which allow for manual control of gene expression (218). Indeed, one study demonstrated that using 4-hydroxytamoxifen, zinc finger transcriptional activation of an anti-CD20 CAR could achieve titratable CAR expression *in vitro* and *in vivo* (219). However, to extrapolate druggable approaches such as this to expression of transcription factors may be unfeasible, as it may prove to be time consuming and challenging to determine dosing strategies.

Several novel strategies are emerging to achieve inducible gene expression. One such strategy is the design of 'logic-gated' CARs, which upregulate gene programs in response to specific environmental stimuli (220–222). For instance, synthetic Notch CAR-T cells have been engineered to upregulate CAR expression

upon recognition of tumour antigens by a synthetic Notch receptor. This approach circumvented issues associated with tonic CAR signalling, generating more persistent cellular products that exhibited enhanced anti-tumour control in preclinical solid tumour models (222). Synthetic Notch receptors are extremely versatile tools. The intracellular domain of such receptors have been engineered to contain transcription factors directed towards reporter genes to provide functional readouts (223). Another strategy is knocking-in or inserting a gene of interest into the locus of an endogenous gene, such that the expression of the inserted gene is under the control of the promoter of the endogenous gene. This was successfully performed by Kim et al. The IL-12 gene was inserted into the PD-1 locus in T cells to achieve IL-12 expression upon T cell activation and target-cell recognition whilst simultaneously disrupting the wildtype PD-1 receptor, overall enhancing the effector function and anti-tumour responses of T cells (224). Degron technologies that exploit protein degradation pathways to rapidly target and ablate proteins can also be used. Jan et al. tagged a CAR construct with the degron of the IKZF3 protein, which can be targeted using lenalidomide, a pharmacological mediator of IKZF3 and several other proteins degradation, to induce rapid degradation of the CAR (225). Another form of degron technology called the bioPROTAC, which binds and tags a degron to a target protein, has been combined with synthetic Notch receptors in a CAR construct, to generate an inducible system modulating endogenous levels of a protein of interest in tandem with CAR expression (226). Repurposing inducible systems such as synthetic Notch receptors, degron technologies, and site-specific knock-ins, to modulate the expression of transcription factors that drive superior anti-tumour $CD8^+$ T cell responses may be promising approaches for achieving dynamic transcriptional modulation.

9.3 Tissue specificity

Another interesting prospect for the field is the possibility of engineering tissue-specific CAR-T cells. As highlighted previously, transcription factors such as RUNX3 can be targeted to drive accumulation of T cells in tumour sites (59). Transcriptional programs driving T_{RM} cells have been shown to overlap with programs in TILs, which is coherent with these populations sharing a common propensity for tissue accumulation and retention, as opposed to recirculation. Interestingly, it has been shown that immune cells in different tissues have specific transcriptional and metabolic programs that promote their residency within these distinct niches. Whether this is due to specific trafficking processes, survival capabilities within their unique metabolic milieu, or other phenomena, is unknown. These unique, tissue tropic transcriptional modules have been investigated in a range of immune cells including macrophages (227) and ILC2s (228). Recently, a study identified a transcriptional network that governed tissue-specific residency of $CD8^+$ T cells in the small intestine (229). Targeting tissue tropism may be one strategy to enhance ACT towards tissue specific tumours. Genetic engineering strategies could co-opt tissue-specific transcriptional networks in immune cells to generate cells that specifically hone to a cancer's tissue of origin. For instance, could engineering of liver-specific tissue residency transcription factors create liver-tropic CAR-T cells that more effectively control hepatocellular carcinomas? Could this strategy be used to target metastatic disease that is almost exclusively observed in one organ, such as uveal melanoma metastases present in the liver? As the field unravels the complexities of residency within the diverse and distinct niches of the body, new molecular targets to create tailored CAR-T cell therapies may emerge.

9.4 Looking beyond CAR-T cell therapy and cancer

Finally, although CAR-T cell therapy for cancer draws immense attention in preclinical research and in the clinic, other forms of cell therapies exist for the treatment of cancers and other diseases. The rewiring of transcriptional circuitry may be a powerful tool across these various platforms. TIL therapy is a prime example of another T cell-based therapy that may be enhanced by reshaping the transcriptional landscape. TIL therapy is used for the treatment of relapsed or refractory metastatic melanomas, outperformed anti-CTLA treatment in a Phase 3 clinical trial, and has recently been approved for the clinic by the FDA (230–232). TILs from patient data sets and preclinical murine models provide valuable tools for unravelling tumour-specific T cell exhaustion (233, 234). As such, the targets identified from these data sets may be directly applicable to TIL therapy, counteracting or reinforcing highly relevant biological circuits in these cells. For example, CRISPR/Cas9 KO of *PRDM1* in human TILs can restore polyfunctional cytokine production and expression of memory surface markers CD62L and CCR7 (142). In addition, other immune cells such as NK cells and macrophages are also emerging as promising alternatives to T cell therapies, and transcriptional rewiring may be an attractive approach to further enhance their respective anti-tumour capabilities (235–237). Recently, ID3 expression was found to program effective anti-tumour macrophages (238). Thus, transcriptional modulation to enhance cell therapies may be a versatile strategy that extends beyond cancer, and beyond T cells. CAR-T cell therapies are also under development for pathologies beyond cancer (239–241). Recent clinical trials have demonstrated therapeutic efficacy using CAR-T cells for HIV and various autoimmune diseases (242, 243). Importantly, many major discoveries regarding T cell fate decisions and their governing transcriptional networks are owed to pioneering fundamental research in the anti-viral immunity field. Anti-viral cell therapies may benefit from exploiting these transcriptional circuits that have already been characterised in a viral context, to provide superior future outcomes for patients with chronic infections such as HIV.

10 Conclusions

Major advances in powerful sequencing technologies have been instrumental in unravelling the intricate transcriptional networks that govern the phenotype and function of $CD8^+$ T

cells. This accelerated discovery of genetic targets is met with concurrent developments in genetic engineering platforms that allow us to leverage these molecular drivers with increasing efficiency and accuracy. Preclinical screening pipelines have demonstrated that targeting the expression of transcription factors in CAR-T cell products is an effective approach to overcome existing barriers in the treatment of solid tumours, with emerging clinical data demonstrating that this strategy is feasible in the clinic. Thus, emerging strategies to enhance CAR-T cell therapy hold promise for improving clinical outcomes to patients with cancer.

Author contributions

SS: Conceptualization, Writing – original draft, Writing – review & editing. JA: Conceptualization, Supervision, Writing – original draft, Writing – review & editing. JN: Supervision, Writing – review & editing. JW: Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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