

Programming CAR-T cells to kill cancer

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T cells engineered to express chimeric antigen receptors (CARs) that are specific for tumour antigens have led to high complete response rates in patients with haematologic malignancies. Despite this early success, major challenges to the broad application of CAR-T cells as cancer therapies remain, including treatment-associated toxicities and cancer relapse with antigen-negative tumours. Targeting solid tumours with CAR-T cells poses additional obstacles because of the paucity of tumour-specific antigens and the immunosuppressive effects of the tumour microenvironment. To overcome these challenges, T cells can be programmed with genetic modules that increase their therapeutic potency and specificity. In this Review Article, we survey major advances in the engineering of next-generation CAR-T therapies for haematologic cancers and solid cancers, with particular emphasis on strategies for the control of CAR specificity and activity and on approaches for improving CAR-T-cell persistence and overcoming immunosuppression. We also lay out a roadmap for the development of off-the-shelf CAR-T cells.

The surface of virtually every cell in the body displays endogenous peptides, derived from intracellular proteins, as complexes with major histocompatibility proteins. This ensures that if a cell is infected with a pathogen or undergoes malignant genetic transformation, peptides derived from the pathogen or from the mutant proteins will be exposed to patrolling T cells. Each T cell has an exquisitely sensitive and specific T-cell receptor (TCR) that constantly surveys the organism in search of ‘non-self’ signals, triggering a cascading immune response when a foreign peptide is identified. T cells are therefore potent and precise killers of foreign entities, be these pathogenic invaders or malignant cells.

Tumours are frequently invaded by immune cells as part of the body’s response to malignant growth. Among these immune infiltrates, tumour-infiltrating lymphocytes (TILs) include T cells specific for mutated proteins that are present in cancer cells. These TILs can be isolated from a patient’s tumour tissues, expanded *ex vivo* to high numbers and adoptively transferred back into the patient to elicit a durable antitumour response¹. However, this procedure carries several limitations: TILs need to be harvested during surgery, are not found in sufficient numbers for all cancers, and in many cases expand poorly². A potentially broader therapeutic approach involves collecting polyclonal T cells from the blood and, through genetic engineering, inserting in them genes encoding a TCR that is specific for a known tumour antigen. For instance, T cells bearing an engineered TCR targeting the NY-ESO-1 antigen have yielded promising results in the treatment of patients with multiple myeloma and synovial cell sarcoma^{3,4}.

Using tumour-specific TCRs enables the targeting of both membrane and cytoplasmic proteins; unfortunately, cancers can evade this endogenous recognition-and-response program by downregulating major histocompatibility complex (MHC) proteins or other factors required for antigen processing and presentation, rendering cancer cells invisible to patrolling T cells. To overcome this evasion strategy by the tumours, T-cell specificity can be directed to tumour-associated antigens (TAAs) in a MHC-independent manner. This can be achieved by employing chimeric antigen receptors (CARs).

CARs combine the specificity of a high-affinity recognition domain, most often derived from a monoclonal antibody (mAb), with the cytolytic properties of T cells. T cells modified using CARs

(CAR-T cells) have shown unprecedented potency against haematologic malignancies, as exemplified by the several clinical trials of CD19-CAR-T-cell therapy that have reported complete response rates of 50–90% for refractory B-cell malignancies^{5–13}. These results led to approval from the US Food and Drug Administration (FDA) in the second half of 2017 for the first CAR-T therapies^{14,15}. Such early success has ignited a revolution in cancer immunotherapy, and intensified the focus on the development of strategies that extend the use of CAR-T cells to other haematological malignancies and, in particular, to solid tumours, which presents substantial challenges with regard to efficacy and safety. In this Review Article, we survey several next-generation approaches for the engineering of CAR-T cells that hold potential for overcoming these challenges. In what follows, we discuss the design of CARs for increased tumour specificity, and how functional modules and pathways can be implemented to regulate the activity of CARs for enhanced safety, including options available for enhancing the delivery of transgenes into T cells. We also discuss approaches for overcoming immunosuppression mechanisms that protect the tumour from the immune system, and describe how modulation of the tumour microenvironment can promote antitumour therapies.

The molecular architecture of CARs

CARs transduce antigen recognition events into a signalling cascade that evokes T-cell effector functions, such as the secretion of cytotoxic factors and pro-inflammatory cytokines. The essential components of these synthetic receptors are an extracellular antigen-targeting moiety, such as a single-chain variable fragment (scFv), a transmembrane and hinge domain that anchors the receptor on the cell surface and projects the scFv out to the extracellular space, and intracellular signalling domains that are triggered on antigen engagement (Fig. 1a).

The antigen-binding domain. The antigen specificity of a CAR is most often conferred by the scFv module (the smallest functional domain of a mAb), which contains only the variable regions of the light and heavy chain fused via a flexible linker. The advantages of using scFvs as the CAR antigen-binding domain include their high specificity, the fact that they can be readily generated against

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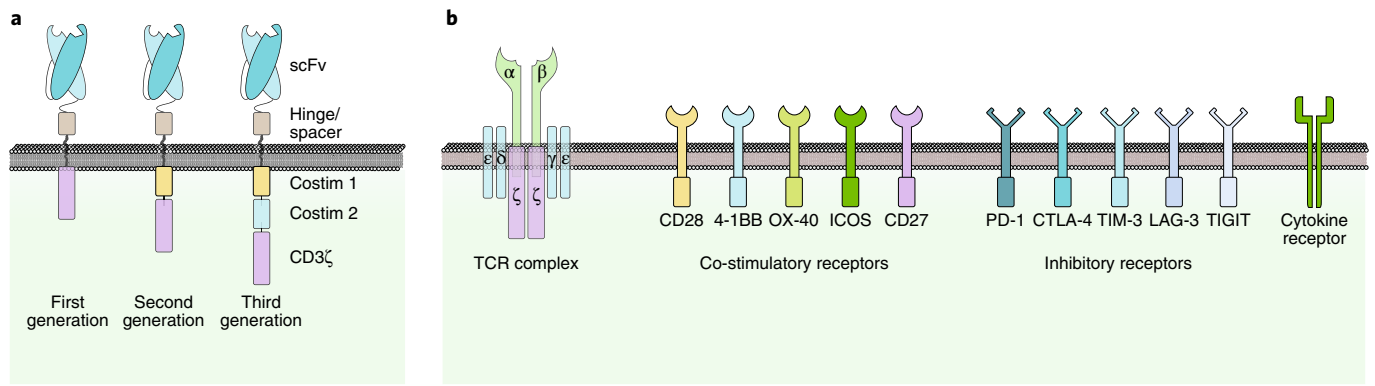


Fig. 1 | CAR and TCR structure. **a**, CARs comprise an extracellular antigen-targeting moiety, such as an scFv, a transmembrane and hinge domain that anchors the receptor to the cell surface and projects the scFv into the extracellular space, and intracellular signalling domains that activate the T cells following antigen engagement. First-generation CARs contain only a CD3 ζ signalling domain. Second-generation and third-generation CARs incorporate one or more co-stimulatory domains (costim), respectively. **b**, The TCR complex is composed of variable α - and β -chains associated with CD3 invariant chains. Full T-cell activation requires signal 1 and 2 from the TCR complex, as well as co-stimulatory receptors. Signal 3, which is transmitted by cytokine receptors, is important for survival, differentiation and memory formation. TIM-3, T-cell immunoglobulin and mucin domain-containing protein-3; TIGIT, T-cell immunoreceptor with Ig and ITIM domains.

most antigens of interest through well-established methods, and how easily they can be grafted into the CAR design¹⁶. Conversely, a disadvantage of using scFv-binding domains is their tendency for oligomerization, which can lead to tonic signalling and T-cell exhaustion¹⁷. Alternative binding domains have been used in pre-clinical studies, including receptors¹⁸, ligands¹⁹, cytokines^{20,21}, DARPins (designed ankyrin repeat proteins)²², adnectins²³, Fc receptor fragments²⁴, nanobodies²⁵, peptides²⁶ and variable lymphocyte receptors²⁷. The relative efficacy, safety and immunogenicity of these alternative binders have not yet been fully investigated and are the focus of substantial ongoing work.

The ability of a CAR to transduce a strong activating signal to the T cell is influenced by many factors, including the binding affinity to the antigen, the level of expression, antigen density on the target cell, and epitope proximity. The relationship between binding affinity and efficacy is more nuanced in the context of CARs as compared with mAbs, for which higher affinity is typically desirable. For example, on the basis of observations from preclinical studies, whereas a receptor tyrosine kinase-like orphan receptor 1 (ROR1)-CAR derived from a high-affinity scFv (with a dissociation constant of 0.56 nM) resulted in increased therapeutic index when compared with a lower-affinity variant^{28–30}, other examples have reported that engineering the scFv for lower affinity improves the discrimination among cells with varying antigen density^{31–33}, which could be useful for enhancing the therapeutic window for antigens differentially expressed on tumour versus normal tissues. However, this approach might increase the risk of immune evasion, owing to the emergence of low-antigen-expressing tumour cells.

Other factors such as the proximity of the target antigen-binding site to the cell surface can also have strong effects on lytic activity and cytokine production^{34,35}, which in some cases supersedes the effects of affinity. In the context of CD22-CARs, a comparison between two scFvs targeting the same epitope but with varying affinities did not reveal a significant impact of affinity on CAR function, whereas an alternative CD22 binder (m971) with lower affinity proved most efficacious, possibly because of better accessibility of the targeted epitope^{34,35}. Thus, for a given antigen density on a target cell, there is likely to be an optimal range of scFv affinities and CAR expression levels required for a specific and effective antitumor response^{36–38}.

Co-stimulatory domains. First-generation CARs, which relied solely on the intracellular domain of CD3 ζ for signalling, had limited

efficacy in clinical trials owing to the weak expansion potential of the CAR-T cells *in vivo*³⁹ and despite observations of significant antitumor responses with CARs of this kind that targeted the GD2 protein in neuroblastoma^{40,41}. Incorporation of one or more co-stimulatory domains to provide additional activating signals (Fig. 1a) has resulted in enhanced clinical responses and persistence^{5,6,42–44}. The choice of co-stimulatory domain influences the phenotype and metabolic signature of T cells. For example, CD28 co-stimulation yields a potent, yet short-lived, effector-like phenotype, with high levels of cytolytic capacity, interleukin-2 (IL-2) secretion and glycolysis⁴⁵. By contrast, T cells modified with CARs bearing 4-1BB co-stimulatory domains tend to expand and persist longer *in vivo*, have increased oxidative metabolism, are less prone to exhaustion, and have an increased capacity to generate central memory T cells^{17,44–48}. Whereas the vast amount of studies to date are based on CD28 or 4-1BB CARs, several other co-stimulatory domains are currently being evaluated (Table 1)^{48,49}. Preclinical studies have reported that

Table 1 | CAR co-stimulatory domains

Domain	Properties	Refs
CD28	Potent effector function, rapid initial expansion, high IL-2 secretion and increased glycolysis	45–48,245
4-1BB	Increased persistence and central memory formation, increased oxidative metabolism and decreased exhaustion	17,44–48
OX-40	Similar persistence and pro-inflammatory cytokine secretion as 4-1BB <i>in vitro</i> ; decreased secretion of IL-10	246–248
ICOS	High IFN- γ production; increased IL-17A, IL-17F and IL-22 production; TH17 phenotype	248,249
CD27	Increased persistence and expression of Bcl-XL	250,251
MyD88/CD40	Inducible MyD88/CD40 resulted in improved cytokine secretion, proliferation and survival	49,77

ICOS, inducible co-stimulator; TH17, T-helper 17 cell; Bcl-XL, B-cell lymphoma-extra large protein.

third-generation CARs — bearing both CD28 and 4-1BB domains — have superior efficacy when compared with second-generation constructs^{50,51}; however, others reported opposite findings^{52,53}. Expression of the 4-1BB ligand in CD28 ζ -based CARs has also been shown to extend CAR-T-cell persistence, and is currently being tested in clinical trials¹⁷ (NCT03085173).

Hinge and transmembrane domains. The flexible hinge domain is a short peptide fragment that provides conformational freedom to facilitate binding to the target antigen on the tumour cell. It may be used alone or in conjunction with a spacer domain that projects the scFv away from the T-cell surface^{28,54}. Previous studies have suggested that the optimal length of the spacer depends on the proximity of the binding epitope to the cell surface, with proximal epitopes requiring longer spacers and distal epitopes requiring shorter ones^{28,55}.

Besides promoting binding of the CAR to the target antigen, achieving an optimal distance between the T cell and the cancer cell may also help to sterically occlude large inhibitory molecules from the immunological synapse⁵⁶. Long spacers typically include the CH2CH3 domain (~220 amino acids) of immunoglobulin G1 (IgG1) or IgG4 (refs 34,57), whereas the CH3 region can be used on its own to construct an intermediate spacer²⁸ (~120 amino acids). Shorter spacers are derived from segments (<60 amino acids) of CD28 (refs 30,34,58), CD8 α (refs 30,34,50,58,59), CD3 (ref. 60) or CD4 (ref. 61). Several CARs with long spacers comprised of the CH2CH3 domain were reported to have diminished efficacy and persistence in vivo despite potent effector function in vitro, a disparity that was shown to result from binding of Fc receptors expressed on macrophages, monocytes and natural killer (NK) cells. These off-target interactions resulted in increased activation-induced cell death of CAR-T cells and cross-activation and lysis of these innate immune cells⁶². Following these observations, deletion and mutation of CH2CH3 residues that resulted in abrogated Fc receptor binding led to a significant improvement of in vivo persistence and efficacy in murine models^{57,63}.

The properties of the transmembrane domain have not been as meticulously studied, but they can potentially affect CAR expression and association with endogenous membrane proteins^{58,64,65}.

Improving safety

Unlike traditional cancer drugs (such as small molecules and antibodies) that rely on passive targeting, T-cell-based therapeutic agents can actively home to disease sites, sense the microenvironment and integrate multiple inputs to mount a complex and dynamic immune response. These cells are also capable of self-amplification, renewal and differentiation into distinct effector subsets. Despite all these advantages, such complex behaviour can pose challenges with regard to safety. Recent clinical trials have reported CAR-T-associated toxicities, such as cytokine-release syndrome (CRS), neurotoxicity, off-tumour effects and acute respiratory distress syndrome, all of which are potentially fatal^{66–68}. These adverse effects highlight the need for developing control programs to modulate the activity of CARs, and numerous approaches have been explored to program these genetically engineered systems with feedback control systems and safety modules, by leveraging recent advances in synthetic biology (Figs. 2 and 3).

Control switches. One strategy to control severe CAR-T-cell toxicity is to include a mechanism for rapid ablation of the transplanted cells through a suicide switch (Fig. 2). For example, cells expressing inducible caspase 9 (iCasp9) can be killed by the addition of a small-molecule drug that dimerizes and activates the protein to induce programmed cell death⁶⁹. This system was used to eliminate 90% of T cells expressing iCasp9 within 30 minutes in patients receiving a hematopoietic stem cell transplant, and reversed

graft-versus-host disease (GVHD)⁷⁰. Alternative suicide genes include epitope tags that are recognized by FDA-approved mAb therapeutics such as cetuximab^{71,72} or rituximab^{73,74}, which induce T-cell death through endogenous antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity. These epitope tags can also serve as handles for purification of transduced T-cell populations^{11–13}. Neither drug-induced nor mAb-mediated suicide switches have been tested clinically in the context of CAR-based therapeutics, and therefore it remains unclear how effective they may be in mitigating both on-target and off-target toxicity.

Suicide switches are effective at stopping the toxicity of the engineered cells, but result in the irreversible termination of the therapeutic treatment. Alternatively, non-cytotoxic reversible systems may be useful for controlling adverse toxicity without deleting the therapeutic cells (Fig. 3). For instance, the CAR gene can be placed under the control of inducible expression systems that toggle the transcription of the CAR between on and off states through small molecules such as doxycycline and tetracycline. These safety mechanisms may have limitations, such as a relatively slow induction or reduced receptor expression, and inherent leakiness of the system can lead to residual CAR expression even in the absence of the inducer⁷⁵. Chemical inducers can also be used to control the assembly of the CAR signalling domains at the protein level through drug dimerization domains. In this context, a small molecule that brings together the ζ -chain and CAR to modulate CAR activation (Fig. 3a)⁷⁶ and another similar model involving a MyD88/CD40 inducible co-stimulatory molecule^{49,77} have been reported.

Modular and switchable CAR (sCAR) designs in which the specificity is determined by a soluble antigen-binding fragment (Fab), administered in parallel to the sCAR-engineered cell, add important safety and specificity functionalities (Fig. 3b)^{78,79}. Rather than recognizing an antigen, the sCAR binds to a bioorthogonal tag placed on a Fab derived from a mAb such as Herceptin. In this design, the Fab acts as an adapter that bridges the T cell and the TAA-expressing cell. Alternatively, CARs specific for Fc domains can be directed at TAAs through co-administration of commercially available or proprietary mAbs^{24,80}. These approaches are only now being tested in the clinic, but preclinical results suggest that CAR activity and specificity can be tuned according to the type and dose of the bridging antigen-binding molecule administered, which may provide a means to both limit the activity of CARs for safety and target multiple antigens, thus hindering antigen escape by the tumour.

Combinatorial antigen sensing. An ideal tumour antigen is exclusively expressed by tumour cells and not by normal tissues, as in the case of epitopes derived from tumour-specific mutated or glycosylated proteins^{59,81}. Although a great effort is being applied to discovering such antigens, the vast majority of candidate antigens are also expressed on healthy tissues, albeit at lower levels. Consequently, T-cell-based therapies targeting these antigens carry the potential for on-target, off-tumour toxicities^{82–84}. One strategy to mitigate these effects and increase specificity is to require multiple TAAs to be engaged for full T-cell activation (AND gate, Fig. 4a). This strategy was used in a system that employed a synthetic notch receptor (synNotch) that releases a transcription factor on binding antigen 'A', which in turn drives expression of a CAR specific for antigen 'B'⁸⁵. These AND-gated T cells were able to clear tumours bearing both antigens in a xenograft murine tumour model while sparing cells expressing only one of the antigens. However, once the synNotch induced expression of the second CAR, that expression was sustained for some time; therefore, such cells could travel throughout the body and subsequently react against normal tissues expressing antigen B. Although this type of toxicity was not observed in mouse models, these were short-term experiments that do not fully replicate the effects of long-lived T cells in humans.

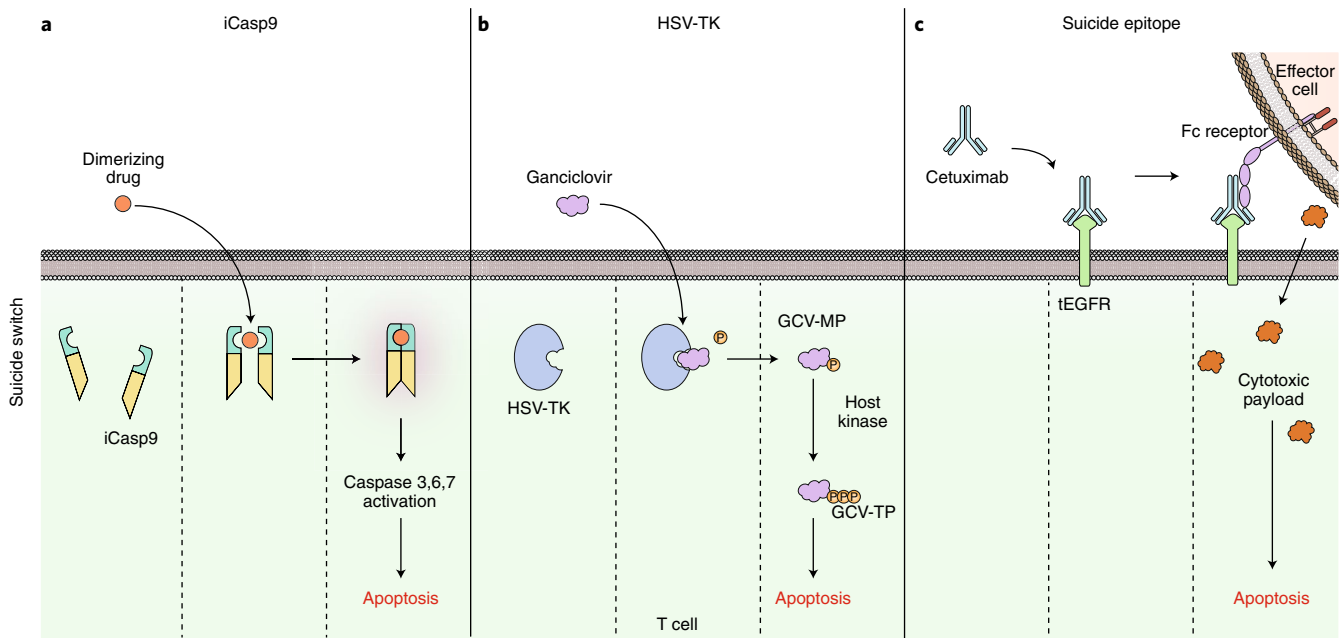


Fig. 2 | Suicide switches. **a**, The addition of a dimerizing drug activates iCasp9 signalling and leads to apoptosis⁶⁹. **b**, T cells expressing HSV-TK convert the prodrug ganciclovir into a cytotoxic molecule that results in cell death²⁴⁴. **c**, A truncated form of EGFR acts as an epitope handle that can be used to target T cells with a mAb such as cetuximab⁷¹. T cells that are tagged with cetuximab are killed through antibody-dependent cell-mediated cytotoxicity or complement-dependent cytotoxicity. HSV-TK, herpes simplex virus thymidine kinase; GCV-MP, ganciclovir monophosphate; GCV-TP, ganciclovir triphosphate; tEGFR, truncated EGFR.

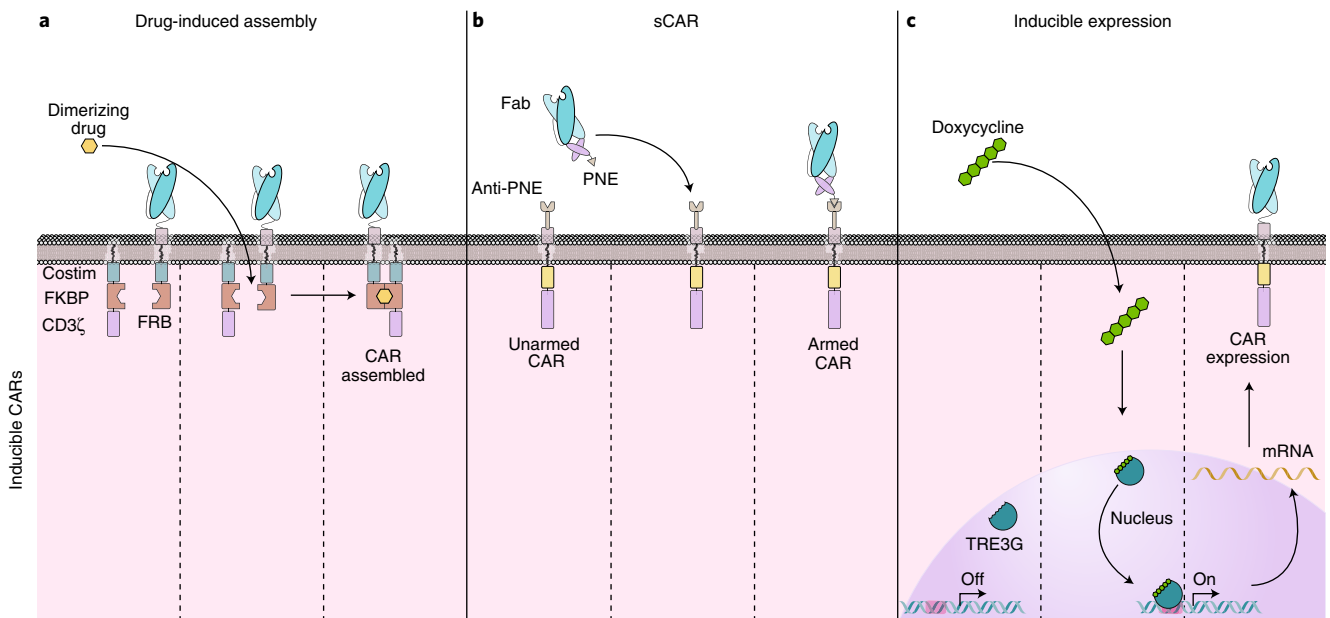


Fig. 3 | Inducible CARs. **a**, CAR domains can be split into separate non-functional receptors. Assembly of the CAR can be induced through the addition of a dimerizing drug⁷⁶. **b**, sCARs are specific for a bi-orthogonal tag, such as a peptide neo-epitope (PNE). sCAR-T cells can be directed to a TAA through the addition of a Fab tagged with PNE⁷⁸. **c**, CAR expression can be controlled by TRE3G-based expression systems. The addition of doxycycline induces a conformational change in TRE3G that enables it to turn on the expression of the CAR⁷⁵. FKBP, FK506 binding protein; FRB, FKBP12-rapamycin binding domain; TRE3G, tet response element 3G.

CARs can also be made to discriminate between cancer and healthy cells through co-expression of an inhibitory CAR (iCAR) that dampens the T-cell response when a healthy antigen is present (AND–NOT gate, Fig. 4b)⁸⁶. In iCARs, an antigen-binding domain against a ‘healthy tissue’ antigen is fused to inhibitory intracellular

signalling domains, such as those in programmed cell death protein 1 (PD-1) or cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). Other combinatorial antigen approaches are based on segregation of the CD3ζ domain and the co-stimulatory domain on two different CARs^{87,88}; in this way, the ‘signal 1’ and ‘signal 2’ necessary

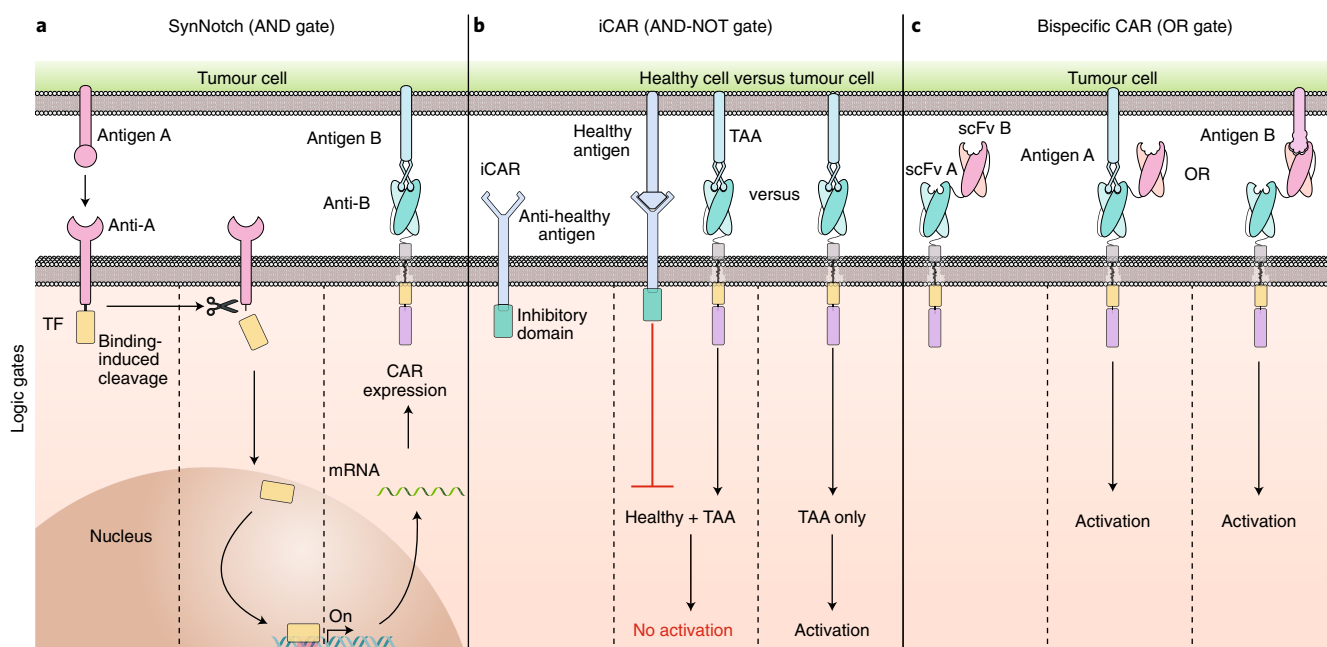


Fig. 4 | Logic gates. **a**, On binding antigen A, the synNotch receptor undergoes a conformational change that leads to the release of a transcription factor (TF), which subsequently translocates to the nucleus and turns on the expression of a CAR for antigen B⁸⁵. **b**, iCARs dampen the T-cell response when a healthy antigen is encountered. T-cell activation only occurs in the absence of the healthy antigen⁸⁶. **c**, Bispecific CARs can be activated in response to a cell expressing antigen A or antigen B.

for T-cell activation (Fig. 1b) are only combined in response to the engagement of both antigens. A potential disadvantage of this approach is that it assumes that individual CD3 ζ or co-stimulatory CARs will not function alone; however, activity has been detected in CD3 ζ -only CARs^{30,40}, which could mediate deleterious effects if co-stimulatory signals from an inflammatory tumour microenvironment are provided.

The specificities of natural TCRs can provide additional input for CAR-T cell sensing. $\gamma\delta$ T cells comprise a small fraction of circulating and tissue-resident lymphocytes that recognize antigens in a MHC-independent manner. A subset of these cells expressing the V γ 9V δ 2 TCR are thought to recognize transformed or metabolically dysfunctional cells by binding to specific phosphoantigens that act as ‘danger signals’ presented on the cell surface⁸⁹. This property has been explored by inserting GD2-CARs into $\gamma\delta$ T cells so that cytotoxicity towards GD2⁺ tumour cells was restricted only to those expressing these danger signals⁹⁰. Furthermore, by employing a CAR that contained only a DAP10 co-stimulatory signalling domain, both the $\gamma\delta$ TCR and CAR needed to be engaged for lytic activity — an approach that could potentially limit off-tumour effects.

Although targeting TAAs also expressed on normal tissues risks on-target, off-tumour toxicity, evidence from preclinical models suggests that CAR-T cells may not react to low-level antigen expression in healthy tissues^{33,36–38}. Instances of human epidermal growth factor receptor 2 (HER2)-CAR off-tumour toxicity⁹² have been largely overshadowed by safe trials with a distinct HER2-CAR that demonstrated clinical efficacy in patients with sarcomas^{91,92}. Combinatorial antigen-sensing programs have the potential to provide increased safety by adding an extra layer of specificity to avoid adverse toxicities; however, increasing the stringency of antigens required for CAR activation could facilitate cancer cell evasion via antigen escape.

Autonomous sensing and activation. CAR safety modules can also be designed to exert spatial control over targeting by activating the

CAR-T cells only at the site of the tumour (Fig. 5). Masked CARs achieve this by including an inhibitory peptide, tethered extracellularly via a cleavable linker, that reversibly blocks the scFv and keeps the T cells in an off state until they reach the tumour microenvironment⁹³. Once there, proteases specifically secreted locally cleave the inhibitory peptide and unmask the CAR, priming it for targeting tumour cells. One report showed that a masked CAR had comparable efficacy to non-masked CARs when targeting the epidermal growth-factor receptor (EGFR) in a murine xenograft model, but further studies are required to demonstrate that the strategy leads to decreased on-target, off-tumour effects given that the scFv used was derived from cetuximab, which has minimal cross-reactivity with mouse EGFR⁹⁴. Another feature of the tumour microenvironment that can be explored for context-dependent activation of CARs is the hypoxic milieu generated by inadequate tumour vascularization⁹⁵. This feature has been taken advantage of by the design of a CAR with oxygen-sensitive domains that is degraded in normoxic conditions but remains stabilized in hypoxic conditions⁹⁶. Although the surface expression of this construct was low and the system had a limited dynamic range, it nevertheless demonstrates that the concept of engineering T cells with microenvironmental sensors can enable control over their activity.

If CAR therapy is to become widely applicable, it is without doubt that further developments of CAR-T technology need to address the potential for toxicity. The on-target, off-tumour effect observed with the CD19-CAR, which results in killing of healthy B cells, is generally well tolerated and can be managed with intravenous immunoglobulin therapy. However, these effects may be detrimental when targeting TAAs in solid tumours, as most of these are also expressed on essential tissues. Several cell-engineering strategies have provided possible means to improve specificity and control of basic CAR-T function in vivo. Future developments may include autonomous feedback loops and control mechanisms that enable engineered T cells to continuously sense key biochemical cues in the local microenvironment and to adjust their response accordingly. For instance, a CAR-T cell could potentially be programmed

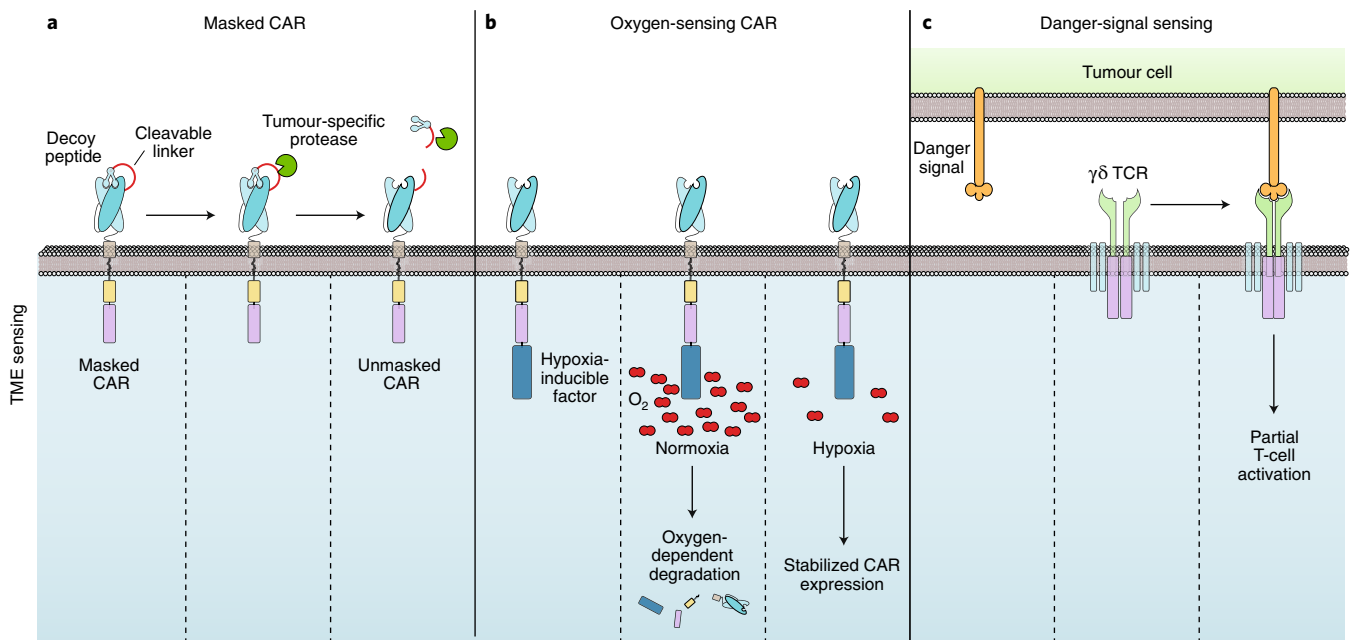


Fig. 5 | Sensing the tumour microenvironment. **a**, Masked CARs are kept in an off state through a decoy peptide that is tethered via a cleavable linker. Once the T cell reaches the tumour microenvironment (TME), specific proteases liberate the decoy peptide and unmask the CAR for targeting⁹³. **b**, Oxygen-sensing CARs are selectively degraded under normoxia, yet stably expressed in hypoxic conditions. **c**, $\gamma\delta$ TCRs can recognize transformed cells through danger signals expressed on the cell surface⁹⁰.

to secrete proteins that neutralize the effects of cytokines associated with CRS when inflammatory cytokines surpass a specific threshold. With many unresolved challenges left in the field, it is probable that CARs will continue to drive development and clinical testing of novel synthetic biology tools.

Improving efficacy and overcoming immune suppression

Improving the efficacy of CAR-T therapies to extend their application beyond blood cancers has met several formidable hurdles. After infusion into a patient, the T cells must traffic to the tumour site, infiltrate the tumour stroma and engage the specific TAA on malignant cells. Then, they must rapidly expand to high numbers in response to an antigen and mount the attack on cancer cells within a harsh tumour microenvironment that features multiple physical barriers^{97–99}, a biochemical and metabolic milieu that is unfavourable to T-cell effector function^{100–102}, and immunosuppression signals from an arsenal of inhibitory molecules displayed or secreted by cancer cells and supporting stromal cells alike^{103–105} (Fig. 6). Additionally, T-cell activation must be sustained to enable clearing of the tumour before the cells become dysfunctional or exhausted^{106,107}. Ideally, the CAR-T cells should then persist after tumour elimination and form memory cells to provide long-lasting protection. Engineering T cells with additional functional modules, along with their use in combination therapies, can help circumvent these obstacles (Table 2).

Homing and trafficking. To leave blood vessels and accumulate within solid tumours, circulating T cells must undergo extravasation, a highly coordinated process mediated by an array of factors such as adhesion molecules, chemokines and chemokine receptors¹⁰⁸. The tumour vasculature, however, has a deregulated chemokine profile and downregulates key adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) (ref. ¹⁰⁹). In addition, natural T cells may not express the appropriate chemokine receptors to enable trafficking into specific tumour tissues, leading to poor homing and opening the door to tumour immunoevasion¹¹⁰. One strategy to overcome this deficit is to engineer T cells to express

chemokine receptors such as CCR2 (refs ^{111,112}), CCR4 (ref. ¹¹³) and CXCR1 (ref. ¹¹⁴). Tumours often secrete chemokines for these receptors to attract supporting stromal cells; thus, ectopic expression of these receptors or other factors that may enhance tumour trafficking in adoptively transferred T cells provides a potential means to preferentially direct their migration to the tumour site, which has been shown to improve antitumour efficacy in animal models.

Expansion and persistence. After trafficking to the tumour site and encountering their cognate antigen, T cells must undergo rapid expansion to attain the appropriate numbers relative to the tumour burden. The efficacy of CAR-T-cell treatment in clinical trials has been highly correlated with the T cells' capacity for in vivo expansion and persistence^{3,44,115}. Preconditioning treatments with chemotherapeutics that deplete native lymphocytes^{12,13} or small-molecule inhibitors such as ibrutinib have been shown to improve CAR-T-cell engraftment in haematologic malignancies^{115,116}. For CAR-T cells to achieve optimal activation and proliferation, they require not only CD3 ζ and co-stimulatory domain activity, which provide signals 1 and 2, but also a third signal provided by immunostimulatory cytokines. However, these activating cytokines are often downregulated in the microenvironment of solid tumours. To overcome this hurdle, several transgenic-based strategies have been developed to deliver those missing cytokines. Preclinical studies have shown that T cells engineered to secrete IL-12 (refs ^{117,118}), IL-15 (ref. ¹¹⁹), IL-18 (ref. ¹²⁰) and IL-21 (ref. ¹²¹) can help to promote the cells' proliferation and reshape the tumour microenvironment. However, exogenous expression of such cytokines can ultimately hinder ex vivo expansion and result in increased toxicity in some cases¹²². In addition, several inflammatory cytokines may potentially counteract CAR-T-cell therapy by triggering negative feedback regulatory loops to dampen inflammation via indirect induction of immunosuppressive responses^{123–125}.

Chimeric cytokine receptors — engineered chemokine receptors with modified functions — can be used to achieve selective expansion of T cells by an administered cytokine¹²⁶. However, systemic administration of cytokines can result in significant toxicities^{127–130}.

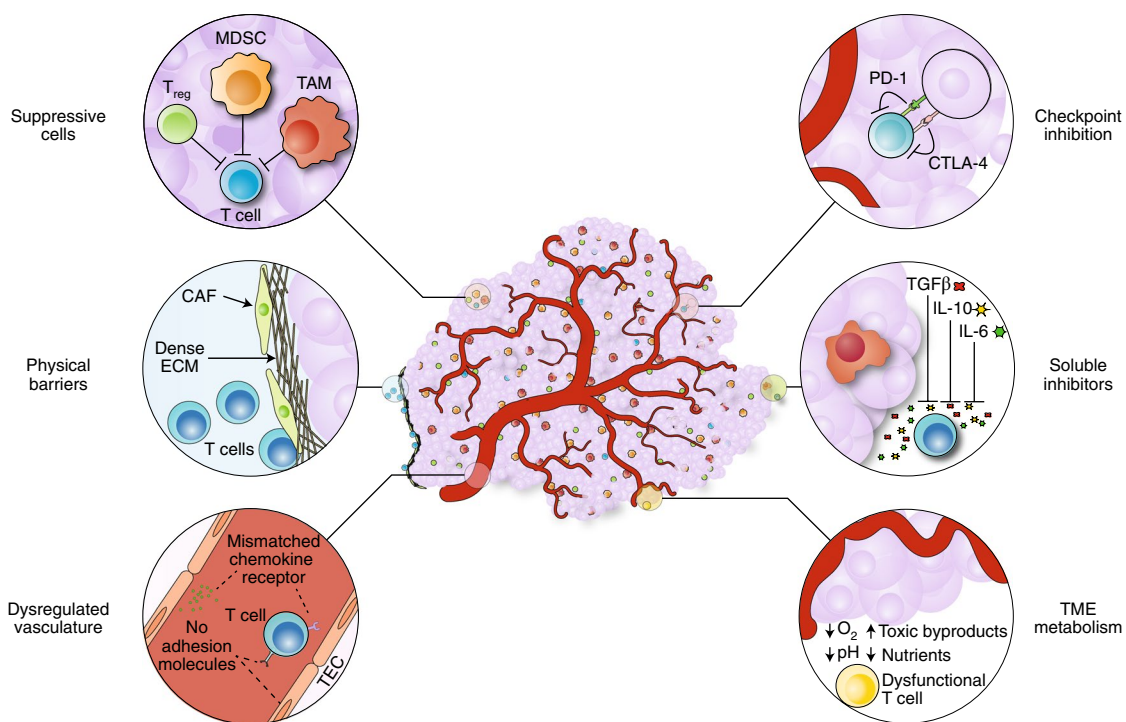


Fig. 6 | Mechanisms of immune suppression in the tumour microenvironment. T-cell homing and infiltration is impeded by mismatched chemokine receptors and by the lack of adhesion molecules in the dysregulated tumour vasculature. CAFs create a physical barrier to T-cell infiltration by maintaining a dense ECM that traps T cells at the periphery of the tumour. Tumour-associated cells such as T_{reg} cells, MDSCs and TAMs support the tumour by suppressing immune responses. T cells can be inhibited through checkpoint receptors such as PD-1 and CTLA-4, which are engaged by cognate ligands expressed on tumour cells or tumour-associated cells. Soluble inhibitors in the form of suppressive cytokines (TGF- β , IL-10 and IL-6) disrupt T-cell effector functions. The metabolic milieu of the tumour microenvironment is unfavourable to effector T cells owing to a lack of nutrients, acidic conditions and toxic byproducts.

Table 2 | Functional modules for improving efficacy

Module	Examples	Purpose	Refs
Chemokine receptor	CCR2, CCR4, CXCR1	Improve homing and trafficking	111-114
Secreted cytokines	IL-12, IL-18, IL-21	Improve proliferation and persistence	117,120,121
Constitutively active cytokine receptor	IL-7R	Improve proliferation and persistence	131
Viral antigen + virus-specific T cells	EBV, CMV	Improve proliferation and persistence; reduce GVHD	136,139,151
ECM-degrading enzymes	Heparanase	Breakdown tumour stroma	140
CARs against tumour-associated cells	Targeting CAFs, TAMs, TECs and T _{reg} cells	Weaken immunosuppressive barriers in the tumour microenvironment	118,141-144
Dominant-negative receptors	TGF- β	Counteract immunosuppression	151
Switch receptors	PD-1-CD28, IL-4/IL-7	Counteract immunosuppression	152,252
Secreted antibodies	Anti-PD-L1, anti-PD-1	Locally delivered checkpoint blockade	253,254
Custom response modules	SynNotch	Programmable response in response to antigen engagement	185
T-cell backpacks	Nanoparticles loaded with TGF- β inhibitor and IL-15	Counteract immunosuppression and improve proliferation	193-195

EBV, Epstein-Barr virus; CMV, cytomegalovirus; PD-L1, programmed death-ligand 1.

It has been shown that an engineered, constitutively active IL-7 receptor (IL-7R) does not require extracellular IL-7 for the antitumor effects of GD2-CAR-T cells against metastatic neuroblastoma and orthotopic glioblastoma xenograft models¹³¹. The receptor, derived from a naturally occurring gain-of-function IL-7R mutant found in lymphoblastic leukaemias^{132,133}, has a cysteine in the

juxtamembrane domain that promotes dimerization of the receptor and initiates signalling independently of IL-7. In another example of this strategy, transgenic expression of cytokines tethered to their receptors can deliver stimulatory signals to the T cell while diminishing the risk of systemic toxicities associated with the administration of soluble cytokines¹³⁴.

Vaccine-based approaches can also be used to enhance T-cell proliferation by using CAR-T cells that keep an identified native TCR, such as one that is specific for a viral antigen^{135–138}. In this context, administration of an attenuated virus, such as vaccinia, or viral antigens present in host cells, provides stimulation through the native TCR and promotes T-cell activation. However, clinical trials evaluating virus-specific CARs have not shown a clear advantage compared with standard second-generation CAR-T therapy^{41,136,139}. In fact, preclinical studies have shown that signalling through both the TCR and the CAR can accelerate T-cell exhaustion and result in diminished persistence⁴⁶.

Targeting the tumour stroma and supporting cells. It has been well established that solid tumours coerce non-transformed stromal and immune cells, such as myeloid-derived suppressor cells (MDSCs), tumour-associated macrophages (TAMs), cancer-associated fibroblasts (CAFs), tumour endothelial cells (TECs) and regulatory T cells (T_{regs}), to support and maintain the tumour⁹⁷. Importantly, these conditioned cells can directly suppress CAR-mediated anti-tumour effects in the tumour microenvironment⁵³. In some cases, malignant cells can recruit these supporting cells to perform various functions, such as remodelling of the extracellular matrix (ECM), enhancement of angiogenesis and secretion of growth factors, and evasion of immune surveillance. Targeting supporting cells can thus help promote lymphocyte infiltration, and may weaken the immunosuppressive barriers of the tumour microenvironment. CAR-T cells have not only been administered concomitantly with agents that modulate the tumour microenvironment⁵³, but have also been engineered to directly modulate it, for example through overexpression of heparanase for degradation of the ECM, which resulted in enhanced therapeutic efficacy in a neuroblastoma tumour model¹⁴⁰. CAFs involved in maintaining the ECM can also be targeted using CARs specific for fibroblast activation protein¹⁴¹, and other cancer-associated cells such as TAMs, TECs and T_{regs} can be targeted using CARs directed against tumour-microenvironment-specific antigens^{118,142–144}.

Immunosuppression in the tumour microenvironment. Tumour cells, together with tumour-associated stromal cells, suppress T-cell function through an array of immunosuppressive signals, such as transforming growth factor- β (TGF- β), IL-10 and the Fas ligand (refs 97,145). Tumour cells can also activate immune checkpoint receptors on T cells, such as the receptors PD-1 and CTLA-4, via expression of their ligands. Immune checkpoints are part of the physiological mechanisms for attenuating T-cell activation. Blocking the activation of immune checkpoint receptors by using mAbs, a strategy for reinvigorating T cells termed checkpoint blockade, has recently emerged as an effective immunotherapy for several cancers¹⁰⁶. Preclinical studies have shown a synergistic effect when CAR-T therapy is combined with checkpoint blockade^{146,147}, and recent clinical reports have suggested that anti-PD-1 agents enhance the efficacy of CD19-CAR-T therapy in patients with refractory diffuse large B-cell lymphoma and acute lymphoblastic leukaemia^{148,149}. Despite these encouraging reports, a phase-I study that used a third-generation GD2-CAR for patients with neuroblastoma reported absence of improvements in efficacy when combined with anti-PD1 administration¹⁵⁰. Therefore, the potential benefits of combining CAR-T-cell therapy with checkpoint blockade have to be tested further by taking into account the specific CAR used and the patient's clinical history. Moreover, whereas combining checkpoint blockade with CAR-T cells may improve their potency, it could also increase toxicity, owing to the dampening of T-cell safety mechanisms.

Immunosuppression in the tumour microenvironment can also be circumvented with engineered dominant-negative receptors, which are truncated receptors for inhibitory molecules (such as

TGF- β) that lack intracellular signalling domains¹⁵¹. These decoy receptors compete with endogenous receptors by binding to and depleting the ligand, resulting in decreased signalling of inhibitory pathways in immune cells. Taking this approach to the next level, the immunosuppressive tumour microenvironment can also potentially be used against the tumour itself through switch receptors: using this approach, inhibitory signals can be reversed into stimulatory signals with fusion receptors composed of the extracellular domain of inhibitory receptors (such as PD-1) and the signalling domains of stimulatory molecules (such as CD28)¹⁵².

Selectively knocking out endogenous genes through targeted nucleases, a powerful technique that is revolutionizing T-cell engineering, can also be applied to prevent tumour-derived immunosuppression. Checkpoint molecules such as PD-1 (ref. 153), CTLA-4 (ref. 154) and lymphocyte activation gene 3 (LAG-3) (ref. 155) have been efficiently deleted by CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats-CRISPR-associated system 9) or TALENs (transcription activator-like effector nucleases), rendering T cells less prone to checkpoint inhibition and exhaustion, and resulting in improved antitumour efficacy and persistence in preclinical models. Advances in multiplexed assays have enabled targeting of multiple gene targets for simultaneous deletion^{154,156}. However, caution must be exercised with such approaches as these inhibitory receptors are part of physiological processes involved in modulating T-cell activity, and permanently unleashing T cells with no feedback and attenuation mechanisms may increase the risk of toxicity owing to uncontrolled activation. Importantly, recent work demonstrated that PD-1 is a tumour suppressor gene, raising the possibility that deletion of PD-1 in engineered T cells could ultimately increase the risk of malignant transformation¹⁵⁷.

Metabolism and suppression. Following activation, T cells must adapt their metabolism to meet the energetic demands associated with rapid proliferation and effector function, such as the production of cytokines and cytotoxic payloads. Consequently, the metabolic profile of T cells is intimately linked to the regulation of T-cell function and differentiation stage. The aberrant metabolic milieu in tumour microenvironments, as a result of the high metabolic activity of tumour cells and dysfunctional tumour vasculature, is both hypoxic and acidic, depleted of nutrients such as glucose and glutamine, and lacks key amino acids such as arginine and tryptophan due to the upregulation of the inhibitory enzymes indoleamine 2,3-dioxygenase-1 (IDO-1) and arginase by the tumour stroma¹⁰⁰. This unfavourable environment inhibits T-cell effector functions and promotes a defective T-cell state. Metabolically reprogramming T cells by gene editing can potentially help them resist these conditions. Ectopic expression of PPAR- γ co-activator 1 α (PGC1 α) can rescue mitochondrial function and lead to increased cytokine production in T cells, resulting in enhanced antitumour efficacy¹⁵⁸. Modifying cholesterol metabolism by knocking out acyl-CoA:cholesterol acyltransferase 1 (ACAT1), an enzyme involved in esterification of cholesterol, can also potentiate the effector functions of CD8⁺ T cells¹⁵⁹, whereas resistance to reactive oxygen species can be conferred by expression of catalase, which relieves oxidative stress in the tumour microenvironment¹⁶⁰.

The choice of CAR co-stimulatory domain can also drive T cells towards distinct metabolic profiles. Whereas co-stimulation through CD28 enhances aerobic glycolysis, 4-1BB drives mitochondrial biogenesis and oxidative metabolism, which may account for the phenotypic differences seen between CARs with these different domains^{17,45}.

Avoiding antigen escape with bispecific CARs. Resistance to CAR-T therapy has been observed in the form of antigen escape, a process by which the tumour escapes CAR targeting by down-regulating or mutating the CAR's target antigen¹⁶¹ via selection of

antigen-negative cells⁷ or via lineage switch^{162,163}. Although clinical trials with the CD19-CAR in B-cell acute lymphoblastic leukaemia (B-ALL) have reported high complete response rates (70–90%), relapse with CD19-negative tumours occurred in >20% of responders^{5,7,11,164–167}. In the context of solid tumours, antigen escape poses a potentially even greater challenge given their extensive heterogeneity in antigen expression. A recent trial of ten patients with glioblastoma treated with peripherally infused EGFR variant III (EGFRvIII)-specific CAR-T cells reported that five of seven patients who underwent tumour resection after treatment had reduced EGFRvIII expression in tumour tissue¹⁶⁸. EGFRvIII antigen escape has also been implicated as a resistance mechanism for a peptide vaccine¹⁶⁹. In a separate trial, a patient with glioblastoma treated with an IL-13 receptor $\alpha 2$ (IL13R $\alpha 2$)-specific CAR had recurrent tumour with decreased antigen expression¹⁷⁰. These clinical data, alongside preclinical studies demonstrating the emergence of antigen loss variants, underscore the need for targeting multiple antigens^{119,161,164,171–173}.

Bispecific CARs have the potential to mitigate this escape mechanism by targeting two antigens simultaneously (OR gate, Fig. 4c)¹⁷². Tandem bispecific CARs can be constructed by linking two scFv domains in series^{174,175}; these constructs may require optimization of the orientation of the heavy and light chains to achieve correct pairing and a favourable conformation for binding, and the linker length needed for targeting each antigen must be taken into consideration. For a bispecific CAR targeting CD19 and CD20, a CD19-proximal and CD20-distal scFv configuration was found to be ideal as these antigens require a short and long spacer, respectively¹⁷⁴; in this format, the CD19 scFv effectively served as a linker to project the CD20 scFv away from the surface. In another example, a CD19–CD22 bispecific CAR required a loop configuration, with the CD19 variable heavy (V_H) and light (V_L) chains flanking the CD22 scFv (CD19 V_L –CD22 V_H –CD22 V_L –CD19 V_H); this CD19–CD22 bispecific CAR is currently being tested in clinical trials for both paediatric and adult B-ALL and lymphoma¹⁷⁶ (NCT03241940 and NCT03233854).

Alternative formats of multi-antigen CAR therapies involve co-expressing two or more CARs of different specificities in the same cell using ribosomal skip sequences, or treatment with mixed CAR-T cell populations^{177–179}. This combinatorial approach circumvents the need for engineering and optimizing a new CAR construct; yet it is important to note that dual expression of two full-length CARs substantially increases the genetic payload to be delivered to cells, whereas adding an additional scFv module has only a minimal impact on the payload. This is an important consideration when devising cell therapies: for viral-based gene delivery, for example, larger constructs are associated with decreased transduction efficiencies. In the only study that has directly compared multi-antigen CAR platforms to date, pooled infusions of mono-specific CARs were inferior to the tandem CAR or to dual CAR expression on the same cells in a preclinical model of glioblastoma multiforme¹⁷².

The immune response induced by CAR-T-cell killing of tumour cells could also play an important part in preventing the rise of antigen-loss variants by inducing ‘epitope spreading’, which occurs when lysis of tumour cells in an inflammatory milieu leads to presentation of additional tumour antigens to lymphocytes, inducing parallel cell-mediated and humoral antitumour immune responses^{180,181}. A preclinical study utilizing immunocompetent mice supported this model: mice whose EGFRvIII-positive tumours were cleared by treatment with EGFRvIII-CAR-T cells could reject EGFRvIII-negative tumour cells in subsequent re-challenge experiments, whereas tumour naive mice could not reject EGFRvIII-negative cells¹⁸². In clinical studies, patients receiving mesothelin-specific CAR-T cells developed elevated antibody responses against self-proteins, including mesothelin¹⁸³. These data also raise the prospect

of potentially enhancing the generation of physiological immune responses through combination therapies with checkpoint inhibitors that could potentially unleash tumour-reactive T cells and enable antitumour immunity beyond the specificity determined by the CAR. The contribution of epitope spreading to antitumour activity in patients undergoing CAR-T therapies remains unknown, as this phenomenon has not yet been thoroughly evaluated in clinical trials.

Other strategies for increasing efficacy. An alternative approach for cancer immunotherapy is based on the use of T cells as vehicles to deliver therapeutic agents to the tumour site. T cells engineered to secrete a soluble form of herpes virus entry mediator that inhibited B-cell lymphoma proliferation through the B and T lymphocyte attenuator signalling axis augmented the efficacy of CD19-CAR-T therapy¹⁸⁴. A separate study reported a versatile system, termed ‘synNotch’, for the integration of signals from molecular stimuli in the local microenvironment into cellular responses¹⁸⁵. Using this approach, T cells could be programmed to express antibodies, adjuvants, cytotoxic molecules or cytokines in response to binding to target cells.

MicroRNAs (miRs) are another attractive target for improving CAR-T therapy, as these non-coding RNAs are involved in the physiological regulation of T-cell development and effector function^{186–189}. However, the immunosuppressive tumour microenvironment can downregulate key miRs such as miR-17-92 (ref. ¹⁹⁰). Transgenic expression of miR-17-92 in CAR-T cells, for instance, led to enhanced proliferation, interferon- γ (IFN- γ) production and increased the durability of treatment in a murine glioma xenograft model¹⁹¹. miR engineering is a promising strategy to augment CAR-T-cell function, potentiated by its capacity for genetically encoded transcriptional control of endogenous or exogenous genes^{191,192}.

Advances in biomaterials and drug delivery offer promising alternative avenues for increasing the efficacy of adoptively transferred cells. T cells can be outfitted with nanogel or liposome ‘backpacks’ loaded with therapeutic agents such as TGF- β inhibitors^{193,194} or IL-15 (ref. ¹⁹⁵), endowing the treatment with time-limited release functionality. The release properties of these depot formulations can be fine-tuned by chemical means or even configured to unload during cytolysis through perforin-mediated rupture of the particles¹⁹⁶. T cells can also be loaded inside biopolymers¹⁹⁷, along with adjuvants, and implanted directly onto solid tumours¹⁹⁸. In this approach, the combination of debris from dying cells and the adjuvant promotes activation of immune cells directly at the tumour site.

Towards an off-the-shelf T-cell product

The first two CAR-T therapies to be approved by the FDA, Kymria and Yescarta, are currently priced at US\$475,000 and US\$373,000, respectively^{199–202}. Although some consider this a steep cost to pay, others deem it conservative given the robust potency, response rates and curative potential against otherwise refractory conditions. Nonetheless, providing personalized CAR-T treatment to the millions of patients with cancer worldwide may not be economically sustainable with current technologies. Furthermore, manufacturing autologous T cells *ex vivo* may be problematic when patients have low T-cell counts, as occurs with those who have been heavily pre-treated with chemotherapy regimens. Therefore, the development of universal, off-the-shelf T-cell products that are effective and persistent could dramatically reduce manufacturing costs and increase accessibility to this revolutionary cancer therapy.

An alternative — and potentially less costly — approach is to use T cells from an allogeneic source^{46,203–205}, but this strategy bears additional complications in the form of potential cellular rejection and GVHD²⁰⁶. In this context, disruption of key genes through gene-editing tools can offer solutions¹⁵⁶: a TALEN-based editing approach was used to create an allogeneic CD19-CAR-T treatment

for two infants with B-ALL (ref. ²⁰⁷), whereby native TCRs from the donor cells were depleted by disrupting the TCR- α constant locus (*TRAC*) to render them non-reactive to the recipient's endogenous cells. The CD52 locus was also targeted for deletion so that the infused cells could be spared from lymphodepletion by the anti-CD52 antibody administered to deplete the host T cells. The infants achieved molecular remission after treatment but developed GVHD, possibly due to residual TCR-expressing cells present in the donor product²⁰⁷. This strategy is currently being investigated in clinical trials for CARs targeting CD19 (NCT02808442) and CD123 (NCT03190278 and NCT03203369). Another strategy to avoid GVHD is to express the CAR in haematopoietic stem cells or other T-cell precursors that can be rendered tolerant to host antigens through the natural process of T-cell selection and maturation in a functional thymus^{208–210}. Alternatively, cells may be sourced from induced pluripotent stem cells by using cellular reprogramming techniques, providing a potentially unlimited supply of T-cell precursors that can be expanded indefinitely and differentiated *in vitro* into T-cell subsets expressing specific CARs^{211,212}.

Knocking out the TCR effectively avoids GVHD, but the infused cells can still be rejected by the host immune system. A possible solution involves the disruption of the β -2 microglobulin gene, which can ablate human leukocyte antigen I (HLA-I) expression to help donor CAR-T cells evade host CD8 T cells^{154,156}. However, the CAR-T cells could still be vulnerable to rejection via CD4 cytolytic cells or lysis by NK cells as part of the 'missing-self' response^{213,214}. Inserting non-classical HLA-E molecules, which are much less polymorphic than HLA-I, may help to avoid NK-cell-mediated rejection by the missing-self response^{215,216}.

An alternative approach to overcome the costs of *ex vivo* individualized cell generation is to transduce the T cells *in vivo*. In this regard, a recent study has provided proof-of-concept evidence of nanoparticle carriers loaded with DNA encoding the CAR and a transposase that facilitates transgene integration²¹⁷. The modified cells showed significant expression levels and expression frequencies, and mediated antitumor effects in a preclinical model. T cells can be targeted in this approach by using anti-CD3 ϵ antibodies decorated on the nanoparticle surface.

Delivery of CAR genes

Delivery of CAR-encoding genes to T cells *ex vivo* is most commonly achieved with viral vectors^{5,6,218}. Gamma-retroviral- and lentiviral-based delivery result in high expression and transduction efficiencies, but these have a limited payload (~8 kilobases (kb)) and pose the theoretical risk of insertional oncogenesis²¹⁹. The potential for this risk arises from the random nature of integration into the host genome, although no instances of this occurring in clinical trials using CAR-T cells engineered through these methods have been reported. Alternatively, transposon-based systems are capable of delivering a larger payload (~100 kb), cost effective, scalable and potentially less immunogenic than viral vectors^{220–222}. This approach requires delivery of plasmid DNA encoding the transgene and transposase components, which is most commonly achieved through electroporation²²¹, a method that can be toxic to the cells and is not as efficient as viral-based delivery. Hybrid systems that utilize non-integrating viral vectors to deliver the transposase and transgene are currently under investigation^{223–225}.

Non-integrating transient expression systems such as mRNA electroporation avoid the potential for insertional mutagenesis, but expression of the CAR is short-lived and is diluted with each cell division¹⁸³. The development of targeted nucleases such as CRISPR-Cas9²²⁶, TALENs²⁰⁷, zinc fingers²²⁷ and homing endonucleases²²⁸ enable the targeting of the CAR gene to specific sites in the genome. Recent work has also demonstrated that delivering the CD19-CAR gene into an endogenous TCR locus by CRISPR-Cas9 resulted in uniform CAR expression, reduced tonic signalling and TCR-like

regulation of expression, and enhanced T-cell potency²²⁶. The ideal target locus for other CARs may differ depending on the level of expression needed for optimal function.

Clinical results

The majority of CAR-T cell clinical trials conducted thus far have targeted CD19 to treat B-cell malignancies. CD19 is an ideal target, owing to it being highly expressed in the B-cell lineage and not being expressed in other tissues. Moreover, the on-target, off-tumour toxicity that results in B-cell aplasia can be managed with intravenous immunoglobulin. Remarkable responses have been observed with CD19-CAR-T therapy in patients with refractory B-ALL, chronic lymphocytic leukaemia, non-Hodgkin lymphoma, follicular lymphoma and diffuse large B-cell lymphoma²²⁹. Additionally, the first results of a CAR targeting CD22 on B-cell malignancies, published within the past few years, showed impressive activity in CAR-naïve patients and patients who relapsed with CD19-negative disease following CD19-CAR-T therapy^{230–232}. Clinical trials of targeting other B-cell antigens, such as B-cell maturation antigen (refs ^{233,234}), CD20 (refs ^{235,236}), Igk (refs ^{237,238}) and ROR1 (refs ^{239,240}), are underway.

Adverse effects. The primary adverse effects observed with CAR-T therapies have been CRS and neurotoxicity. Neurotoxicity occurs in approximately 40% of patients and is characterized by aphasia, seizures, ataxia, delirium and other disturbances of the nervous system. In the vast majority, the symptoms are fully reversible, but in a small number of patients it led to cerebral oedema and death. Although the pathophysiology of this syndrome has remained enigmatic, a recent study proposed a plausible mechanism based on clinical data from patients receiving CD19.BBz-CAR-T cells⁶⁸: CAR-T cells can induce endothelial cell activation in the central nervous system, resulting in increased permeability and coagulopathy; a low ratio of angiopoietin 1 (ANG1) to ANG2 then results in dysregulated endothelial cell activation and a breakdown of the blood-brain barrier. These data raise the prospect that approaches for normalizing the ANG1/ANG2 ratio could prevent or reverse the syndrome, and that agents such as recombinant BowANG1 could potentially be tested for this indication in the context of a clinical trial²⁴¹. Less potent CARs might induce cytotoxicity but secrete lower cytokine levels. One such CAR targeting CD19 with a low-affinity binder is already in clinical trials, and in a series of nine patients, none experienced grade 3 or above neurotoxicity²⁴². In summary, neurotoxicity remains an important concern following CD19-CAR-T therapy, but the majority of occurrences are fully reversible, and mortality rates are below 5%, which is in line with other treatments for aggressive B-cell malignancies.

Tackling solid tumours. Preliminary clinical results using CAR-T cells to treat solid tumours have not been as striking as those in blood cancers, but are only the first steps towards antitumor efficacy for this more challenging subset of tumours. One trial reported that 3 out of 11 patients treated with a GD2-CAR achieved complete remission^{40,243}. In another, 4 out of 17 patients with sarcomas achieved stable disease⁹¹ with a HER2-CAR and no lymphodepletion, and 1 out of 6 patients achieved a complete response⁹² with a lymphodepleting regimen. Additionally, a patient treated with IL13R α 2-CAR-T cells delivered intracranially to treat glioblastoma showed regression of disease for at least 7.5 months²¹. These results are just the beginning of what will probably be a long road ahead for CAR-based therapies against solid tumours. The challenges include toxicities and limited efficacy in the face of a suppressive tumour microenvironment, but the engineering of new safety mechanisms as well as efficacy-enhancing elements will pave the way to success in the treatment of these cancers.

Outlook

The immune system has a remarkable ability to identify and eradicate dysfunctional cells, but acquired and inherited mutations in transformed cells enable their evasion from immune recognition. The engineering of CAR-T cells that redirect immune function against malignant cells marks the beginning of a new era in cancer therapy, marked by the encouraging initial success against B-cell malignancies with the CD19-CAR, which provides a proof-of-principle system for extending this therapeutic paradigm to other cancers. The primary hurdles limiting the success of CAR-T therapy thus far are treatment-associated toxicities and relapse of antigen-negative tumours. Tackling solid tumours poses several additional challenges, owing to a paucity of tumour-restricted antigens, high levels of tumour heterogeneity, poor T-cell trafficking and persistence, and the immunosuppressive effects of the tumour microenvironment. The next generation of CAR technology is moving towards the development of multifaceted smart T-cell machines that can: (1) simultaneously target multiple antigens; (2) be regulated either via small molecules or via intrinsic sensors; (3) be modified by gene editing to augment potency and endow resistance to suppressive factors present in the tumour microenvironment; (4) be equipped with recognition programs to discriminate between cancer and healthy cells; and (5) have intrinsic fail-safes and/or suicide switches. Bridging principles of immunology with recent developments in synthetic biology and genetic engineering will drive the development of the next generation of CAR-T therapies.

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Author contributions

All authors contributed to writing and editing the manuscript.

Competing interests

C.L.M. is an inventor on a patent for a CD22-directed CAR licensed by JUNO Therapeutics, receives research funding from Bluebird Bio and Obsidian Therapeutics, and serves on the advisory boards of Unum Therapeutics, GlaxoSmithKline and Vor Pharmaceuticals. L.L. and R.G.M. declare no competing interests.

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