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Genetic engineering of T cells for immunotherapy

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Abstract

Genetically engineered T cell immunotherapies have provided remarkable clinical success to treat B cell acute lymphoblastic leukaemia by harnessing a patient's own T cells to kill cancer, and these approaches have the potential to provide therapeutic benefit for numerous other cancers, infectious diseases and autoimmunity. By introduction of either a transgenic T cell receptor or a chimeric antigen receptor, T cells can be programmed to target cancer cells. However, initial studies have made it clear that the field will need to implement more complex levels of genetic regulation of engineered T cells to ensure both safety and efficacy. Here, we review the principles by which our knowledge of genetics and genome engineering will drive the next generation of adoptive T cell therapies.

Through natural selection, our immune system has evolved the ability to recognize and eliminate a wide array of pathogens and tumours while sparing healthy tissue. Advances in gene and cell engineering mean that one no longer has to wait for natural selection to devise ways to successfully control pathogens and tumours. Rather, immune interventions, often called 'immunotherapies', have been developed that collaborate with the immune system to reverse or prevent a disease state. Vaccines were the earliest immunotherapy and have been by far the most impactful, saving millions of lives[1]. Additionally, infusion of antibodies, the product of B cells, can block undesired immune interactions, flag the immune system to remove cells coated with particular antibodies or enhance effector responses by blocking

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negative regulators of T cell activation (checkpoint therapies), a particularly effective therapeutic for a wide spectrum of diseases spanning autoimmunity and cancer[2–4]. The newest form of immunotherapy is cell and gene therapy, whereby immune cells are modified to express synthetic molecules and are genetically altered to generate a tailored immune response for a specific disease (FIG. 1). This is an emerging field in which all immune cells could be utilized, although T cells have been the most studied to date.

T cells are a key component of the adaptive immune system and can be subdivided on the basis of function, co-receptor expression (CD4 or CD8), trafficking, metabolism and lifespan[5,6]. T cells arise from haematopoietic stem cells and mature in the thymus. During this process of development, T cells undergo a complex gene-editing programme by which gene segments within the T cell receptor (TCR) locus are randomly rearranged to generate a TCR of unique specificity that enables a population of T cells to recognize a wide array of peptides presented by major histocompatibility complex (MHC) molecules. When a TCR recognizes cognate peptide presented via an MHC molecule on an antigen-presenting cell, a signalling complex is recruited to the TCR (signal 1), which, when coupled with one or more co-stimulatory signals (signal 2), initiates a second phase of development that leads to the expansion of T cell numbers, differentiation and, ultimately, the development a memory pool of cells poised to eliminate the pathogen or tumour on secondary exposure.

Some pathogens and tumours deftly avoid the T cell response; for example, Ebola virus sterically interferes with T cell–MHC interactions[7], and some tumours overexpress the immune regulator PDL1, resulting in a higher signalling threshold to induce the full complement of T cell effector functions[8]. By using genetic tools, investigators can take advantage of what is known about a particular pathogen or tumour to generate synthetic approaches to sidestep evasion mechanisms and redirect T cells towards a desired target. This approach has led to durable, complete cures in the case of CD19-expressing tumours[9] (BOX 1), begetting many other potential therapies that are in development for infectious disease, autoimmunity and cancer.

A host of strategies have been developed to prolong T cell function, protect allogeneic T cells from immune rejection, thwart viruses from infecting the T cells programmed to recognize and eliminate them, and augment effector functions to clear a particular tumour or pathogen. However, until recently, the field lacked the tools to implement complex gene therapy approaches that would take full advantage of our knowledge of T cell function. Only single genetic alterations of T cells had been tested, and the only FDA-approved therapies to date, which treat some leukaemias and lymphomas, use T cells engineered to harbour randomly placed genomic insertions of a single synthetic gene that redirects T cells to kill. Additional engineering, beyond redirecting the T cell to a target, is likely required to increase the number of indications for which genetically modified T cells provide durable cures. Recently, results from a phase I clinical trial of TCR transgenic triple-gene-edited T cells were reported[10], demonstrating feasibility and preliminary safety, ushering in a new phase of adoptive T cell therapy involving multiple genetic manipulations. While this initial trial found off-target gene edits and chromosomal translocations, a long-term in vitro culture assay used before infusion did not find T cell transformation[10]. To minimize off-target gene editing, a variety of improvements to CRISPR gene-editing systems, including base editing,

are being developed (reviewed in REF.[11]). With an ever-expanding and ever more precise toolbox with which to engineer T cells, investigators are now broadening the scope of T cell alterations to cure a variety of disease states.

Here, we review why T cells are attractive cells to genetically engineer, approaches to engineer T cells, how these engineered T cells are being used to combat chronic disease and outline how future multifaceted genetic engineering strategies can increase safety and efficacy. We focus on cancer therapy and refer readers interested in engineering T cells for autoimmunity and infectious disease to other in-depth reviews[12,13].

Why are T cells attractive to engineer?

Although cell types such as haematopoietic stem cells, B cells and macrophages have unique properties that make them attractive for immune-based therapies[14–16], T cells are on the leading edge of this exciting new pillar of medicine. They are readily available via a simple venipuncture; culture and delivery systems compatible with good manufacturing practice (GMP) have been devised to expand T cells ex vivo and safely reinfuse them into patients[17]; lessons learned from HIV have generated high-efficiency vectors that readily transduce T cells; T cells are fully mature cells that resist oncogenic transformation[18]; and engineered T cells have the potential to be very long-lived, with some studies showing decade-long persistance[19]. Moreover, owing to natural genomic rearrangement and editing that occurs to generate a unique TCR, it is tempting to speculate that they are well equipped to perform and survive all forms of genome engineering. Lastly, the mobility and functional range of T cells make them excellent agents for cellular engineering.

Sources of T cells

The first wave of approved gene-engineered adoptive T cell therapies uses bulk autologous T cells harvested by apheresis[20,21], which minimizes the risk of graft-versus-host disease (GVHD) and avoids immune clearance of therapeutic T cells[22–25]. This approach has notable disadvantages, including the turnaround time needed for the individualized manufacturing and logistics process (known as the ‘vein-to-vein’ time) during which a patient’s disease may progress[23,25], the risk of manufacturing failure[23,25] and high cost[26]. For these reasons, significant effort is being expended to develop gene-engineered products made from allogeneic T cells as ‘off-the-shelf’ therapies. Three sources of allogeneic gene-edited T cells are being explored: healthy adult donors[27–29], umbilical cord blood[30,31] and induced pluripotent stem cells[32,33].

Allogeneic genetically engineered T cells pose unique risks and drawbacks that are not encountered with autologous T cell products. To counter these challenges, the optimal use of third-party T cells may require additional gene engineering steps that are not required for autologous products (FIG. 1a). From a safety perspective, allogeneic T cells can mediate serious or fatal GVHD[34], so either they need to be obtained from low-risk sources such as partially or fully HLA-matched donors[29,35], virus-specific T cells[28] or umbilical cord blood[36,37], or the endogenous TCR must be deleted[27,38,39]. Finding the optimal combination of gene engineering approaches to enable allogeneic T cells to avoid GVHD and survive in

patients of different HLA types will be key to establishing off-the-shelf replacements for autologous cell therapies.

Engineered T cell types

Transgenic TCR T cells and CAR T cells.—The most common approaches to T cell engineering are the introduction of either a transgenic TCR or a chimeric antigen receptor (CAR), which enable the T cell to recognize a new target (FIG. 1b). Expansion and adoptive transfer of these cells enables the killing of cancerous or infectious cells en masse. Both approaches have their strengths and weaknesses. TCRs are able to access the full proteome of the cell, but are confined by the requirement for antigen processing and presentation of peptide targets[17]. CARs bind their antigens directly, and are not limited to the proteome but expand to other macromolecules such as glycans, which can differ markedly between normal and tumour cells[40]. However, only surface-accessible or secreted antigens can act as targets for CARs[17].

TCR T cells are predominantly engineered to express one transgenic TCRα chain and one transgenic TCRβ chain in addition to or in place of their endogenous chains. The concept stemmed from cloning TCRs from tumour-infiltrating lymphocytes, which could redirect bulk T cells to have tumour specificity[41]. On binding of transgenic TCR to a therapeutically relevant peptide–MHC of interest — for example, the 9-mer peptide derived from amino acids 157–165 of the highly immunogenic cancer testis antigen NY-ESO-1, which is presented by HLA-A*02:01 (REF.[42]) — natural TCR signalling activates T cell function and expansion.

The endogenous TCR can limit the efficacy of TCR T cells in two major ways: through competition for the CD3 complex, reducing transgenic surface TCR expression[43], and through mispairing between endogenous and transgenic TCR chains[43]. TCR chain mispairing can generate novel TCRs with autoreactivity and alloreactivity[44], reduces the amount of the desired transgenic receptor, limiting potency[45,46], and poses a risk of autoimmune toxicity, which fortunately so far has been observed only in mice[47]. Numerous strategies have been used to avoid mispairing between the endogenous and transgenic TCR chains as well as to increase expression of the transgenic TCR (reviewed in REF.[48]) and include equimolar expression of chains via 2A ribosomal skip sequences[49]; modifications facilitating intramolecular bonding between transgenic α and β chains, fusing the chains to CD3ζ; and knockout of endogenous TCR expression via nuclease editing[50] or small interfering RNAs[51]. Recently, the first clinical trial of adoptively transferred TCR T cells with edited endogenous TCR α and β chains demonstrated safety and feasibility of the approach[10], building upon a decade of work following the first TCR T cell trial[52].

CAR T cells harbour a customizable transgenic receptor that is a chimera of a ‘binder domain’, typically derived from an antibody, with T cell-derived transmembrane and intracellular signalling domains (FIG. 1b). CARs harness the exquisite MHC-independent binding specificity of antibodies to activate T cells via TCR (signal 1) and co-stimulatory signalling domains (signal 2)[53–55]. Affinity tuning of binder domains and selection of signalling domains are important considerations in CAR T cell therapy. High-affinity binder domains prevent tumour escape by antigen-low cancer cells[56], whereas lower-affinity

domains confer increased cytotoxicity and proliferation[57] and can spare normal cells expressing physiological levels of target antigen[56]. The choice of intracellular signalling domain should match the optimal character of the desired immune response. For example, CAR T cells expressing the ICOS co-stimulatory domain secrete more T helper 17 cell cytokines compared with the CD28 or 4–1BB domains[54].

Chimeric autoantibody receptor (CAAR) T cells (FIG. 1b) are a subset of CAR T cells that assuage antibody-mediated autoimmune diseases by reversing the antibody–protein binding paradigm of CAR T cells. CAARs contain autoantigen as their extracellular binder domain, eliminating autoantibody-secreting B cells by binding cognate B cell receptors. CAAR T cells with a desmoglein 3 binder domain kill pathogenic B cells to ameliorate pemphigus in animal models of disease[58], which became the foundation for a phase I clinical trial (https://clinicaltrials.gov/ct2/show/NCT04422912).

Regulatory T cells.—Regulatory T cells (T_{reg} cells) are anti-inflammatory $CD4^+$ T cells tasked with maintenance of immunological homeostasis with self and commensal antigens. Adoptive transfer of T_{reg} cells is envisioned to ameliorate the scores of autoimmune diseases in which T_{reg} cell paucity or dysfunction contributes to the cause[59–61] or to prevent disease where dominant tolerance fails to block the initiation of an unwanted immune response[12]. Clinical-scale adoptive transfer of in vitro expanded, unmodified T_{reg} cells was safe and decreased the incidence of GVHD following allogeneic bone marrow transplantation[62], paving the way for engineered T_{reg} cells. T_{reg} cells are broadly suppressive; stimulation via transgenic TCR or CAR can simultaneously suppress an array of inflammatory cell types targeting multiple autoantigens. T_{reg} cells seem to have a lower threshold for signal 1, as CD8-derived TCRs readily work in $CD4^+$ T_{reg} cells but not effector $CD4^+$ T cells[63]. CAR T_{reg} cells that recognize MHC class I are protective in animal models of allotransplantation and GVHD[64–67], whereas transgenic TCR T_{reg} cells have been designed to recognize pancreatic β-cell antigens for the treatment of type 1 diabetes mellitus[68,69].

T_{reg} cell engineering has benefited from progress in effector T cell engineering, yet fundamental differences in biology between the two cell types present unique design and manufacturing challenges. Even the most successful T cell modifications must be assessed for translation to T_{reg} cells both in vitro and in vivo. For example, the 4–1BB and CD28 co-stimulatory domains, which are major contributors to the efficacy of Kymriah and Yescarta CAR T cells, signal differently in T_{reg} cells; 4–1BB co-stimulation causes T_{reg} cells to lose suppressive function[70,71]. Moreover, the relative paucity of T_{reg} cells in peripheral blood necessitates GMP sorting followed by serial stimulation ex vivo, neither of which is part of the proinflammatory CAR T cell manufacturing process[72,73]. The major safety concern of engineered T_{reg} cells is the loss of T_{reg} cell identity by conversion into IL-17-secreting or interferon-γ-secreting ‘ex-FOXP3 cells’ within inflammatory milieus, which hastens disease progression[74]. Mitigating strategies include stringent isolation of T_{reg} cells[72], reinforcement of T_{reg} cell identity by overexpressing FOXP3, or redirecting effector T cells into suppressor ‘edited T_{reg} cells’ by placing endogenous *FOXP3* under the control of an active promoter[75].

Genome engineering approaches

At their core, T cell therapeutics rely on the ability to engineer the genome. The strategies used can be broadly stratified into viral or non-viral types and can have non-specific or targeted effects (FIG. 2). As we discuss the pros and cons of putative approaches, it is important to realize that one size will not fit all. Disease cause, severity, corporeal location and pathophysiology should inform the design and approach of the intended knock-ins, knockouts or other genetic mutations. As the toolbox of methods to engineer DNA expands, the fidelity and efficiency of each method must be thoroughly characterized in preclinical and clinical studies to inform the optimal approach for each type of therapeutic challenge.

Non-targeted genome editing

Viral gene delivery.—Viral vector transduction is a reliable means of integrating a transgene into the T cell genome (FIG. 2a). The large packaging size enables delivery of CARs or TCRs, in addition to multicistronic suites containing additional transgenes to enhance the behaviour of the T cell product. However, larger provirus inversely correlates with titre, imposing an upper limit to cargo size[76]. Integration is semi-random, with Moloney murine leukaemia virus-based gammaretroviral integration clustering near transcriptional start sites[77], and HIV-based lentiviral integration showing a propensity for active genes. Insertional mutagenesis of oncogenes or tumour suppressor genes has the potential to cause oncogenic transformation via dysregulation of these genes. Although gammaretroviral vectors were used for the first CAR T cell trials in the late 1990s[78–80], lentiviral vectors have since become the virus of choice, owing to their higher transduction efficiency and transgene expression in T cells[81,82].

Tuning of viral transgene expression can be modulated via the promoter and 3′ untranslated region sequences accompanying the coding region. Often, low-affinity CAR or TCR and/or low expression of target antigen can render some cancer cells invisible, necessitating maximum receptor expression for sufficient therapeutic effect. Constitutively active promoters combined with optimized 3′ untranslated regions[83] endow stable, high-level transgene expression in primary human T cells[81,84]. However, overexpression of CAR can lead to T cell exhaustion[85] and may render the CAR more reactive against low-expressing, healthy tissue targets. Thus, more regulated kinetics may be favourable that allow the CAR T cell to eliminate tumours, ignore healthy tissue and have physiological activation strength and kinetics. Incorporation of activation-dependent or tissue-specific regulatory elements may provide an additional layer of genetic control[86].

In contrast to oncogenic gene integration events, insertional mutagenesis causing overexpression or knockout of other proteins in a therapeutic T cell product is a phenomenon being actively exploited. Analysis of virally transduced T cells that are selected by their thriving in the hypoglycaemic, hypoxic, cytokine-laden tumour microenvironment could identify genes that are vital for survival and therapeutic function among such pressures. Since each T cell’s unique TCR acts as a barcode, deep sequencing of the TCR locus can characterize the therapeutic response as polyclonal, pauciclonal or monoclonal and track a functional clonal population from the infusion product through the peak of response to long-term maintenance. In a patient with chronic lymphocytic leukaemia participating in a

CD19 CAR T cell clinical trial, TCR deep sequencing revealed that a clonal population derived from a single CAR T cell was responsible for remission, constituting 94% of all peripheral blood T cells at the peak of response[87]. Integration site analysis revealed insertion of the CAR transgene into the methylcytosine dioxygenase *TET2* locus, causing loss of gene function. CAR T cells with knockdown of TET2 showed a skewing towards a central memory phenotype, increased growth in vitro and perturbed cytokine secretion. Fortunately, this massive clonal expansion of $TET2^{CAR/-}$ cells retracted to set point levels, absent of oncogenic transformation[87], cementing TET2 knockdown as a testable candidate for intentional knockdown in future CAR T cell therapy. Similar molecular analyses of responders and non-responders alike will unearth other processes fundamental to clinical efficacy.

Non-viral gene delivery.—Non-viral gene delivery offers an alternative method of T cell engineering free from the high cost of production and safety testing of cells modified using viral vectors. T cells are readily electroporated, allowing DNA, RNA, proteins and ribonucleoprotein complexes to cross the plasma and nuclear membranes. In vitro transcribed mRNA can be introduced into both resting and activated T cells, inciting transient expression of a protein of interest[88]. The magnitude and persistence of transgene expression can be regulated by varying the amount of RNA electroporated[88], the length of the poly(A) tail[89] and the inclusion of a 5′ cap.

Co-transfection of *piggyBac* or *Sleeping Beauty* transposase with transgene-containing DNA leverages ‘jumping genes’ to non-virally integrate genetic information into engineered T cells[90,91] (FIG. 2a). As with viral vectors, *piggyBac* prefers integration into genes and thus carries the risk and reward of insertional mutagenesis[92,93]. By contrast, *Sleeping Beauty* integrates genes favourably for therapeutic application in a close-to-random fashion. However, toxicity associated with template DNA delivery and lower transduction efficiency compared with lentiviral vectors limits engineered T cell expansion in vitro[94], and care must be taken to avoid integration of the transposase itself, leading to unconstrained remobilization of the transgene. The development of novel forms of *Sleeping Beauty*[95,96] along with methods for delivery of both transposase and transgene template[97] continue to support the clinical translation of this therapeutic modality. Clinical trials of transposon-based CD19-specific CAR T cell adoptive therapy demonstrated safety and feasibility of this approach[98,99].

Targeted genome editing

A new crop of site-specific nucleases grants the ability to select where a construct integrates into the genome (BOX 2). Each method in this class shares an underlying tenet — the ability to shepherd a DNA nuclease to a predetermined region of interest. DNA double-strand breaks can be repaired by non-homologous end joining or homology directed repair (HDR) processes. Non-homologous end joining repairs DNA double-strand breaks without end processing, occasionally inducing insertion and deletion (indel) frameshift mutations, leading to premature stop codons and engineered knockout of unwanted protein expression.

HDR exploits homology found on undamaged sister chromatids to resolve damage. This pathway can be commandeered by cellular engineers to deliver transgenes to a specific locus by delivering DNA repair templates containing the transgene enveloped by homology arms specific for either side of the DNA lesion (FIG. 2Ba). In contrast to semi-random integration with viral vectors or transposases, the integration of a transgene at a precise genomic location via HDR allows avoidance of integration near an oncogene or tumour suppressor gene, finer control of transgene by an endogenous promoter and/or simultaneous knockout of another protein. Since the only prerequisite for HDR is a DNA double-strand break, multiple nucleases[85,100–102] have been used for HDR-mediated knock-ins in T cells. Donor DNA can be delivered to T cells by either adeno-associated virus serotype 6 (REFS[100,103]) or DNA electroporation[104,105]. To increase safety, DNA can be inserted into a ‘safe harbour’ site identified as unlikely to cause insertional mutagenesis[106]. Alternatively, the HDR template can be targeted to a gene, generating a two-for-one benefit: knockout of an undesired protein of interest and control of transgenic protein expression under a novel promoter. For example, in TCR T cells or CAR T cells, integration of a transgene into the TCRα-encoding *TRAC* locus concurrently eliminates TCR expression, increasing efficacy by decreasing crosspairing with endogenous TCRα, and places the transgenic receptor under endogenous control of the *TRAC* promoter[85] (FIG. 2Ba). Such regulation improves function versus constitutive expression by reducing T cell exhaustion in the absence of tonic signalling[85]. This approach highlights that controlling expression can be as important as the character of the transgene. Along the same lines, targeted integrations can help customize a cell product for a particular disease indication. For example, targeted insertion of HIV-specific CARs into the *CCR5* locus renders the resulting HIV CAR T cells HIV resistant as well by eliminating a viral co-receptor[107].

Each method of engineering has its own benefits and limitations (TABLE 1). Because many are in the nascent stages of characterization, the overall safety profile of each is still being delineated. A balance between on-target and off-target editing must fall within the therapeutic range whereby enough cells are engineered correctly to have an impact on disease without causing oncogenesis or dysfunction due to editing of an unintended locus.

Engineering approaches to increase efficacy

How a T cell is engineered alters its trafficking, function, survival and, ultimately, ability to cure disease. For example, within a single CAR framework, constructs using the CD28 or 4–1BB co-stimulatory domains have marked differences, with CD28-based CARs displaying enhanced effector function and 4–1BB-based CARs having enhanced survival and expansion[24,81,108,109]. Given that even subtle changes to how T cells are programmed can have dramatic effects on function and persistence, correctly programming a T cell to meet the challenges of a given disease setting is a daunting task. In the oncology field, where the exploration of gene-engineered T cells is concentrated, no two cases of a given cancer histology are identical in their genetic and epigenetic aberrations, and tumours acquire further heterogeneity within an individual patient as they evolve, further complicating the engineering challenge[8]. In keeping with the heterogeneity of tumours, preclinical models and early clinical studies of gene-engineered T cell therapies have revealed a panoply of mechanisms that potentially contribute to insufficient or transient efficacy. Such insights

provide a premise for the rational design of ‘next-generation’ gene-engineered T cell therapies equipped either through addition (‘armoured’) or deletion of specific regulatory factors to overcome the principal immune resistance mechanisms of tumours while avoiding toxicity (see the section entitled Engineering approaches to increase safety). As more advanced forms of genetic engineering emerge, the ultimate goal is to programme T cells to act in a more discriminating and context-dependent fashion to optimally harness both the potency and the selectivity of T cells against cancer.

Defining the problem

While barriers to the efficacy of engineered T cell therapies can be categorized in numerous ways, here we consider them in the oncology setting using a well-known framework from the broader drug development field, the ‘three pillars’ of survival of candidate medicines in mid-phase clinical testing[110]: (1) exposure of a drug at the site of action, which for T cell therapies refers to the active process of trafficking to the tumour; (2) target engagement, which for T cell therapies can be inferred to mean addressing diversity in target antigen expression levels or antigen loss as a result of immune editing; and (3) expression of functional pharmacological activity, which in this context equates to the sustained function of the therapeutic T cell once it has engaged its target, which is strongly influenced by the tumour microenvironment. The three-pillar framework may be especially useful given that even phase I trials of CAR T or TCR T therapies are closely scrutinized for signs of clinical benefit, and regulatory approval has been based on single-arm studies of fewer than 100 patients[20,21], akin to the typical phase II study size for traditional pharmaceuticals. FIGURE 3 summarizes some of the specific strategies that have been used to enhance the efficacy of engineered T cells that are discussed in the following sections.

Exposure at the site of action.—T cell trafficking is a complex, active, multistep process that requires the engagement of chemokine receptors on T cells by chemokines released in the tumour microenvironment; the attachment, rolling and arrest of T cells on activated endothelium; and subsequent extravasation and migration through the extracellular matrix of the tissue stroma[111]. There is often a mismatch between the types of chemokines released by tumours and the chemokine receptors expressed by effector T cells, resulting in diminished chemoattraction[111]. Furthermore, the endothelium of tumour-associated blood vessels is often anergic to activation by inflammatory stimuli[111], poorly conducive to T cell attachment and extravasation[112], and tends to overexpress FAS ligand (FASL), triggering apoptosis in effector T cells[113,114]. Even when effector T cells overcome these hurdles and make it into the tumour stroma, they typically face a dense extracellular matrix as an additional physical barrier through which they must migrate[115]. The culmination of the various barriers results in fewer than 1% of adoptively transferred T cells successfully arriving at the sites of action to exert a pharmacological effect[116,117].

To improve the trafficking of gene-engineered T cells to tumours, numerous strategies have been demonstrated in preclinical models (FIG. 3A). Engineering T cells to express a chemokine receptor that responds to the chemokines produced by the tumour facilitates T cell infiltration of tumour lesions[118]. T cells equipped with a dominant negative version of FAS were able to resist FASL-induced apoptosis[114]. Those armoured to overexpress

heparanase were able to digest the dense extracellular matrix of solid tumours[119]. Each of these approaches showed benefit in preclinical models, resulting in improved tumour control, which warrants potential exploration in patients. However, differences between patients and even between different sites of tumour metastasis may be a key hurdle for implementation.

Target engagement.—To address the intrinsic antigen diversity of tumours[120] and the tendency of both haematological tumours[121] and solid tumours[10,122,123] to undergo antigen loss on treatment with engineered T cells, efforts to target multiple antigens or to enhance the endogenous immune response by promoting epitope spreading have been proposed (FIG. 3B).

Conceptually, perhaps the most straightforward approach to address antigen diversity or escape is a cocktail or 'CAR pool' in which traditional CAR T cells of distinct specificity are mixed[124]. However, to meet GMP standards, CAR pools will likely require the manufacture of each component separately before mixing at defined ratios, multiplying cost considerations. Alternatively, T cells can be engineered to express two kinds of CAR molecule[125]; that is, a dual CAR, or both a CAR and a TCR[126]. Either approach imparts on T cells the function of a classical OR Boolean logic gate, such that encountering either antigen is sufficient to activate the T cell. Dual CARs were found to have superior activity compared with CAR pools of the same individual specificities as well as being more straightforward to prepare[125]. In addition, our group recently demonstrated that dual CAR T cells transduced with two separate CARs containing a CD28/CD3ζ endodomain or a 4–1BB/CD3ζ endodomain had improved effector function, increased survival and increased expansion versus CAR T cells containing a single CD28/4–1BB/CD3ζ CAR[53].

An alternative to expressing two separate CAR molecules is the bispecific or tandem CAR (TanCAR), in which both binders are engineered into a single CAR molecule[127,128]. When both antigens are present, this approach has the advantage of superior activity compared with dual CARs owing to enhanced immune synapse formation, and TanCARs require less nucleotide coding capacity than dual CARs[127,128]. However, TanCARs are inferior to CAR pools or dual CARs when antigen escape at one antigen has already occurred[129]. They also have the disadvantage of needing to determine the optimal order and spacing of the binders, and the potential of single-chain variable fragment (scFv) binders to mispair[130]. Expanding on the dual CAR concept, a trispecific CAR T design has also been reported in which three separate CAR constructs are delivered via one lentivirus and expressed in a single T cell; the trivalent CAR T cells were able to recognize any of three independent antigens[131]. While even greater valency may be possible with future designs using gene delivery systems with sufficient nucleotide coding capacity, a significant limitation to increased valency may be the issue of compounding the on-target off-tumour toxicity (discussed in the section entitled Engineering approaches to increase safety) to unacceptable levels.

Two alternatives to address antigen diversity beyond merely increasing the valency of gene-engineered T cells have been proposed: the 'universal' CAR T cell paired with a library of targeting modules that can be infused separately, and engineering T cells to optimize the

induction and/or recruitment of an endogenous immune response, resulting in epitope spreading against the tumour.

Universal CAR T cells make use of a single T cell product that can be infused in multiple disease settings, followed by infusion of distinct targeting moieties where the variable end recognizes the selected tumour antigen and the constant end is recognized by the universal CAR. The simplest form of this platform borrows the CD16-dependent antibody-dependent cellular cytotoxicity mechanism from natural killer cells, allowing T cells expressing a CD16-based CAR to be redirected by existing therapeutic monoclonal antibodies (mAbs)[132]. Alternative universal CARs can be redirected using tagged or hapten-conjugated mAbs or antibody fragments, bispecific adaptors[133] or natural binding partners such as streptavidin–biotin (reviewed in REF.[134]) or SpyTag-SpyCatcher[135]. To implement universal CAR systems clinically, each targeting ligand needs to demonstrate adequate pharmacokinetics, safety and low immunogenicity, and the degree to which universal CAR T cells can persist in the absence of targeting a ligand will need to be determined.

Knowledge of the antigen landscape in a given patient's tumour, especially with respect to the variation between metastatic sites, is likely to remain limited. Hence, the concept of engineering T cells to promote the induction of endogenous immune responses is a worthy one. To this end, CAR T cells engineered to secrete IL-7 together with either CCL19 (known as 7×19 CAR T cells)[136] or CCL21 (known as 7×21 CAR T cells)[137] have been reported (FIG. 3B). IL-7 and CCL19 were selected on the basis of their importance in maintaining T cell zones in lymphoid organs[136], whereas CCL21 was selected owing to its ability to attract naive T cells and dendritic cells to T cell zones, as well as for its antiangiogenic effects[137]. Mice treated with 7×19 CAR T cells demonstrated an increase in dendritic cell and T cell tumour infiltration with improved resolution of large established tumours compared with CAR T cells alone[136]. Efficacy was reduced if host T cells were eliminated before 7×19 CAR T cell therapy, and endogenous CAR-negative T cells formed memory responses to tumour[136]. Meanwhile, 7×21 CAR T cells outperformed both 7×19 CAR T cells and CAR T cells in several mouse models, with evidence of enhanced dendritic cell and T cell infiltration, reduced angiogenesis and activity against tumours composed of a mixed population of cells that were positive or negative for the target antigen[137].

With a number of clinical trials under way, or already starting to produce results[138–140], testing various strategies to counteract antigen diversity and escape, we should soon learn which approaches are most appropriate in given settings. Many B cell malignancies have fairly low mutational burdens[141] and high expression of lineage-specific markers, such as CD19, CD20 and CD22, which may be ideal indications for multitargeting CAR T cells. At the other end of the spectrum, tumours with high mutational burdens such as non-small-cell lung carcinoma and melanoma[141] might be better substrates for approaches that seek to break tolerance and induce epitope spreading.

Achieving sustained pharmacological activity.—The suppressive tumour microenvironment is a key barrier to immunotherapy in general and a challenge to study using engineered human T cells[142]. Numerous mechanisms are involved, often with significant crosstalk, which include a lack of nutrients, hypoxia, the presence of toxic

metabolites, suppressive cytokines and immune checkpoints, and an abundance of suppressive stromal, myeloid and lymphoid cells (reviewed in REF.[143]). FIGURE 4 provides examples of some of the notable approaches that have been deployed to enable gene-engineered T cells to resist the effects of the suppressive tumour microenvironment and thereby sustain their pharmacological activity.

The emergence of PD1–PDL1 checkpoint antagonists as a major class of immunotherapies has stimulated a number of approaches to tackle this pathway and similar immune checkpoints in the setting of gene-engineered T cells (see the section entitled Challenges in implementing solutions). Notably, these approaches have included the first published examples of CRISPR-engineered T cells in patients, in which the gene for PD1, *PDCD1*, was deleted in NY-ESO-1-specific TCR T cells[10] or in bulk T cells[144] (FIG. 4a).

TGFβ is also emerging as a key immunosuppressive factor in many tumours[145]. To counter this pathway, the dominant negative receptor approach has been used (FIG. 4b), for example, in CAR T cells targeting prostate-specific membrane antigen (PSMA), where it enhanced their persistence and function in vitro and in vivo[146]. The dominant negative receptor lacks an endogenous signalling domain and thus fails to propagate the typical signalling pathways downstream of the receptor complex, acting as a sink for the cytokine and reducing the amount of suppressive TGFβ signalling for bystander T cells as well as the gene-engineered T cells[147]. In a clinical study of Epstein–Barr virus (EBV)-specific cytotoxic T lymphocytes for the treatment of EBV-positive Hodgkin lymphoma, a patient whose tumour resisted therapy with non-engineered cytotoxic T lymphocytes achieved an objective response on subsequent therapy with cytotoxic T lymphocytes bearing the dominant negative TGFβ receptor[148]. With numerous trials of TGFβ-resistant gene-engineered T cells in progress, we may soon learn the degree to which efficacy can be safely enhanced using this approach.

Beyond the plethora of suppressive checkpoints and cytokines, metabolites such as adenosine, acting via adenosine A_{2A} receptors, suppress T cell activity. To counter this challenge, CAR T cells were engineered to express a short hairpin RNA molecule to trigger the degradation of the mRNA for the adenosine receptor (FIG. 4a). This approach succeeded in reducing expression of the adenosine receptor by CAR T cells and improved their in vitro and in vivo function in preclinical models[149].

Suppressive cells, including T_{reg} cells, M2-type macrophages and myeloid-derived suppressor cells, are key regulators of effector T cell function in the tumour microenvironment[143]. T_{reg} cells are an especially pernicious challenge since activated effector T cells secrete IL-2, promoting T_{reg} cell expansion[150]. To counter T_{reg} cells, CAR T cells with both CD28 and 4–1BB as co-stimulatory molecules were engineered with a mutation in the CD28 element preventing the recruitment of the SRC kinase LCK, thus avoiding IL-2 induction (FIG. 4b). In mouse models, CAR T cells engineered in this fashion were resistant to the effects of T_{reg} cells[151]. Since second-generation CARs with 4–1BB and CD3ζ alone were not tested, the study primarily supports a method to improve on CD28-containing CARs rather than demonstrating superiority to constructs utilizing alternative co-stimulatory domains.

Aside from the numerous immunosuppressive cells, checkpoints and cytokines in the tumour microenvironment, the metabolic demand of the tumour cells themselves, coupled with inadequate perfusion, contributes to creating an environment that is poor in many essential nutrients as well as oxygen, representing significant challenges to the sustained effector function of gene-engineered T cells[152]. While many design solutions are possible that may help circumvent these challenges, the choice of co-stimulatory domains for CAR T cells is a basic and impactful one. Compared with CD28 co-stimulation, 4–1BB co-stimulation results in enhanced mitochondrial spare respiratory capacity in gene-engineered T cells and thus an enhanced capability to function in hypoxic microenvironments[153] (FIG. 4b). More complex mechanisms to enhance the function of gene-engineered T cells in the tumour microenvironment include equipping T cells to express enzymes involved in the resynthesis of the critical amino acid arginine[154] (FIG. 4a). CAR T cells engineered to express the enzymes argininosuccinate synthase and ornithine transcarbamylase were self-sufficient in arginine and maintained function in the tumour microenvironment, showing enhanced activity[154].

As the above examples show, countermeasures that have been deployed in the preclinical or clinical setting have largely been achieved via the simple addition or deletion of specific regulatory elements. However, given that specific countermeasures may increase the potency of gene-engineered T cells to potentially toxic levels, scientists are increasingly seeking more complex engineering approaches to counter immune suppression in specific contexts. For example, the cytokine IL-12 is a potent immunostimulatory cytokine that is fairly toxic when administered systemically[155]. When constitutively expressed from engineered T cells, it prevents the manufacture of an adequate product by dysregulating T cell expansion and promoting apoptosis[156]. In an attempt to effectively harness IL-12, a copy of the encoding gene was added to TCR T cells under the control of an NFAT-inducible promoter to link expression to tumour antigen recognition. While this approach worked well in the preclinical setting[156], a clinical trial testing the approach using tumour-infiltrating lymphocytes (https://clinicaltrials.gov/ct2/show/NCT01236573) was terminated due to toxicity, suggesting that either the NFAT system was too leaky or the on-target activity of the tumour-infiltrating lymphocytes and their expansion in patients resulted in intolerable levels of IL-12 production. Such a result underscores the need for improved control of gene-engineered T cells especially when armoured to increase their potency.

Challenges in implementing solutions

The challenges for successful clinical application of efficacy enhancement strategies include finding an indication where the resistance mechanism being addressed is a primary barrier to efficacy, selecting an optimally designed countermeasure and establishing that the enhanced T cell is sufficiently safe.

While much of our existing knowledge of bypassing resistance mechanisms comes from preclinical models and interrogation of patient biopsy samples from early clinical studies, these sources suffer from the potential bias in what the investigators chose to study and how they measured it. CRISPR screens have been used as a non-biased way to uncover pathways involved in enhanced function, stability or persistence of engineered T cells. In this method,

libraries of guide RNAs targeting the whole of the genome or curated subsets of the genome are incorporated into T cells along with Cas9 or a catalytically inactive ('dead') Cas9 (dCas9)[157]. After application of a relevant method of selection — high proliferation[158], low FOXP3 expression[159] or presence in the tumour microenvironment[160], for example — proviral guide RNAs are amplified and sequenced to uncover targets that represent important nodes in pathways relevant to the selected process. Incorporation of more than one guide RNA per cell allows the identification of collections of knockout targets which would otherwise be masked by epistasis or complementarity. Alternatively, CRISPR knock-in in the presence of a pool of HDR templates can rapidly select for transgenes that improve CAR T cell and TCR T cell function in a high-throughput manner. Via this method, a TGFβ receptor–4–1BB switch receptor was found to enhance TCR T cell-mediated clearance of melanoma[161]. By discovering functional T cells that endure the selective pressures of the tumour microenvironment, engineered T cells can be designed to better match that tumour microenvironment.

Once the major immune-resistance pathway relevant to a given gene-engineered T cell therapy in a specific disease setting has been selected, the appropriate countermeasure is required. In general, numerous design solutions can be proposed to address any given challenge. Taking the PD1 pathway as an example, one may focus at the genetic level on knocking out the *PDCD1* gene[10] or knocking down its expression via vector-encoded RNA interference[162]. Alternatively, the endogenous PD1 can be left untouched and its effects eliminated by overexpressing a dominant-negative version of PD1 (REF.[163]), overexpressing a 'switch' receptor that converts the signal to a co-stimulatory signal[164] (FIG. 4b), disabling downstream signalling pathways triggered by PD1 ligation[165] or equipping the T cell to secrete its own PD1 pathway-blocking agents such as antibodies[166] or soluble PD1 (REF. [167]). Moreover, beyond these mechanisms directly targeting PD1, the sensitivity of T cells to PD1-mediated dysfunction depends on many other factors, which can also be modulated. For example, specific T cell subsets seem to be intrinsically more resistant to PD1-mediated suppression[168], and exhaustion in general resulting from any checkpoint pathway might be targeted via countering the transcription factors involved in establishing or maintaining T cell exhaustion, such as TOX[169], TCF1 (REF.[170]) and NR4A family members[171].

Once the design of the countermeasure to overcome the immune resistance of the tumour has been settled upon, the next hurdle is to establish that the resulting gene-engineered T cell with its enhanced potency will be sufficiently safe and will function as anticipated. In the PD1 example, a T cell intrinsically resistant to PD1 might arguably be safer than one that secretes a PD1 antagonist, as the latter would also block PD1 on bystander T cells. It might also be safer than the use of an approved PD1 checkpoint agent in combination, again due to the restriction of PD1 resistance to the therapeutic T cell. However, the use of a PD1 checkpoint antibody in combination with a therapeutic T cell could be controlled via dosing and withdrawal. Moreover, in a recent clinical trial of PD1-knockout TCR T cells, those lacking PD1 did not persist well[10], in keeping with earlier findings that some level of PD1 signalling may be required to establish T cell memory[172].

As discussed above, thus far the engineering of T cells for enhanced efficacy involves the fairly straightforward deletion or addition of specific genes to counteract the identified

obstacles to achieving deep and sustained clinical responses. The difficulty has largely been in determining which of the barriers are paramount and which are secondary. Much emphasis in the field is on deciphering which changes have the biggest impact for a particular disease. As the ability to perform multiple genetic alterations becomes more mainstream, emphasis will shift to which changes synergize to provide greater therapeutic benefit.

Engineering approaches to increase safety

The ability of T cells to proliferate, act as serial killers of antigen-positive target cells and orchestrate a broader immune response via secreting cytokines and chemokines is a double-edged sword. These mechanisms make T cells a suitable adversary against cancer but can also result in severe or fatal toxic effects[173,174]. The toxic effects associated with gene-engineered T cells have been investigated extensively[173,175–182] and fall into four categories: (1) product risks, (2) on-target toxicity, (3) on-target off-tumour toxicity and (4) off-target toxicity (FIG. 5). Significant toxic effects can also result from drugs that are part of the CAR T cell or TCR T cell regimen[173], but these are beyond the scope of this Review. The potential to achieve safety by design via enhanced genetic engineering of T cell products is promising. The various solutions fall into two categories depending on whether they allow exogenous control (that is, physician-mediated control) or are endogenously programmed, enabling the T cell to respond autonomously to avoid or reduce toxicity (FIG. 6).

Exogenously controlled T cells

Transient or readily depleted products.—A simple method to limit the duration of activity of gene-engineered cells is to deliver the transgene using mRNA electroporation[183] (FIG. 6a). This method limits activity due to the finite half-life of the mRNA transcript and its dilution between daughter cells, with repeated infusions given to overcome the transient persistence[183]. However, this approach may require the production of large quantities of therapeutic T cells to meet the demands of repeated infusions. Moreover, the repeated delivery of a mRNA CAR T cell product based on a murine scFv provoked anaphylaxis owing to the emergence of human anti-murine antibodies of the IgE class[184], thus creating a new product risk.

As an alternative to engineered T cell products with short half-lives, safety or suicide tags and switches are a class of engineering solution intended to enable the rapid depletion of the gene-engineered T cell in the event of unacceptable toxicity (FIG. 6a). Safety tags comprise whole[185] or truncated[186] antigens encoded alongside the CAR or TCR that are recognized by approved mAb therapies, allowing depletion by Fc-dependent mechanisms. However, as mAbs generally do not penetrate the central nervous system, where therapeutic T cells might need to be depleted[187], and Fc-dependent mechanisms might act too slowly, especially in patients with lymphopenia, the exploration of suicide switches is warranted. The inducible caspase 9 (iCasp9) safety switch is activated by a small molecule, rimiducid, that dimerizes the domains of the iCasp9 switch, leading to rapid induction of apoptosis[188]. While it may be possible to partially activate the suicide switch or tag and thereby avoid total elimination of the gene-engineered T cell, an emergent severe toxicity will likely necessitate stringent

intervention and thus depletion of the expensive engineered T cells, likely leaving the patient to face disease progression should they recover from the acute toxicity. For these reasons, alternatives to safety switches are being explored.

On and off switches.—On and off switches act by regulating the transcription, translation or protein stability of the CAR or TCR or, alternatively, by regulating the availability of co-stimulation to promote T cell activation and survival (FIG. 6a).

To regulate activity of the transgene at the transcription level, drug-inducible switches such as the tetracycline-inducible (Tet-On) system[189–191] and the doxycycline-inducible system[192] have been demonstrated. These systems are somewhat limited by ‘leaky’ expression of the transgene, the fairly slow loss of expression on drug withdrawal[189] and immunogenicity of a bacterial-derived antibiotic regulatory system.

Rimiducid-inducible protein dimerization has also been used to create an ‘on switch’ for therapeutic T cells by dimerizing a ‘signal 2’ construct comprising MYD88 and CD40 to provide potent co-stimulation for CAR T cells that were designed to be minimally active without co-stimulation[193]. To enable both an on switch and a safety switch in the same therapeutic cell, an iCasp9 switch using rapamycin as a dimerizer was reported[194]. An alternative on switch design makes use of a split CAR, where dimerization is triggered by a rapamycin analogue acting on rapamycin-binding domains[195]. Aside from small-molecule sensing on switches, a light-responsive element has been demonstrated in the CAR T cell setting, whereby a blue light stimulates expression of the CAR[196]. So far, this system has been demonstrated to work only for locally delivered CAR T cells in subcutaneous tumours in mice and required cycles of 12 h of light induction, and thus applicability to visceral masses and shorter induction periods remains to be demonstrated.

To regulate activity of the transgene at the level of translation and/or protein turnover, ‘off switch’ systems have been developed. In these systems, the transgene is tagged in ways that allow drug-induced degradation via the ubiquitin–proteasome system. This is achieved by dosing with a proteolysis-targeting chimera (PROTAC) molecule[197] or by activating self-degradation domains with small-molecule compounds[198,199].

As an alternative to inbuilt off switches, the approved tyrosine kinase inhibitor dasatinib can switch off CAR T cells by inhibiting LCK-mediated signalling[200]. Indeed, the favourable pharmacokinetic profile of dasatinib supported dose titration to moderate the activity of CAR T cells, and the inhibitory effects were rapidly reversed in vitro and in vivo when the drug was withdrawn[200]. A potential drawback of using a non-selective off switch if applied regularly or for longer durations might be the suppression of epitope spreading, which requires the endogenous immune system and is a desired factor in immunotherapy generally.

Universal CAR T cells.—An alternative approach to enable exogenous control over gene-engineered T cells and simultaneously address the issue of antigen loss is the aforementioned universal CAR that binds to a separately infused targeting moiety. To optimize safety, the targeting moiety should have a limited half-life to enable control of universal CAR T cell activity by the dose and dosing schedule of the targeting ligand (FIG.

6b). This requirement may disfavour the CD16 CAR plus mAb system[132] given the long half-life of mAbs.

A barrier for the application of all exogenously controlled gene-engineered T cell therapy platforms will be in developing the clinical guidelines for regulating the activity of the therapeutic T cells in the patient. This is especially challenging given the lack of methods for real-time analysis of biomarkers, such as cytokine and T cell levels, leaving the physician to react to data that are out of date.

Endogenously programmed T cells

Boolean and IF/THEN logic gates.—Over the past decade, the biological equivalents of the OR, AND and NOT Boolean logic gates have emerged (reviewed in REF.[201]). The OR gate is designed to prevent antigen escape rather than to increase specificity[202] and was discussed previously, but the AND and NOT gates are both designed with increased tumour specificity in mind (FIG. 6c). However, these T cell Boolean logic gates have their drawbacks. For the AND gate, in which ‘signal 1’ (CD3ζ) and ‘signal 2’ are split onto separate CARs, signal 1 alone is sufficient to trigger activation of the memory and effector T cell subsets in the gene-engineered product, while addition of signal 2 boosts the response and enables activation of naive T cells. The loss of either target antigen will diminish the activity of the product, increasing the risk of tumour escape. For the NOT gate, in which a second CAR provides a suppressive signal, it is challenging to find an ideal target associated with healthy tissues that is absent on tumours, and to design a sufficiently potent inhibitory signal for the NOT CAR[203]. Such limitations have stimulated the design of a novel protein logic system and more advanced genetic circuits such as synthetic Notch (synNotch) and hypoxia-inducible systems.

The protein logic system is a true Boolean logic system that combines the combinatorial antigen requirements of the aforementioned Boolean logic CARs with the concept of a universal CAR T cell system recognizing a separately infused logic-programmed targeting ligand. The system relies on synthetically designed colocalization-dependent latching orthogonal cage–key protein (co-LOCKR) switches[204]. These are designed with combinatorial AND, OR and NOT logic in mind, such that after binding to a target cell, they undergo a conformational change to reveal a tag to be recognized by a tag-specific CAR T cell only if the required logic conditions are met. Precise control of CAR T cell activity has been shown in vitro with this system[204], but it will be vital to establish whether co-LOCKR switches, which are non-human synthetic proteins, can be designed with the necessary pharmacokinetics and low immunogenicity to work in patients, and it will remain a challenge to find appropriate antigen targets for use in NOT logic components just as it was for the earlier NOT CARs[203].

The synNotch and hypoxia-inducible CAR systems are ‘IF/THEN’-type logic systems where an encounter with a specific antigen or condition induces functional expression of a CAR to a second antigen that is capable of mediating full T cell activation. The objective with these systems is to compartmentalize activity of gene-engineered T cells to the tumour while reducing or preferably eliminating the activity in healthy tissues.

For the synNotch system, it was shown that on-target off-tumour toxicity could be avoided in animal models as long as healthy cells expressing the target of the second ‘traditional’ CAR were not co-located with the tumour[205,206]. Where healthy and tumour cells were co-located, once primed by the synNotch CAR, gene-engineered T cells were able to kill antigen-positive healthy and tumour tissues alike[205], which is a drawback of this type of system that will need further attention. Moreover, current synNotch systems are limited by the use of immunogenic sequences of non-human origin, potentially limiting product persistence in the patient.

An alternative fully human conditional expression system links CAR expression to hypoxia by including elements of the oxygen-sensitive subdomain of HIF1α in the CAR molecule[207]. With use of this system, it was found that CAR expression could be induced by hypoxia and dropped by 80% within 2 h when normoxia was restored. The resulting CAR showed a significant hypoxia-dependent differential in cytotoxicity towards target cells, although this shift was not binary[207]. Future studies may determine whether such designs sufficiently limit on-target off-tumour toxicity by focusing T cell activation to hypoxic environments common to solid tumours.

Alternative approaches.—Alternative approaches to logic-gated or inducible systems include the tuning of the expression or affinity of the transgenic receptor, or modulating its function by altering the linker length between the antigen-binding and transmembrane domains (FIG. 6d,e). For approaches that modulate the expression or affinity of the immune receptor, the ultimate goal is to calibrate the immune receptor to recognize antigen densities typical on tumour cells while sparing healthy cells, with the possibility of also reducing or avoiding tonic signalling, which is detrimental to T cell function and persistence[85]. The expression of transgenes can be regulated at the level of mRNA transcript stability via inclusion of microRNA-response elements[208] or at the protein level by inclusion of destabilizing tags known as ‘degrons’[209]. So far, working model systems for both approaches have been described, and their application to CAR T cells or TCR T cells is pending. Affinity tuning of the antigen-binding domain has been shown to enable gene-engineered T cells to avoid activation by low antigen levels on healthy cells while retaining anti-tumour activity[56]. Finally, changing the hinge length in a CD19 CAR T cell was shown both in preclinical models and in the clinic to uncouple efficacy from cytokine release syndrome and immune effector cell-associated neurotoxicity syndrome by reducing cytokine production while maintaining cytotoxicity[210]. Iterations and improvements to immune receptor design using the concepts of logic-gating, conditional induction and/or fine-tuned expression or binding affinity will likely increase the inherent safety of gene-engineered T cell therapies, thereby simplifying toxicity management.

Concluding remarks

Genetic engineering of T cells is opening new avenues to treat a wide array of disease states. Successful approaches to date rely on the constitutive expression of a single protein. To reach the full potential of engineered T cells, numerous genetic alterations will likely be necessary. Moreover, engineering approaches that allow T cells to adapt to multiple challenges simultaneously, such as adapting to antigen escape, poor nutrient environments

and T cell exhaustion, will likely be required to enable durable successful clinical outcomes. The ease of engineering and the impressive array of both pro-immune and anti-immune response functions of T cells opens seemingly limitless possibilities for how T cells can be altered to fight a particular disease. However, numerous examples of engineered T cells having caused significant morbidity and in some cases death have been reported. Thus, toxicity remains the biggest barrier to widespread use of genetically engineered T cells. Improved tools such as prime editing[211], controlled expression of immune modulators and safety switches will hopefully allow both effective and safe engineered T cell-based therapies.

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Competing interests

J.L.R. is a co-founder and shareholder of Tmunity Therapeutics. He has been in receipt of research funding from Tmunity Therapeutics. N.C.S. is a shareholder of Tmunity Therapeutics and Fate Therapeutics. G.I.E. declares no competing interests.

Glossary

Allogeneic
A term to denote a genetically different individual of the same species

Antigen loss
A mechanism of cancer or pathogen recurrence in which the target (tumour or pathogen) escapes engineered T cell recognition, typically via exon skipping, downregulation or alteration of the target antigen

Epitope spreading
Diversification of the immune response by endogenous immune cells against new targets following engineered T cell therapy.

Boolean logic gate
AND, OR and NOT functions, which can describe the response of combinations of immune receptors to multiple antigens

Immune synapse
The location of physical interaction between an immune cell and its activator, either another immune cell or a non-haematopoietic target cell. The strength and the type of signals exchanged at the immune synapse can modulate an immune response

Single-chain variable fragment
(scFv). A linear transposition of an antibody’s heavy and light chains separated by a flexible linker, which retains the antigen binding capacity of the original antibody and can be used as the binder domain of a chimeric antigen receptor molecule

Monoclonal antibodies

(mAbs). Antibodies purified from a single B cell hybridoma that can be used as a diagnostic or therapeutic product

Fc-dependent mechanisms

A series of effector modalities by which targets bound by antibodies are depleted via recognition of the constant region of the antibody molecule.

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Box 1 |

The evolution of CAR T cell therapies

Some of the first iterations of engineered T cells were designed to combat viral infections. Chimeric antigen receptor (CAR) T cells expressing a CD4 extracellular binder domain and the CD3ζ signalling domain (signal 1 alone) capitalized on HIV's dependency on CD4 for viral entry[229]. Although these CD4ζ CAR T cell clinical trials were unable to show long-term clinical benefit, they were able to demonstrate the long-term safety and feasibility of engineered adoptive T cell transfer[19]. The addition of co-stimulatory signalling domains (signal 2) to CARs enhanced in vivo function[70,230] and persistence[109], and has enabled T cell engineers to design collections of co-stimulatory signalling domains that tailor CAR T cells to a particular malady[53,54]. A major clinical breakthrough for engineered T cell therapeutics came in 2011, when a CAR T cell therapy targeting CD19 achieved durable remission of paediatric B cell acute lymphoblastic leukaemia[231]. This report was swiftly followed by other reports of clinical activity of CD19 CAR T cells in B cell malignancies[232,233], and together these findings contributed to the regulatory approval of Kymriah (Novartis) and Yescarta (Kite) and the near-term approval of lisocabtagene maraleucel (Bristol Myers Squibb), all of which target the CD19 lineage antigen on B cells. The resulting proliferation of transgenic T cell receptor T cell[123] and CAR T cell[234] trials for other haematologic malignancies has been met with enthusiasm. However, due to chemical and mechanical barriers in the tumour microenvironment, T cells engineered for solid malignancies have been less effective and require further engineering to overcome the tumour microenvironment and tumour immune evasion mechanisms.

From lessons learned from cancer CAR therapy[235], a new generation of HIV-specific CAR T cells have been generated[81,107,236–239], some of which are currently being tested in phase I clinical trials (https://clinicaltrials.gov/ct2/show/NCT03617198 and https://clinicaltrials.gov/ct2/show/NCT03240328). Preclinical models have shown that CAR T cells can be developed to oppose other infectious diseases, including hepatitis B virus infection[240], hepatitis C virus infection[241] and opportunistic fungal infections[242], suggesting that CAR T cells could be an important therapeutic for many infectious diseases.

Box 2 |

Genome-editing technologies

ZFNs, TALENs and megaTALs

Zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) are chimeric proteins that fuse site-specific DNA binding with the non-specific cleavage domain of the endonuclease FokI. The obligate dimer character of FokI[243] necessitates ZFN or TALEN binding to both plus and minus strands of DNA, decreasing off-target cleavage at the expense of on-target efficiency. The ease in generating TALENs for virtually any genomic sequence is an advantage over ZFNs, and a collection of TALENs targeting every protein in the human genome has been created[244]. However, TALENs are about threefold larger than ZFNs, making them more difficult to package in viral vectors. When TALEN and ZFN editing were directly compared at the *CCR5* and *IL2RG* loci, TALENs were as specific as ZFNs and provoked less cytotoxicity[220]. Infusion of ZFN-modified and TALEN-modified T cells into patients has demonstrated safety and efficacy, including a lack of product tumorigenesis, despite the presence of rearranged chromosomes generated by DNA double-strand breaks (DSBs)[212,218].

MegaTAL nucleases share DNA-binding domains with TALENs but have a meganuclease replacing FokI, allowing cutting with a single molecule rather than a pair[245]. In comparison with TALENs, megaTALs induced more non-homologous end joining insertions and deletions (indels) at *TRAC*[246] and fewer at *CCR5* (REF.[103]).

CRISPR–Cas9

CRISPR–Cas9 uses a guide RNA to ferry the nuclease Cas9 to a region of interest to induce gene knockout by DSB and indel formation following non-homologous end joining repair. dCas9 is an enzymatically inactive variant that retains its ability to be guided by RNA[247] and can be tethered to transcriptional activators[248], repressors[249] or methyltransferases[250] to influence transcription of DNA, often in non-coding regions. Other Cas molecules are in development that have different protospacer adjacent motifs, enabling CRISPR-mediated DSBs in areas inaccessible to Cas9 (REF.[251]). A clinical trial of multiplex CRISPR T cell receptor-engineered T cell adoptive therapy demonstrated feasibility and persistence of cells for up to 9 months, albeit with chromosomal translocations between CRISPR sites[10].

Base editing

Base editing uses a fusion protein consisting of either a cytidine deaminase or an adenosine deaminase and dCas9 to impart RNA-guided transition point mutations without DSBs[252], reducing the chance of chromosomal translocation. Mutations induced by base editing can correct genes that contribute to a disease phenotype or knock out protein expression by alteration of RNA splice donor–acceptor pairs or introduction of a premature stop codon. In T cells, triplex base editing of *B2M*, *CIITA* and *TRAC* occurred at more than 98% of sequencing reads and eliminated surface expression of β2M and HLA-DR[225]. CD3 expression due to *TRAC* editing was downregulated but not

extinguished, emphasizing that nonsense-mediated decay is not sufficient to knock out protein expression for some loci[225]. Additionally, ribosomal stop codon readthrough can circumvent novel stop codon mutations.

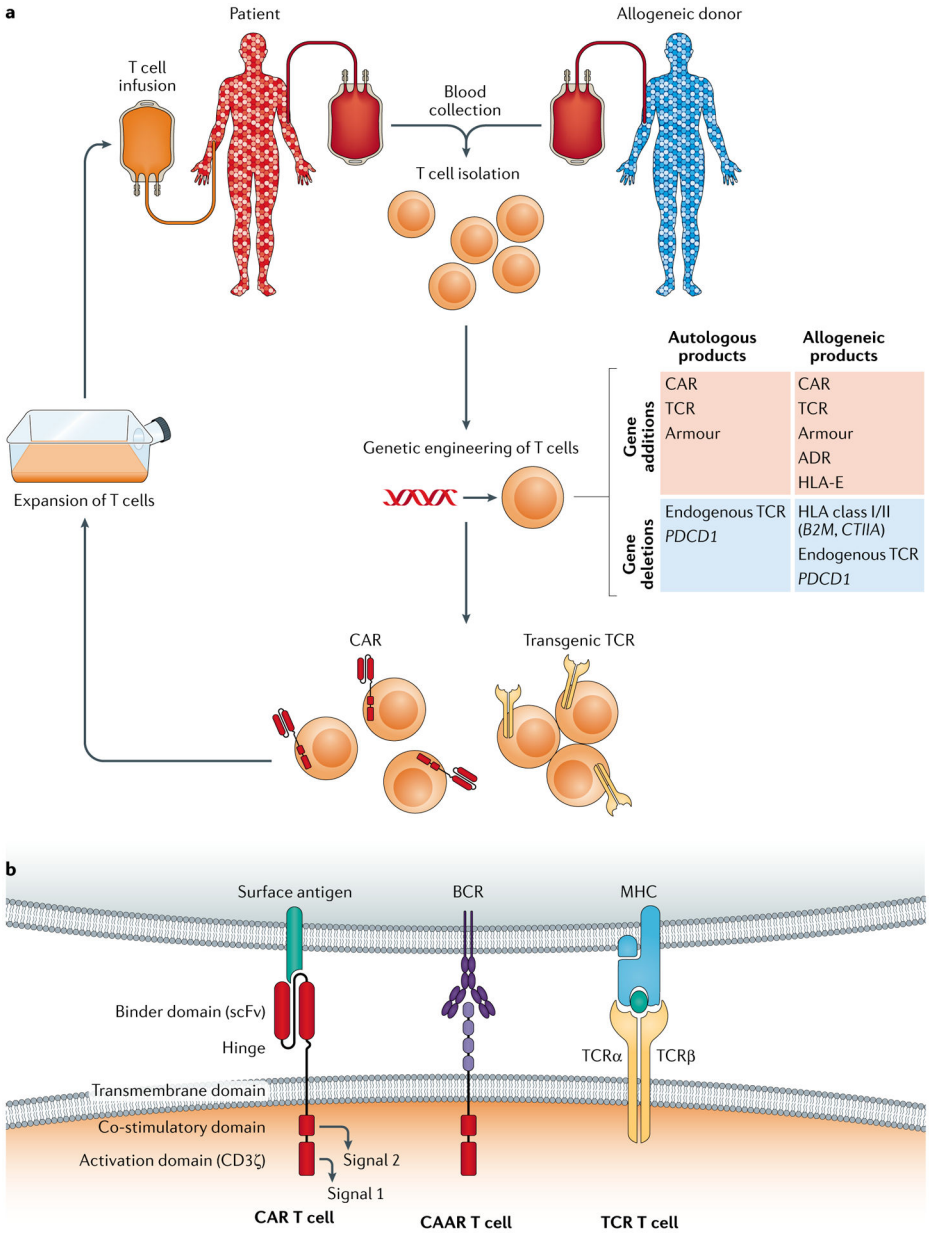

Fig. 1 |. Autologous and allogeneic T cell immunotherapy.

a | Manufacturing process. Blood is collected by venipuncture or apheresis, and T cells are isolated either from the patient (autologous donor) or from an allogeneic donor. Purified T cells undergo engineering to introduce a transgene using viral vectors or non-viral strategies and/or genome editing to eliminate protein expression. Following stimulation, engineered T cells are expanded to increase cellular dosage and infused into the patient. Site-specific transgene insertion may be preferred for allogeneic products to reduce batch-to-batch variation (not shown). T cells can undergo genetic engineering (middle right), for example, to endow antigen specificity, evade the host immune response or confer resistance to the tumour microenvironment. From an efficacy perspective, allogeneic T cell products are vulnerable to immune-mediated rejection[34]. This can be ameliorated by genetic deletion of

both HLA class I and HLA class II expression by targeting *B2M* and *CIITA*, respectively, which extends the survival of chimeric antigen receptor (CAR) T cells in preclinical models[27]. However, allogeneic CAR T cells without HLA molecules can be killed by the recipient's natural killer cells, which may necessitate the overexpression of HLA-E or other non-classical major histocompatibility complex (MHC) molecules as a remedy[226]. Deleting multiple genes and adding HLA-E in addition to a CAR or T cell receptor (TCR) is fairly complex, and thus recently an alloimmune defence receptor (ADR) approach was described whereby a CAR is used to trigger cytotoxicity of alloreactive host T cells and natural killer cells[227]. **b** | Composition of CARs, chimeric autoantibody receptors (CAARs) and transgenic TCRs. CARs and CAARs recognize antigen via a binder domain — typically a single-chain variable fragment (scFv) or a protein target of a B cell receptor (BCR), respectively. Each also has a flexible hinge domain, a transmembrane domain and a CD3ζ activation domain (signal 1), along with a choice of co-stimulatory domains (signal 2) tailored to fit the therapeutic task. Alternatively, a pair of TCRα and TCRβ chains dimerize to form a transgenic TCR, which recognizes MHC-presented antigens. Wild-type chains can be engineered to facilitate their dimerization preferentially over cross-pairing with the T cell's endogenous chains.

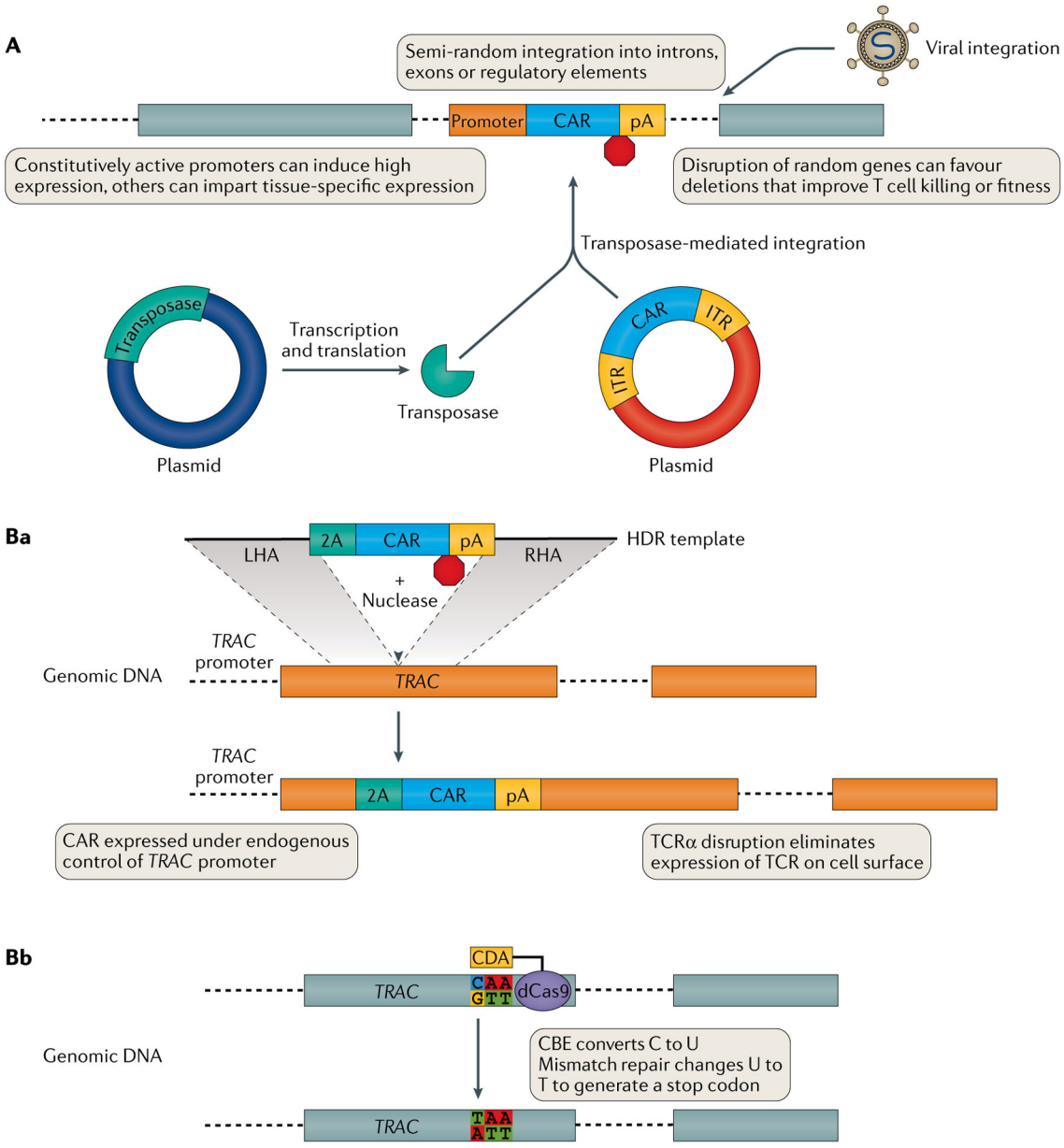

Fig. 2 |. The genetic outcome of modifying the T cell genome.

A | Viral vectors (top) or co-delivery of transposase and transposon (bottom) can integrate transgenes into the genome in a non-targeted fashion. **Ba** | Nuclease-targeted DNA double-strand breaks in the presence of homology directed repair (HDR) template DNA facilitates integration of template DNA at a specific genomic locus. Depending on the design of the template and the location of the DNA double-strand breaks, the transgene can be placed under the control of an endogenous promoter and/or can knock out expression of a gene into which it integrates. For example, integrating a transgene expressing a chimeric antigen receptor (CAR) into the *TRAC* locus, which encodes the T cell receptor (TCR) α-chain constant region, eliminates endogenous TCR expression. **Bb** | Cytidine deaminase (CDA) or adenine deaminase tethered to catalytically inactive ('dead') Cas9 (dCas9) forms a cytosine

base editor (CBE) or adenosine base editor, respectively. Base editing can repair damaged genes or generate functional knockouts through introduction of a novel stop codon (shown) or by disrupting RNA splice acceptor–donor pairs. 2A, ribosomal skip peptide; ITR, inverted terminal repeat; LHA, left homology arm; pA, poly(A) tail; RHA, right homology arm; TCR, T cell receptor.

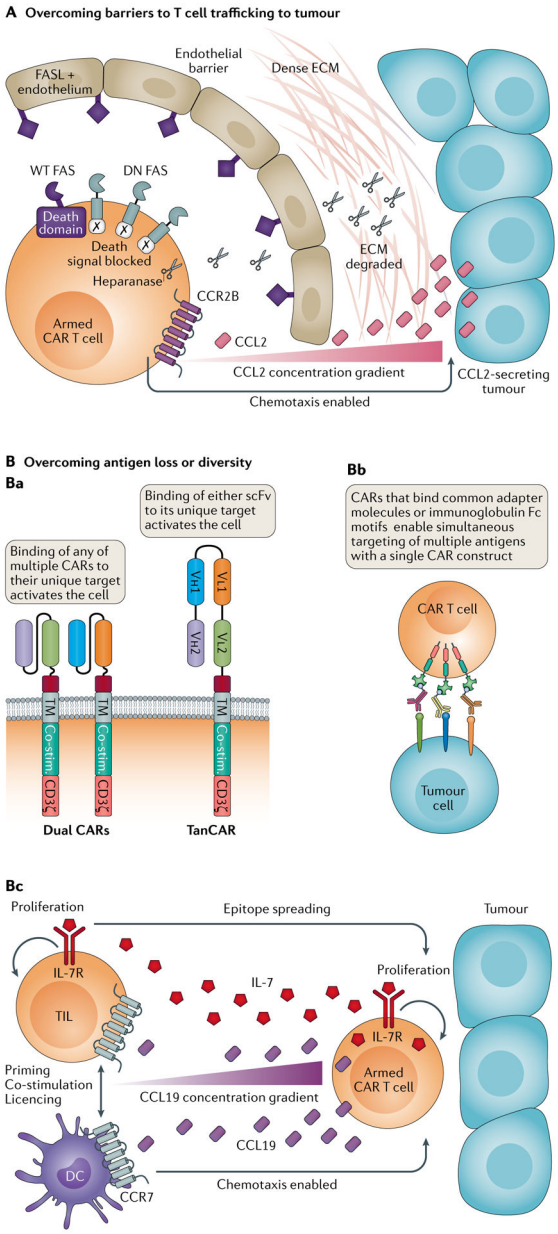

Fig. 3 |. Gene-engineered T cell products to enhance efficacy.

A | Overcoming barriers to T cell trafficking to tumour. The addition of an appropriate chemokine receptor matched to sense chemokines released by the target tumour has increased T cell infiltration in animal models. Addition of a dominant negative (DN) FAS receptor has enabled engineered T cells to avoid FAS ligand (FASL)-mediated apoptosis induction. Equipping T cells to secrete heparanase has enabled them to counter the dense extracellular matrix (ECM) of tumours in preclinical models. **B** | Overcoming antigen loss or diversity can be achieved by transduction with two or more chimeric antigen receptors (CARs) (OR gate logic) or via tandem CARs (TanCARs) (panel **Ba**), using a universal CAR and separate tumour-targeting ligands (panel **Bb**) or equipping T cells to express both growth factors and chemokines to enhance infiltration of tumours by T cells and dendritic

cells (DCs) (panel **Bc**). Co-stim., co-stimulatory domain; scFv, single-chain variable fragment; TIL, tumour-infiltrating lymphocyte; TM, transmembrane domain; WT, wild type.

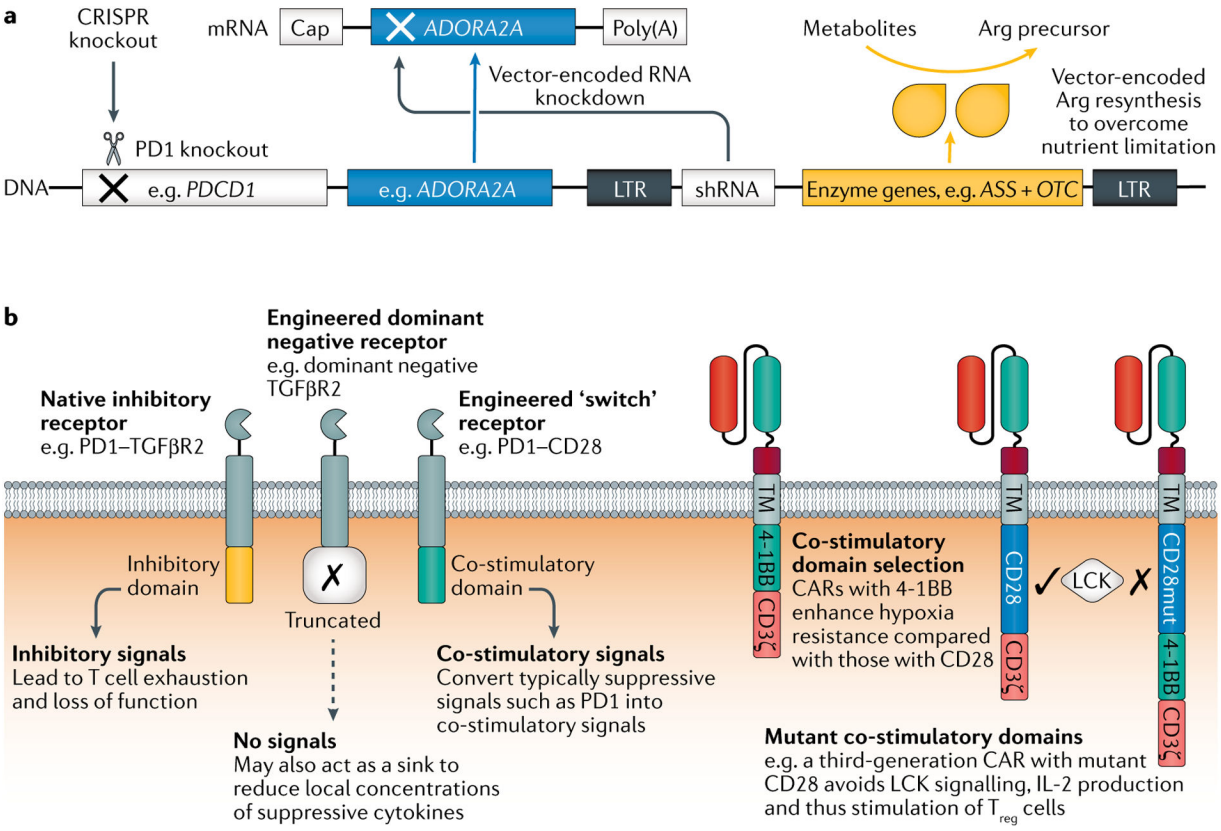

Fig. 4 |. Addition of armour or subtraction of suppressive genes or their transcripts enables TME resistance.

a | Many potential design solutions are available, including gene deletion (for example, using CRISPR–Cas9 genome editing to knock out *PDCD1*), RNA interference (for example, knockdown of the adenosine A_{2A} receptor gene *ADORA2A* to confer adenosine resistance) and addition of genes to enable metabolic self-sufficiency (for example, addition of *ASS* and *OTC*, which encode the enzymes argininosuccinate synthetase and ornithine transcarbamylase, respectively) or that counter specific checkpoints and suppressive cytokines. **b** | Receptor-based approaches to resist tumour microenvironment (TME) suppression include expression of a dominant negative form of an inhibitory receptor to act as a sink. Alternatively, the receptor's inhibitory domain can be switched to a co-stimulatory domain to change the inhibitory signal into an activating one. Chimeric antigen receptor (CAR) design considerations include the choice of co-stimulatory domains; both natural and mutant co-stimulatory domains may enhance the ability of the engineered T cell to survive in the suppressive TME. Multiple strategies may be used in parallel and should be customized to the specific TME roadblocks encountered. LTR, long terminal repeat; shRNA, short hairpin RNA; TM, transmembrane domain; T_{reg} cell, regulatory T cell.

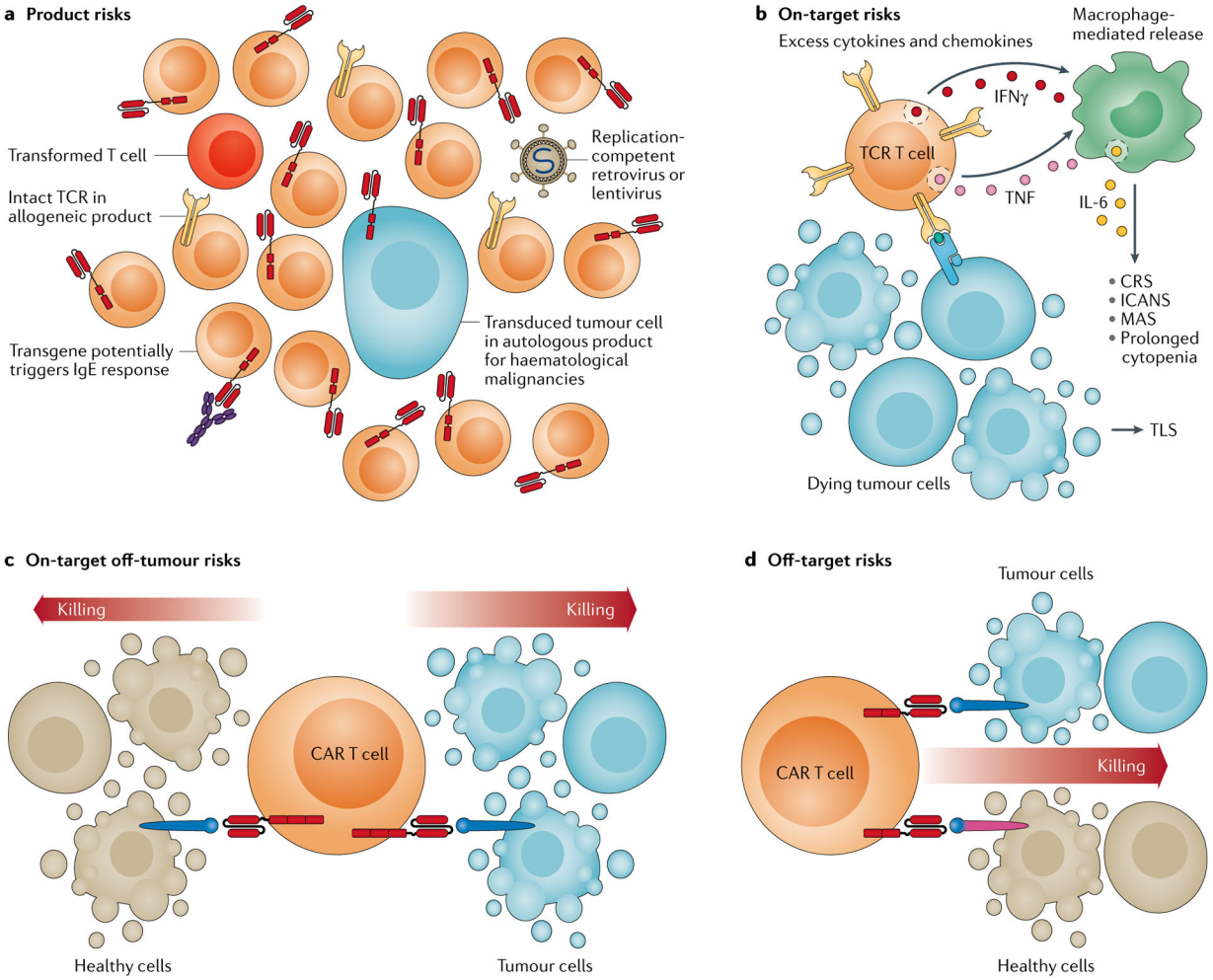

Fig. 5 |. Toxicity risks associated with gene-engineered T cells.

a | Product risks are the inherent liabilities associated with the manufactured product such as unwanted contaminants or by-products of the process, including replication competent vectors, chimeric antigen receptor (CAR) transformed residual tumour cells, T cells with DNA damage that may become cancerous themselves and allogeneic T cells that have escaped T cell receptor (TCR) ablation and thus pose a graft-versus-host disease risk. These risks also include the potential immunogenicity of the transgenic construct, which may provoke infusion reactions. **b** | On-target toxicity derives from a surplus of target cell killing resulting in tumour lysis syndrome (TLS) or a surplus of cytokine and chemokine production by the therapeutic T cells alone or in concert with myeloid cells that results in cytokine release syndrome (CRS), immune effector cell-associated neurotoxicity syndrome (ICANS), macrophage activation syndrome (MAS) or prolonged cytopenia. **c** | On-target off-tumour toxicity occurs if the target antigen is also found on healthy cells at levels sufficient to trigger the therapeutic T cells. **d** | Off-target toxicity occurs if the transgenic immune receptor cross-reacts with an antigen found on healthy tissues. IFNγ interferon-γ; TNF, tumour necrosis factor.

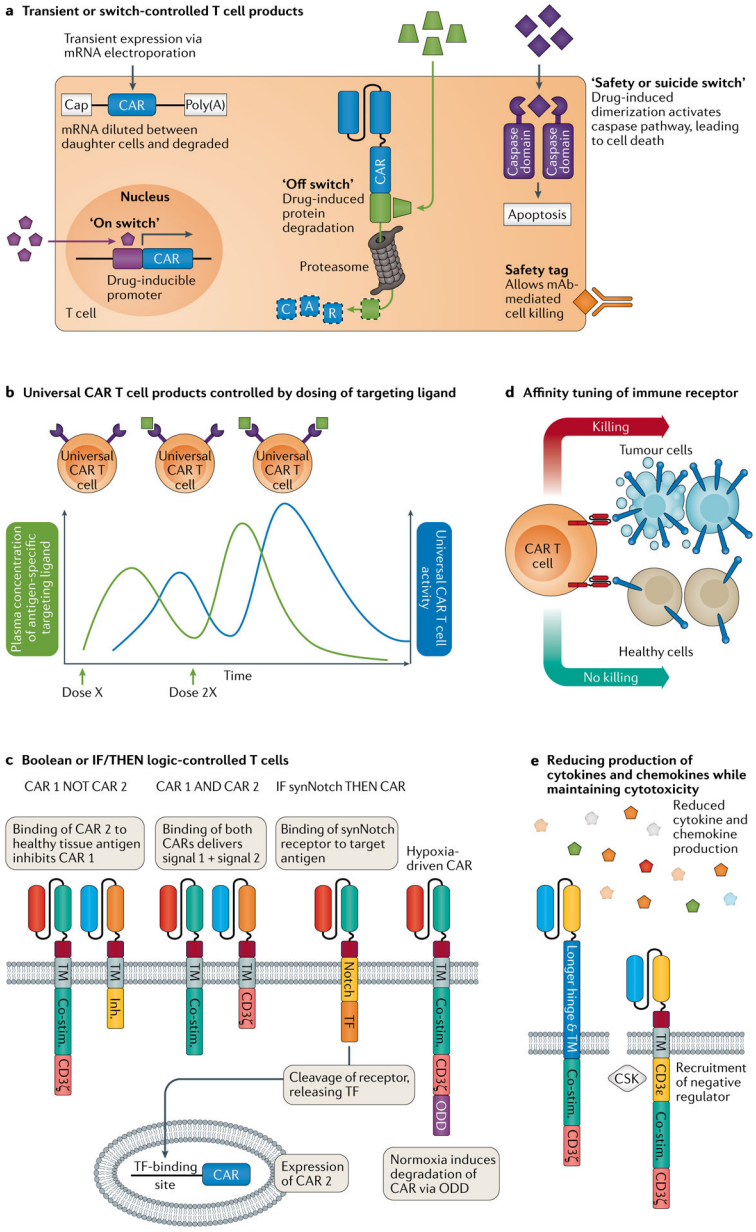

Fig. 6 |. Gene-engineered T cell products for enhanced safety.

a | Exogenous control of gene-engineered T cells can be achieved via transient delivery of the transgene using mRNA electroporation or via application of a variety of on, off or suicide switches. The transgene may be switched on or off at the level of transcription or via induced protein degradation, or the therapeutic T cell can be eliminated either by a caspase-activation system or via the inclusion of specific tags recognized by approved therapeutic monoclonal antibodies (mAbs). **b** | Exogenous control can also be achieved via a 'universal' chimeric antigen receptor (CAR) system in which infusion of separate antigen-specific targeting ligands redirects the CAR T cells to the target, while the dose and schedule of the antigen-specific targeting ligand can be modified as required. **c** | Endogenous control can be achieved via Boolean logic-type CAR designs that require CAR T cells to recognize two

antigens for full activation (AND gate, via a split CAR) or inhibit CAR T cell activation via a second inhibitory CAR (NOT gate). The synthetic Notch (synNotch) and hypoxia-inducible systems provide conditional 'IF/THEN' logic for endogenous control of T cell activity. In the synNotch system, the first CAR is an artificial Notch-type receptor wherein antigen recognition stimulates proteolytic cleavage of a transcription factor (TF), which in turn triggers expression of a second 'traditional' CAR recognizing a separate antigen, making sensing of the second antigen dependent on first sensing the first antigen. In the hypoxia-inducible system, the CAR includes oxygen-sensing elements of hypoxia inducible factor 1α, which promote its degradation via the ubiquitin pathway under normoxia but allow full expression in hypoxic conditions such as those found in solid tumours. **d** | Affinity tuning of the immune receptor provides another form of endogenous control whereby the affinity is set to enable the therapeutic T cell to be activated by tumours expressing high levels of the antigen but not by healthy tissues that express low levels. **e** | Extending the hinge and transmembrane (TM) domain of a CAR enabled maintenance of cytotoxicity but a significant reduction in the production of cytokines and chemokines, and subsequent cytokine release syndrome and immune effector cell-associated neurotoxicity syndrome[210]. Similarly, engineering CARs to express the CD3ε domain recruited the negative regulator CSK and resulted in more transient signalling with maintained cytotoxicity but reduced cytokine and chemokine expression[228]. Co-stim., co-stimulatory domain; Inh., inhibitory domain; ODD, oxygen-dependent degradation domain.

Table 1 |

Advantages and limitations of technologies for genome editing of therapeutic T cell products

Technology	Mechanism	On-target efficacy	Limitations	Benefits	Targets	Rationale
ZFN	DSB; ZFN DBD + FokI endonuclease domain; operates as dimer	Up to 28% in a clinical trial[212]; up to 90% on a non-clinical scale[213]	ZFN specificity designed by screening then tested empirically; requires pair of ZFNs	Low off-target editing	*CCR5* (REFS[100,212,214])	Resistance to HIV by knockout of viral co-receptor
					CXCL4 (REFS[215,216])	
					TRAC[213]	Knockout of endogenous TCR[a]
					TRBC[217]	
TALEN	DSB; TALE DBD + FokI endonuclease domain; operates as dimer	Up to 64% in a clinical trial[218]; up to 80% on a non-clinical scale[219]	Large protein size; can induce translocations[218]	Low off-target editing	*CD52* (REF.[218])	Resistance to anti-CD52 antibody therapy
					CD40L[102]	Repair of damaged gene causing hyper-IgM syndrome
					CCR5 (REF.[220])	Resistance to HIV by knockout of viral co-receptor
					DCK (encodes deoxycytidine kinase)[219]	Resistance to purine nucleotide analogue drugs
					PDCD1 (encodes PD1)[221]	Reduces T cell exhaustion
					TRAC[219,221]	Knockout of endogenous TCR[a]
MegaTAL	DSB; TALE DBD + meganuclease	Up to 61% on a non-clinical scale[101]	Off-target editing[103]	Small protein size	*CCR5* (REF.[103])	Resistance to HIV by knockout of viral co-receptor
CRISPR-Cas9	DSB; gRNA + Endonuclease Cas9	Up to 45% in a clinical trial[10]; up to 95% on a non-clinical scale[222]	Target must be adjacent to PAM; high degree of off-target editing or translocations	Easy to design and screen guides, high efficiency	*B2M*[222]	Protects universal T cells from alloreactive rejection by host
					CCR5 (REF.[223])	Resistance to HIV by knockout of viral co-receptor
					PDCD1 (REF.[222])	Reduces T cell exhaustion
					RNF20 (REF.[159])	Stabilizes FOXP3 in T_{reg} cells
					TRAC[85,222]	Knockout of endogenous TCR[a]
					TRBC[222]	

Technology	Mechanism	On-target efficacy	Limitations	Benefits	Targets	Rationale
Nuclease + HDR	DSB in presence of ssDNA or dsDNA delivered by AAV or electroporation	Non-viral: up to 61% on a non-clinical scale[105] Viral: 40–60% on a non-clinical scale[85,224]	Immunogenicity of DNA or AAV, off-target integration; dependent on presence of adjacent PAM	Integration of large cargoes	*AAVS1* (REF.[100])	Safe harbour site for genome integration
					CD40L[102]	Repair of damaged gene causing hyper-IgM syndrome
					FOXP3 (REF.[75])	Converts T_{eff} cells into T_{reg}-like cells
					IL2RA[104]	Repair of damaged gene causing autoimmunity
					IL2RG[220]	Repair of damaged gene causing SCID
					TRAC[85,104]	Puts CAR or other transgenes under control of endogenous promoter
Base editing	Nucleotide deaminase + dCas9 + uracil DNA glycosylase inhibitor + single gRNA	>95% on a non-clinical scale[225]	Transition mutations only, no deletions or insertions; bystander edits in editing window, dependent on adjacent PAM; may not eliminate protein expression	No DSB, no detectable translocations in triple editing	*TRAC*[225]	Knockout of endogenous TCR[a]
					CIITA[225]	Protects universal T cells from alloreactive rejection by host
					B2M[225]	

AAV, adeno-associated virus; CAR, chimeric antigen receptor; DBD, DNA-binding domain; dCas9, dead (catalytically inactive) Cas9; DSB, DNA double-strand break; dsDNA, double-stranded DNA; gRNA, guide RNA; HDR, homology directed repair; PAM, protospacer adjacent motif; SCID, severe combined immunodeficiency; ssDNA, single-stranded DNA; TALE, transcription activator-like effector; TALEN, transcription activator-like effector nuclease; TCR, T cell receptor; T_{eff} cell; effector T cell; T_{reg} cell; regulatory T cell; ZFN, zinc-finger nuclease.

[a]Knockout of endogenous TCR improves surface expression of transgenic TCR or generates universal T cells.