'Off-the-shelf' allogeneic CAR T cells: development and challenges

S. Depil[1], P. Duchateau[2], S. A. Grupp[3], G. Mufti[4] and L. Poirot[2]*

Abstract | Autologous chimeric antigen receptor (CAR) T cells have changed the therapeutic landscape in haematological malignancies. Nevertheless, the use of allogeneic CAR T cells from donors has many potential advantages over autologous approaches, such as the immediate availability of cryopreserved batches for patient treatment, possible standardization of the CAR-T cell product, time for multiple cell modifications, redosing or combination of CAR T cells directed against different targets, and decreased cost using an industrialized process. However, allogeneic CAR T cells may cause life-threatening graft-versus-host disease and may be rapidly eliminated by the host immune system. The development of next-generation allogeneic CAR T cells to address these issues is an active area of research. In this Review, we analyse the different sources of T cells for optimal allogeneic CAR-T cell therapy and describe the different technological approaches, mainly based on gene editing, to produce allogeneic CAR T cells with limited potential for graft-versus-host disease. These improved allogeneic CAR-T cell products will pave the way for further breakthroughs in the treatment of cancer.

Leukapheresis
Procedure through which white blood cells are separated and collected from the blood with an advanced centrifuge while red blood cells and other blood components are returned into the circulation.

[1]Centre Léon Bérard and Centre de Recherche en Cancérologie de Lyon, Lyon, France.
[2]Cellectis, Paris, France.
[3]Children's Hospital of Philadelphia and Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA.
[4]King's College London and King's College Hospital, London, UK.
*e-mail: stephane.depil@lyon.unicancer.fr
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One of the most promising approaches in anticancer therapy is chimeric antigen receptor (CAR) T cell therapy, in which T cells are redirected against the tumour after engineered expression of CARs. First-generation CARs are fusion proteins that consist of an extracellular antigen-binding domain (which is usually the single-chain variable fragment of an antobody) linked to an intracellular signalling domain — usually the CD3ζ chain of the T cell receptor (TCR). In second-generation CARs, CAR-T cell activity is enhanced by addition of a costimulatory domain fused to CD3ζ, such as CD28 or CD137 (also known as 4-1BB) to support the expansion and persistence of the genetically engineered cells in vivo. Third-generation CARs, which include several costimulatory domains, have also been developed[1,2]. The use of autologous (patient-derived) second-generation CAR T cells has resulted in frequent complete responses in patients with haematological malignancies that were thus far considered incurable, and has led to the approval of two agents, tisagenlecleucel (Kymriah, Novartis) and axicabtagene ciloleucel (Yescarta, Kite Pharma), for the treatment of relapsed or refractory B cell acute lymphoblastic leukaemia (ALL) and relapsed or refractory diffuse large B cell lymphoma and primary mediastinal large B cell lymphoma[3,4]. Thus, CAR T cells represent a significant breakthrough in the field of cancer immunotherapy.

From an immunological point of view, autologous CAR-T cell therapy is associated with the absence of allogeneic reaction, and the engineered T cells can thus persist for a long time. However, autologous CAR T cell therapies require a bespoke manufacturing process for every patient after leukapheresis. Although this approach has resulted in outstanding clinical data to date, it has certain well-known disadvantages, such as cost of the process, manufacturing failure in some patients and current manufacturing processes of approximately 3 weeks, which translate into a delay in the availability of the treatment[5]. This delay can be particularly problematic in some patients with highly proliferative diseases, such as acute leukaemia, who may show disease progression before an autologous CAR T cell treatment is ready for use, or may lose eligibility for other reasons related to the disease or its therapy. Autologous T cells might not be effective in some patients owing to T cell dysfunction, which is a hallmark of many cancers and is associated with multiple mechanisms of immunosuppression derived from the tumour microenvironment[6]. The biological characteristics of autologous T cells are also negatively impacted by the previous lines of treatment. For these and other reasons, there are cases in which the production of a CAR T cell product from autologous T cells has failed[7]. Finally, the cost of this complex therapeutic approach remains high and is a challenge for health care systems[8].

The ability to use cells from healthy donors, referred to as 'off-the-shelf' allogeneic CAR T cells, could potentially address these issues. Allogeneic CAR T cells have many potential advantages, such as a decreased cost due to the implementation of industrialized and scaled-up

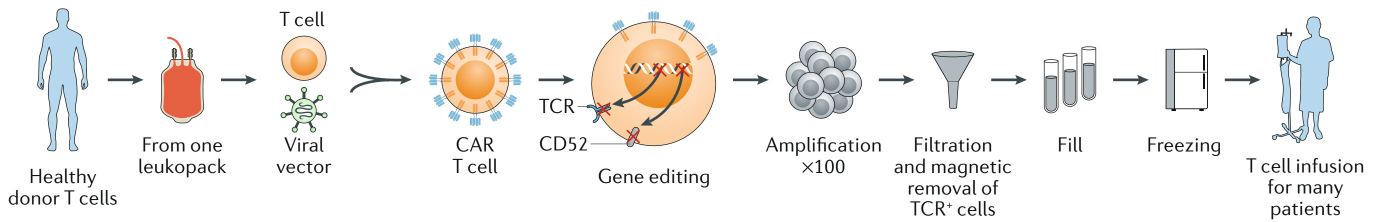

Fig. 1 | **Manufacturing of allogeneic CAR T cells.** Allogeneic chimeric antigen receptor (CAR) T cells from a single manufacturing batch have the potential to benefit multiple patients. The manufacturing process for allogeneic CAR-T cell products starts with a source of third-party healthy T lymphocytes collected by leukapheresis. Technologies such as viral vector-mediated transgenesis or gene knock-in mediated by gene editing enable the permanent insertion of recombinant DNA coding for a CAR and possibly additional genes, such as a suicide gene or a costimulation receptor in said lymphocytes. Technologies can also eliminate expression of αβ T cell receptor (TCR) on said T cells (for example, gene editing-mediated TCRα knockdown) and CD52. T cells are then expanded using anti-CD3/anti-CD28 beads and cytokines. The remaining αβ TCR-positive cells are magnetically removed using anti-αβ TCR antibodies. The vials are then filled with the allogeneic CAR T cells. The product is then stored, frozen and shipped to hospitals when needed.

manufacturing processes, in which a high number of CAR T cells can be produced from a single donor (FIG. 1). In addition, manufacturing allogeneic CAR T cells produces batches of cryopreserved T cells, making treatments immediately available for patients. It simplifies the process of introducing multiple cell modifications in a single cell product as well as the standardization of the CAR T cell product based on donor selection and processing. A key difference here is that a given autologous cell collection is often the only opportunity to make a cell product for that patient, whereas allogeneic cell manufacturing allows the creation of batches of products, which can be used if redosing is necessary. Off-the-shelf allogeneic CAR T cells may also allow combination of CAR T cells directed against different targets (TABLE 1).

However, allogeneic approaches are associated with two major issues. First, the administered allogeneic T cells may cause life-threatening graft-versus-host disease (GVHD). Second, these allogeneic T cells may be rapidly eliminated by the host immune system, limiting their antitumour activity.

In this Review, we present the different sources of T cells for allogeneic T cell therapy and discuss the main challenges associated with this approach and the different ways to improve the current versions of allogeneic CAR T cells. We also describe the different technological approaches, mainly based on gene editing, to produce allogeneic CAR T cells with limited or no potential for GVHD. Finally, as the first clinical trials using allogeneic gene-edited CAR T cells have begun and many companies are moving forward with different allogeneic CAR-T cell strategies, we discuss the perspectives of clinical development in immuno-oncology, both in haematological malignancies and in solid tumours.

Allogeneic CAR T cells sources

The T cells currently used for CAR-T cell manufacturing are mainly derived from peripheral blood mononuclear cells (PBMCs) and, rarely, from umbilical cord blood (UCB). In principle, these T cells could also be derived from renewable stem cells such as induced pluripotent stem cells (iPSCs) or embryonic stem cells.

The manufacture of allogeneic CAR T cells from PBMCs collected from healthy donors is associated with the ability to make multiple vials from a single apheresis product, and therefore with rapid access to previously manufactured products. In addition, there is the opportunity to generate a bank of cells that express the different subtypes of human leukocyte antigen (HLA) complex to potentially select batches that match the HLA type of the patient. Furthermore, because allogeneic CAR T cells are created from healthy donors, they are generated from immune cells that have not been impacted by the immune effects of cancer or by exposure to chemotherapeutic agents, in contrast to autologous T cells from patients. The selection of donors on the basis of their immune characteristics is likely to be a key factor in decreasing the heterogeneity of the final cell product, as discussed below.

The use of UCB-derived CAR-T cell transplantation can be associated with reduced incidence and severity of GVHD, which allows less stringent restrictions on the number of HLA disparities[9]. Indeed, T cells derived from UCB have a unique antigen-naive status that may be related to the decreased alloreactivity of UCB grafts[10]. Furthermore, UCB T cells are also characterized by impaired nuclear factor of activated T cells (NFAT) signalling and reduced reactivity[11], which may also explain the decreased risk of GVHD. Placenta-derived stem cells can be used to generate T cells or natural killer (NK) cells[12]. Placenta is characterized by a unique HLA expression pattern. Unlike all other tissues, extravillous cytotrophoblast cells express only HLA-C, HLA-E and HLA-G, and syncytiotrophoblast cells are HLA negative[13]. The consequences of these specificities on placenta-derived T cells have not been reported yet.

T cells derived from iPSCs can also be a source of CAR T cells[14]. Theoretically, a master iPSC line has unlimited capability to self-renew and can be banked and used indefinitely[14,15]. A bank of iPSCs with common HLA haplotypes can be generated to minimize the risk of allorejection of CAR iPSC T cells. In the context of HLA mismatch, gene editing can also be used to eliminate TCR to avoid GVHD, as discussed in detail later. One advantage of using iPSCs is that CAR T cells are generated from one clonal engineered pluripotent cell line and are therefore homogeneous. Of note, the

HLA disparities
Human leukocyte antigen (HLA) incompatibility between the donor and the recipient. HLA disparities are associated with higher risk of graft failure, delayed immune reconstitution and graft-versus-host disease. New protocols allow donors mismatched for up to six alleles (haploidentical donors) to be used in haematopoietic stem cell transplantation without detrimental graft-versus-host disease.

Antigen-naive
Not previously exposed to foreign antigens.

Extravillous cytotrophoblast cells
The cells of the outermost layer of the fetal component of the placenta.

Syncytiotrophoblast
The epithelial covering of the vascular embryonic placental villi, which invades the wall of the uterus to establish nutrient circulation between the embryo and the mother.

Alloimmunization
Formation of antibodies against non-self antigens (here human leukocyte antigen molecules of the donor).

Stem cell transplantation
(SCT). For allogeneic SCT, haematopoietic stem cells are taken from the bone marrow, peripheral blood or umbilical cord blood of a healthy donor matched for human leukocyte antigen alleles. For haploidentical transplant, a healthy first-degree relative — a parent, sibling or child — serves as a donor, who needs to be only a 50% match to the recipient.

safety and the efficacy of this approach have not yet been clinically assessed.

Strategies for the development of allogeneic CAR T cells must account for the risk of alloimmunization, which may preclude redosing with the same CAR-T cell batch. The presence of donor-specific anti-HLA antibodies (DSAs) is believed to be an important barrier to the successful engraftment of donor cells, and antibody-mediated graft rejection is a well-recognized cause of graft rejection and organ failure in solid organ transplantation[16]. The presence of DSAs is also associated with a higher incidence of engraftment failure in haploidentical stem cell transplantation (SCT)[17]. Thus, assessment of DSAs is required when an allogeneic T cell approach is used, and CAR T cells derived from another donor should be discussed in the case of proven immunization. One theoretical solution to decrease the risk of alloimmunization is the selection of donors with rare HLA alleles or of an ethnic origin different from that of the recipient for CAR-T cell manufacturing. As discussed later, elimination of HLA molecules on donor cells by gene editing is also a possibility.

Approaches to avoid GVHD

Potential risks associated with allogeneic cell therapy have been demonstrated in the context of allogeneic SCT. HLA mismatches between donor and recipient trigger immune recognition, potentially leading to graft rejection, the graft-versus-tumour effect, and GVHD. GVHD is the primary cause of morbidity and death in allogeneic SCT, and studies have shown that αβ T cells are central to the pathogenesis of both acute and chronic GvHD[18–22]. The TCR in αβ T cells recognizes peptides presented by major histocompatibility complex (MHC) molecules (the HLA system in humans). The MHC locus is the most polymorphic region in the human genome, leading to many thousands of expressed MHC variants. Of these MHC variants, any one individual can express, at most, six MHC class I molecules and six MHC class II molecules. Selection processes during thymic education result in a TCR repertoire that is tolerant of self-peptides presented by self-MHC. T cell alloreactivity is mediated by an HLA-restricted TCR repertoire that is able to recognize both structurally similar and structurally dissimilar allogeneic HLA molecules in complex with peptides. Amino acid changes in the peptide-binding region of a given HLA molecule will impact the sequence of the peptides able to be presented. Alternatively, the amino acid changes could directly affect the interaction between the HLA molecule and the TCR. However, numerous studies have clearly shown the existence of alloreactive T cells that are highly peptide specific, illustrating the conventional nature of T cell allorecognition. Alloreactive T cells are able to recognize multiple different peptide–MHC complexes, which explains the high frequency of allorecognition (the precursor frequency of alloreactive T cells was estimated to be 100-fold to 1,000-fold higher than the precursor frequency of T cells that are specific for any single foreign-peptide–self-MHC complex)[23]. In summary, alloreactive αβ T cells, because they interact with foreign MHC molecules in complex with peptides or with shared MHC alleles that present polymorphic peptides, are key mediators of both transplant rejection (T cells from the recipient that are alloreactive against the transplant) and GVHD (T cells from the donor that are alloreactive against the recipient's tissues)[23,24]. In GVHD, T cells cause target tissue cell death mediated by the expression of members of the tumour necrosis factor (TNF) family, such as TNF ligand superfamily member 6 (TNFSF6; also known as FasL) or release of intracellular granule contents, including the serine protease, granzyme B and perforin[24,25].

Several approaches have been developed for administering allogeneic CAR T cells with reduced risk of GVHD: use of donor-derived allogeneic T cells in stem cell transplant recipients, use of virus-specific memory T cells, use of non-αβ T cells and gene editing with TCR deletion in αβ T cells.

Using allogeneic CAR T cells derived from a stem cell transplant donor. This approach is limited to patients who have received an allogeneic SCT but have

Table 1 | **Comparison between autologous and allogeneic CAR T cells**

Characteristic	Autologous CAR T cells	Allogeneic CAR T cells
Origin of the donor	Patient	Healthy donor
Production and manufacturing process	Complex logistics; delay from leukapheresis to CAR-T cell administration; variations of T-cell characteristics according to the patient's immune characteristics and influence of previous treatments	Scaled-up industrialized process in which a high number of CAR T cells can be produced and cryopreserved from a single donor; batches immediately available for patient treatment; possible standardization of T cell characteristics
Clinical indications	Haematological malignancies (demonstrated activity); solid tumours	Haematological malignancies (ongoing trials); solid tumours
Main issues/risks	Cytokine release syndrome; CAR-related gene modifications; potential long-term side effects (B cell aplasia for anti-CD19 CAR T cells)	Cytokine release syndrome; CAR and/or gene editing-related gene modifications; GVHD; rejection of allogeneic cells; toxicity in the case of intense lymphodepletion
Persistence	Intermediate to long (months to years)	Short to intermediate (weeks to months)
Redosing	Limited by the number of cells	Not limited by the number of cells but risk of alloimmunization
Cost	Currently high (may decrease in the future)	Expected to be moderate

CAR, chimeric antigen receptor; GVHD, graft versus host disease.

subsequently relapsed. In this case, CAR T cells can be derived from the original donor. In a recent report, 20 patients with B cell malignancies received CD19 CAR T cells generated from the same donor with no chemotherapy administered before T cell infusion. Six patients achieved complete remission and two patients achieved a partial response. No GVHD was reported[26]. Peak blood CAR T cell levels were higher in patients who achieved remission than in those who did not, and no CAR T cells remained at significant levels after 3 weeks. Overall, these results are in line with previous reports[27,28], and suggest that donor-derived CAR T cells may be included in the strategy of allogeneic SCT to reinforce the graft-versus-tumour effect without increasing the risk of GVHD.

Using virus-specific memory T cells. Another potential approach to decrease the risk of GVHD in allogeneic T cell therapies also comes from observations in the field of allogeneic SCT, in which virus-specific T cells have been used for the treatment of viral infections[29]. Technological advances have made possible the purification of memory virus-specific T cells, which confer protection against viral disease without clinical signs of GVHD[30]. Considering that the risk of alloreactivity is proportional to the quantity of T cells and TCR diversity, one possible explanation for the absence of GVHD is the restricted repertoire of these memory T cells. However, it is not clear how enrichment for T cells with a given specificity would counterselect alloreactive T cells. In preclinical studies, Fanning et al.[31] used spectratyping to demonstrate that leukaemia-responsive T cells displayed a skewed TCR repertoire but that alloreactive cells had a repertoire comparable to that of naive T cells (T_N cells). These data point to the need to increase our understanding of the nature of the molecular mechanisms involved in the recognition of allodeterminants[32]. Adoptive transfer of HLA partially matched virus-specific T cells from healthy donors has had positive results in Epstein–Barr virus-associated malignancies, such as post-transplant lymphoproliferative disease, with response rates of 60–70% and low incidences of toxicity or GVHD)[33]. Therefore, some groups and biotech companies are developing CAR T cells based on allogeneic Epstein–Barr virus-specific T cell lines (TABLE 2). The formal demonstration of antitumour activity without significant GVHD remains to be done in the clinic, but such an approach opens interesting perspectives in the field of off-the-shelf allogeneic CAR-T cell therapies.

Using non-αβ T cells. A second approach consists in avoiding the use of αβ T cells altogether and engineering another cell type to carry a CAR. In theory, a suitable cell type for CAR adoptive therapy must have cytotoxic properties that can be redirected via a cell surface receptor, must be available from accessible sources such as PBMCs or renewable stem cells, and must be relatively easily transduced and expanded. NK cells, which were originally identified for their ability to kill tumour cells, are an integral part of the organism's natural tumour immunosurveillance. NK cells are highly cytolytic towards cells that display the appropriate balance of activating versus inhibitory receptors. Several studies have shown NK cell dysfunction in different types of cancer, illustrating that cancers have evolved mechanisms to escape NK cell killing. Reinforcing the antitumour activity of NK cells by providing them with a CAR is therefore an attractive strategy. Chu et al.[34] demonstrated that transduction of NK cell lines with a CAR construct enhanced the antitumour activity of the NK cells in vitro and in xenogeneic tumour grafts. In another study, NK cell lines and primary NK cells from healthy donors transduced with a CAR directed against epidermal growth factor receptor (EGFR) and the variant EGFRvIII — which is frequently found in glioblastoma — displayed activity against glioblastoma cell lines and against patient-derived glioblastoma stem cells[35]. The relatively low concentration of NK cells in the peripheral blood has triggered the development of strategies to specifically enrich and expand them ex vivo[36]. As an example, the NK92 cell line has been approved for use in humans and would constitute a renewable source of CAR NK cells after expansion under good manufacturing practice conditions with recombinant IL-2 (REF.[37]). Other approaches include the expansion and/or survival of UCB-derived CAR NK cells after infusion by, for example, expressing IL-15 together with a CAR[38]. This strategy demonstrated prolonged survival in a xenograft mouse model of lymphoma using CD19 CAR–IL-15-transduced NK cells compared with CD19 CAR NK cell controls. The preclinical findings on CAR NK cells to date were reviewed recently[39].

NK T cells (NKT cells) are a subset of T lymphocytes that express NK cell surface markers. A subset of NKT cells termed 'invariant NKT cells' (iNKT cells) express a highly restricted TCR that recognizes specific lipid antigens presented by CD1d, a non-polymorphic HLA class I-like molecule expressed on B cells, on antigen-presenting cells and on some epithelial tissues[40,41]. Donor iNKT cells protect from experimental and clinical acute GVHD in the context of allogeneic SCT[42–45]. In preclinical models, iNKT cells engineered with a CD19-directed CAR have strong antilymphoma properties by targeting both CD19 and CD1d expressed on lymphoma cells[46]. Given the protective impact of allogeneic iNKT cells against GVHD, CAR iNKT cells may be an interesting population for off-the-shelf development. The potential limitation is the paucity of these cells, which will require a massive ex vivo expansion.

Another candidate is γδ T cells, which are also naturally capable of cytotoxic responses against tumour cells[47]. γδ T cells constitute only 1–5% of circulating lymphocytes but are predominant in some epithelial sites, such as the intestine, reproductive organs, tongue and skin. Their tissue residency patterns make them an attractive candidate for adoptive therapy because one potential limitation of αβ T cells is their poor access to non-inflamed tumours. Ex vivo, γδ T cells can be expanded to large numbers[48,49]. They are unlikely to induce GVHD because the activation of their TCR is not MHC restricted. CAR γδ T cells directed towards the disialoganglioside GD2, which is frequently overexpressed in gliomas and other tumours of neuroectodermal origin, enhance cytotoxicity against GD2-expressing cell lines[50].

Spectratyping
Technique that measures T cell receptor repertoire diversity.

Using gene editing. Finally, there is the approach of αβ TCR deletion. Because αβ TCR is the determinant of T cell alloreactivity, researchers have developed methods to prevent the expression of a functional TCR at the surface of αβ T cells. Gene editing is one of the most promising such methods today. Since seminal work showed the efficacy of the homing endonuclease I-SceI for inducing site-directed chromosomal recombination in mammalian cell[51,52], a panoply of gene editing tools has emerged (BOX 1). These tools share a common goal of generating a specific DNA double-strand break at a preselected location by introducing a chimeric nuclease into the nucleus while avoiding off-target cleavage as much as possible. Once the DNA break occurs, cellular DNA repair mechanisms will lead to either gene inactivation (gene knockout) through the error-prone non-homologous end joining pathway or to gene insertion or correction (gene knock-in) via the homologous recombination pathway, provided that an exogenous DNA repair template is available[53,54]. The TCR protein complex consists of either an α-chain and a β-chain (in αβ T cells) or a γ-chain and a δ-chain (in γδ T cells), associated with accessory molecules such as CD3 proteins. Whereas the β-chain gene contains two possible constant regions, the gene encoding the α-chain has only one. Therefore, disrupting the gene encoding for the T cell receptor constant α chain (*TRAC*) is the most straightforward approach to disrupt the αβ TCR. The first reported study that evaluated the feasibility of knocking out *TRAC* in CAR T cells was published in 2012 (REF.[55]). In contrast to a previous report that suggested that the presence of TCR was required for optimal CAR function[56], the report authors showed that the abolition of TCR expression through zinc-finger technology (BOX 1) did not impair the antitumour properties of CD19-specific CAR T cells. Following this pioneering work towards universal allogeneic CAR T cells, Poirot et al. used transcription activator-like effector nuclease (TALEN) technology to develop a platform for off-the-shelf CAR T cell production from healthy third-party donors. TALENs are hybrid molecules of DNA-recognition proteins, in this case transcription factors, linked to an endonuclease that can be engineered to cut specific sequences of DNA (BOX 1). These researchers showed that multiplex gene editing could be efficiently achieved by simultaneously electroporating TALENs that targeted *TRAC* and *CD52* into T cells[57]. The resulting TCR-deficient and CD52-deficient donor T cells did not induce GVHD in a mouse model and were resistant to the anti-CD52 monoclonal antibody alemtuzumab, which can be used to eliminate host T cells (which express CD52) and avoid allorejection. More recently, megaTAL nuclease[58] and engineered I-CreI homing endonuclease[59] have also been developed to efficiently disrupt endogenous TCR. Moreover, Ren et al. recently used the CRISPR system to generate allogeneic universal CAR T cells containing two to four disrupted genes[60]. However, this technological performance comes with unknown risks, because simultaneous DNA cleavage at multiple locations may generate multiple translocations[57,61] and does not seem to be applicable for therapeutic products at this point. Similarly, off-target cleavage must be avoided as much as possible because it may trigger adverse effects such as unwanted gene inactivation or rearrangements that may in turn result in a proliferative and survival advantage, as reported by Fraietta et al.[62]. However, this risk seems to be low with allogeneic CAR T cells because universal allogeneic CAR T cells generated from third-party donors are intrinsically bound to be eradicated by the host immune system. This differs from autologous CAR T cells, which in some cases may last for years[3]. One recent study took advantage of TCR gene knockout to create fratricide-resistant (non-cross-reactive against each other) CD3-specific CAR T cells to treat T cell ALL[63].

Multiplex gene editing
Gene editing technology that targets multiple regions in a genome.

The step of gene editing is followed or preceded by the random integration of a CAR after viral vector transduction. By exploiting the cellular homologous recombination pathway, Eyquem et al. developed a strategy to incorporate the CAR construct into the *TRAC* locus[64]. CRISPR–Cas9 technology was used with an adenovirus-associated virus vector carrying the CAR construct flanked by two arms homologous to both sides of the cutting site to insert the CAR-encoding DNA directly into the *TRAC* locus. This targeted integration approach offers several potential advantages compared with the random integration of a CAR after retroviral transduction. First, TCR is inactivated simultaneously with CAR introduction. Second, it may be a safer approach because potential adverse effects linked to insertional mutagenesis are eliminated. Finally, CAR expression is regulated by the endogenous TCR promoter, mimics TCR transcription on exposure to antigen and prevents constant excessive T cell activation that may lead to T cell differentiation and exhaustion. As a result, Eyquem et al. showed that TRAC–CD19 CAR T cells demonstrated greater antitumour potency in a mouse model of ALL than did T cells with a retrovirally encoded CAR[64]. Similarly, megaTAL nuclease and engineered homing endonuclease were also used to introduce a CAR cDNA into the *TRAC* locus using the same strategy[59,65]. CD19 CAR T cells generated with this process are also effective in a B cell lymphoma mouse model[59].

The first clinical trials of CAR T cells generated through TALEN-mediated editing of the TRAC gene are under way[39,40]. Approximately 80% of these cells do not express αβ TCR at the surface after gene editing, and the remaining αβ TCR-positive cells are magnetically removed before storage, using anti-αβ TCR antibodies and a GMP-compatible automated system that had been developed in the context of SCT, in which the removal of T cells could help reduce the incidence of GVHD[57]. No sign of GVHD was observed in in vivo models using these cells. Clinical proof of concept was shown in two paediatric patients with ALL using allogeneic CD19 CAR T cells (UCART19) as a bridge to transplant[66]. Both patients achieved a complete response with negative minimal residual disease (MRD) without significant GVHD. Two phase I/II clinical trials are ongoing to evaluate UCART19 in adult and paediatric ALL[67]. In the paediatric trial, five of six enrolled patients achieved complete remission or complete remission with incomplete haematological recovery with negative MRD at day 28, and proceeded to allogeneic SCT with curative intent as planned in the

Table 2 | **Main programmes of allogeneic CAR-T cell development**

Developer	CAR T cell product	Target antigen	Allogeneic technology	Tools and vectorization	Development phase and trial reference
Allogene Therapeutics	ALLO-715	BCMA	*TRAC* and *CD52* KO	TALEN mRNA (KO)	Preclinical
Allogene Therapeutics and Servier	UCART19	CD19	*TRAC* KO with or without *CD52* KO	TALEN mRNA (KO)	Phase I in relapsed/refractory B cell ALL (NCT02746952, CALM study); phase I in relapsed/refractory B cell ALL (NCT02808442); phase I in lymphoid malignancies (NCT02735083)
Atara Biotherapeutics	Anti-CD19 EBV CTL therapy	CD19	Use of EBV-specific cell lines	Retroviral vector	Preclinical
Cellectis	UCART-123	CD123 (also known as IL-3RA)	*TRAC* KO	TALEN mRNA (KO)	Phase I in AML (NCT03190278); phase I in BPDCN (NCT03203369)
	UCART-22	CD22	*TRAC* and *CD52* KO	TALEN mRNA (KO)	IND
	UCART-CS1	CS1 (also known as SLAMF7)	*TRAC* and *CS1* KO	TALEN mRNA (KO)	Preclinical
	UCART-CLL1	CLL1	*TRAC* and *B2M* KO; CAR at the *TRAC* locus	TALEN mRNA (KO); AAV6 (TI)	Preclinical
Celyad	CYAD-101	NKG2D	Expression of a TRAC-inhibitory molecule peptide consisting of a truncated form of CD3ζ	Retroviral vector (co-expression of TRAC-inhibitory molecule with CAR)	Phase I in CRC (NCT03692429, alloSHRINK)
Chinese People's Liberation Army General Hospital	UCART019	CD19	*TRAC* and *B2M* KO	CRISPR gRNA and Cas9 mRNA (KO)	Phase I in B cell leukaemia and phase II in B cell lymphoma (NCT03166878)
	Mesothelin CAR T cells	Mesothelin	*TRAC* and *PD1* KO	CRISPR gRNA and Cas9 mRNA (KO)	Phase I in solid tumours (NCT03545815)
	Universal dual-specificity CD19 and CD20 or CD19 and CD22 CAR T cells	CD19 and CD22 or CD19 and CD20	*TRAC* KO	CRISPR gRNA and Cas9 mRNA (KO)	Phase I in B cell leukaemia and phase II in B cell lymphoma (NCT03398967)
CRISPR Therapeutics	CTX-101	CD19, BCMA or CD70	*TRAC* and *B2M* KO	CRISPR gRNA and Cas9 mRNA (KO)	Preclinical (B cell malignancies (CD19), MM (BCMA) or solid tumours (CD70))
Fate Therapeutics	FT-819	CD19	*TRAC* KO in iPSC-derived T cells	CRISPR gRNA and Cas9 mRNA (KO)	Preclinical
Memorial Sloan Kettering Cancer Center	CD19 CAR T cells	CD19	*TRAC* KO; CAR at the *TRAC* locus	CRISPR gRNA and Cas9 mRNA (KO)	Preclinical
Poseida Therapeutics	P-BCMA-ALL01	BCMA	*TRAC* and MHC class I KO	CRISPR gRNA and dead Cas9 fused to Clo51 nuclease (Cas-CLOVER™) (KO)	Preclinical
Precision Biosciences and Servier	PBCAR-0191	CD19	*TRAC* KO; CAR at the *TRAC* locus	Meganuclease mRNA (KO); AAV6	Phase I in NHL and phase II in B cell ALL (NCT03666000)
Sangamo Therapeutics	CD19 CAR T cells	CD19	*TRAC* and *B2M* with or without *CISH* KO; CAR at the *TRAC* locus	ZFN mRNA (KO); AAV6 (TI)	Preclinical
Shanghai Bioray Laboratory	CD19 UCART	CD19	*TRAC* and MHC class I KO	CRISPR gRNA and Cas9 mRNA (KO)	Phase I in B cell ALL and B cell NHL (NCT03229876)
Tessa Therapeutics	CAR-transduced Vγ9Vδ2 cells	Undisclosed	αβ T-cell depletion	Cell sorting	Preclinical
University College London	CD19 CAR T cells	CD19	*TRAC* KO	CRISPR gRNA and Cas9 mRNA (KO)	Preclinical
	CD3 CAR T cells	CD3	*TRAC* and CD3 KO	TALEN mRNA (KO)	Preclinical

Table 2 (cont.) | **Main programmes of allogeneic CAR-T cell development**

Developer	CAR T cell product	Target antigen	Allogeneic technology	Tools and vectorization	Development phase and trial reference
University of Ghent	Haematopoietic progenitor-derived CAR T cells	CEA	Monospecific TCR-transgenic cells lacking endogenous rearrangements	Differentiation of haematopoietic progenitors to T cells	Preclinical
University of Minnesota	CD19 CAR T cells	CD19	*TRAC* KO	TALEN mRNA, megaTAL mRNA or CRISPR gRNA and Cas9 mRNA (KO)	Preclinical
University of Pennsylvania	CD19 CAR T cells	CD19	*TRAC* and *B2M* KO; *TRAC*, *B2M* and *FAS* KO; *TRAC*, *B2M*, *PDC1* and *CTLA4* KO	CRISPR shRNA in lentiviral vector and Cas9 mRNA (KO)	Preclinical
University of Singapore	CD19 CAR T cells	CD19	TRAC inhibition via anti-CD3ε PEBLs	PEBLs (inhibition)	Preclinical

AAV6, adeno-associated virus 6; ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; BCMA, B cell maturation protein (also known as TNFRSF17); BPDCN, blastic plasmacytoid dendritic cell neoplasm; CAR, chimeric antigen receptor; CEA, carcinoembryonic antigen; CLL1, C-type lectin-like molecule 1; CRC, colorectal cancer; CTL, cytotoxic T cell; CTLA4, cytotoxic T-lymphocyte-associated antigen 4; EBV, Epstein–Barr virus; gRNA, guide RNA; iPSC, inducible pluripotent stem cell; IND, investigational new drug; KO, knockout; MHC, major histocompatibility complex; MM, multiple myeloma; NHL, non-Hodgkin lymphoma; PDC1, programmed cell death protein 1 (gene); PEBL, protein expression blocker; shRNA, short hairpin RNA; TALEN, transcription activator-like effector nuclease; TCR, T cell receptor; TI, targeted integration; TRAC, T cell receptor alpha constant chain; ZFN, zinc-finger nuclease.

protocol. Two patients were still in remission 1 year later. In the adult trial, 8 of 10 evaluable patients achieved complete remission or complete remission with incomplete haematological recovery. Of note, two patients received a second dose of UCART19 (off protocol) and achieved MRD-negative complete remission or complete remission with incomplete haematological recovery at day 28 after the second dose. Six patients proceeded to allogeneic SCT. No significant GVHD has been observed so far in all treated patients. These preliminary clinical results are encouraging and show that allogeneic CAR T cells can expand in patients. Nevertheless, more patients and a longer follow-up are needed to compare with the results obtained using autologous CD19 CAR T cells. A major question is the duration of response for the patients who will not receive allogeneic SCT.

Challenges

Expansion of allogeneic CAR T cells. Since the seminal work of Steven Rosenberg[68], it has been well established that non-myeloablative and lymphodepletive chemotherapy is required for an efficient in vivo expansion of administered T cells. T cells undergo a process called homeostatic expansion after their transfer into a lymphopenic host[69]. This expansion is driven by homeostatic cytokines such as IL-7 and IL-15 (REFS[70,71]) and by exposure to self-antigens and other antigens[72]. Lymphodepletion is thus included in most CAR-T cell therapy protocols[73]. The optimal conditioning regimen is not defined, but regimens that include the cytotoxic drugs cyclophosphamide and fludarabine have been used in almost all current CAR-T cell studies because of the tolerability and the lymphodepleting properties of the combination. High-dose IL-2 was administered in early trials to enhance T cell expansion but was not used later because of a lack of evidence of benefit[74]. Increasing the intensity of lymphodepletion may lead to the depletion of regulatory T cells and greater engraftment of the infused T cells[75–77]. However, this strategy is limited by the associated toxicity of higher-dose chemotherapy and the higher risk of cytokine release syndrome[78,79]. Of note, the initial proliferation of CAR T cells, which is required to obtain a sufficient ratio of effector to target cells, may be a major factor in the success of this therapeutic approach, and postinfusion CAR T cell expansion has been reported to be associated with response in the case of autologous CD19 CAR T cells in ALL[80].

Non-myeloablative and lymphodepletive chemotherapy
A chemotherapy regimen that does not destroy all the cells of the bone marrow but specifically induces destruction of the lymphocytes.

The expansion of allogeneic CAR T cells with TCR disruption could be driven by the activation of the CAR itself in the presence of the target antigen and/or by the homeostatic cytokines[81,82]. It is still unknown how TCR disruption impacts in vivo T cell proliferation in this context. However, the absence of TCR expression on allogeneic CAR T cells may be an advantage because TCR engagement can negatively affect $CD8^+$ CAR T cell expansion when combined with CAR activation[83]. Nevertheless, the results described above were reported for a syngeneic murine model with controlled TCR reactivity and expression of a cognate TCR antigen, and therefore more translational data from the clinic are needed before a firm conclusion can be drawn. Preliminary data obtained with UCART19 suggest that allogeneic CAR T cells may efficiently expand in patients[67]. It is interesting to note from these early results that interpatient variability is observed despite the use of the same cell product, which suggests that host factors are also involved in the expansion of the injected T cells. Finally, the quality of the lymphodepletion is probably of greater importance in an allogeneic strategy, because persisting host T cells may immediately reject MHC-expressing allogeneic CAR T cells.

Persistence of allogeneic CAR T cells. In autologous CAR T cell strategies, some long-lasting remissions have coincided with the detection of CAR T cells months or years after treatment[84]. However, the optimal duration of CAR T cell persistence may differ according to the nature of the disease, the tumour burden, and other factors,

such as the potency of the administered T cells and the molecular design of the CAR[85]. Theoretically, two disease scenarios can be considered. In the first one, a majority of tumour cells expressing the target antigen are accessible to CAR T cells. If a sufficient ratio of effector to target cells can be achieved after expansion, there may be less need to maintain long-term persistence after the elimination of the tumour cells. In the second case, which is probably more frequent, a population of tumour cells that are intrinsically less sensitive to T cell killing persist into clinical complete remission (as quiescent stem cells[86]). In this case it is necessary to envision a long-term control of the residual disease by CAR T cells. From this aspect, autologous CAR T cells have the advantage of potential long-term persistence. However, as the allogeneic approach is not limited by the quantity of T cells, it allows the ability to readminister CAR T cells in all patients, targeting the same or other tumour antigens (FIG. 2). We can thus envision several courses of allogeneic CAR T cell administration after the first administration as a consolidation treatment aimed at maintaining a sufficient number of CAR T cells and thus eliminating persisting tumour cells. Of note, a proof of concept of redosing with allogeneic CAR T cells has been made in a phase I trial in two patients (one patient who relapsed and one patient who did not respond to the first UCART19 administration)[67]. As mentioned previously, the presence of DSAs should be checked before the readministration of HLA-expressing CAR T cells, and CAR T cells derived from another donor should be used in the case of previous immunization. Another limitation is the toxicity associated with the lymphodepletion that will be required before each CAR T cell administration. It will be necessary to limit the intensity of the lymphodepletion and the number of consolidation cycles, and thus to develop optimized allogeneic CAR T cells, as discussed later.

The therapeutic window of allogeneic CAR T cells to eliminate tumour cells depends on the initial expansion and length of persistence and the ability of the host immune system to reject them. One formal possibility to avoid rejection of allogeneic CAR T cells is the use of a bank of T cells that match the majority of the population in terms of HLA alleles. Experience from organ and UCB transplantation has shown that the most important HLA alleles to match for are HLA-A, HLA-B and HLA-DR[87–89], and that matching for these loci is sufficient to reduce the incidence of allograft rejection. Calculation shows that this may be achieved with a limited number of donors who are homozygous for HLA-A, HLA-B and the HLA class II histocompatibility antigen HLA-DRB1. In theory, and given the availability of the right donors, it has been suggested that a cell bank from 150 selected homozygous HLA-typed volunteers could match 93% of the UK population[90].

A second possibility to increase the persistence of allogeneic CAR T cells is to modify the conditioning regimen to extend the duration of lymphopenia without affecting CAR-T cell activity. It is quite possible that allogeneic CAR T cells may require more intense lymphodepletion than autologous CAR T cells. In this context, gene editing technologies offer many possibilities, such as CAR T cells that are resistant to alemtuzumab by disrupting CD52. Preclinical proof of concept showed that TCR- and CD52-deficient CD19 CAR T cells can be selectively engrafted in the presence of alemtuzumab while maintaining antitumour activity indistinguishable from standard CD19 CAR T cells in an orthotopic mouse model of lymphoma[57]. Preliminary phase I results obtained with UCART19 in the clinic support this rationale[67]. It is also possible to render allogeneic CAR T cells resistant to purine nucleotide analogues used in preconditioning lymphodepleting regimens by disrupting deoxycytidine kinase — a key enzyme involved in the transformation of nucleoside prodrugs to toxic compounds — in these cells. This approach allows the expansion as well as the recovery of a homogeneous population of engineered CAR T cells that retain their proliferative capacity and cytolytic activity against tumour cells in the presence of lymphodepleting doses of different purine nucleotide analogues[91]. However, the use of a conditioning regimen that increases the depth and duration of lymphodepletion can be associated with an increased risk of opportunistic

Box 1 | **Main nucleases for precise gene editing**

Zinc-finger nucleases (ZFNs) are artificial chimeric nucleases that consist of a specific DNA-binding domain fused to the non-specific DNA cleavage domain from the Fok-I type II S restriction enzyme[141]. ZFNs are designed by assembling a pair of zinc-finger arrays specific to the genomic sequence to be cleaved. Usually, five or six base pairs (bp) separate the two zinc-finger binding domains to allow the Fok-I catalytic domain to reach and cleave double-stranded DNA[142,143]. Cleavage by Fok-I generates two 5′-overhang DNA ends. Because each zinc-finger unit recognizes three nucleotides, three to six zinc-finger units are assembled to generate a specific DNA-binding domain that recognizes a 6–18-bp DNA sequence.

Transcription activator-like effector nucleases (TALENs) have structural similarities to ZFNs as they are heterodimeric nucleases that consist of a fusion between the Fok-I catalytic domain and a transcription activator-like effector (TALE) DNA-binding domain[144]. The DNA-binding domain consists of an array of almost identical repeats of 33–35 amino acids. Each of these repeats independently recognizes one nucleotide through two amino acids called repeat variable diresidues (RVDs). A simple 'code' that governs the RVD-nucleotide specificity has been deciphered[145,146], which simplifies the design of a TALEN. Usually, TALENs are designed to bind a DNA target consisting of two sequences of 16 nucleotides separated by a spacer sequence of 15–16 nucleotides. One notable feature of TALENs is the presence of a thymine at the 5′ end of each target sequence (T0), which favours highly efficient cleavage[147]. Monomeric TALEN architectures have been developed by fusing TALE domains to a sequence-specific catalytic domain derived from the homing endonuclease (HE) I-TevI, resulting in a Tev–TALE monomeric nuclease[148].

MegaTALs are monomeric artificial chimeric nucleases derived from HEs[149]. HEs can be engineered to target specific sequences within the genome[59,150,151]. However, this process is highly challenging and requires much expertise. To increase the affinity and specificity of megaTALs, a short TALE domain is fused to the HE. As few as 5.5 TALE repeats can increase megaTAL activity[149]. Similarly to the Tev–TALE nuclease, megaTAL generates 3′-overhang DNA ends, which is believed to favour homologous recombination.

The clustered regularly interspaced short palindromic repeats (CRISPR) system is derived from a microbial adaptive immune system. This system is a combination of a nuclease and a short RNA. In contrast to the nucleases mentioned above, for which specificity is dependent on protein–DNA interactions, the specificity of the CRISPR system relates to complementary RNA–DNA base pairing. A 20-nucleotide RNA called a 'single guide RNA' (sgRNA) is designed to be complementary to the genomic DNA target. However, partial mispairing is tolerated, which may increase the likelihood of off-target cleavage[152,153]. The most commonly used CRISPR system today derives from *Streptococcus pyogenes* and uses the nuclease Cas9[154,155]. A 20-nucleotide target sequence (NGG) directs Cas9 to the target site[156]. In contrast to ZFNs, TALENs and MegaTALs, cleavage by Cas9 generates blunt DNA ends. Recently, a new CRISPR system called 'CRISPR–Cpf1' has been described as highly specific[157].

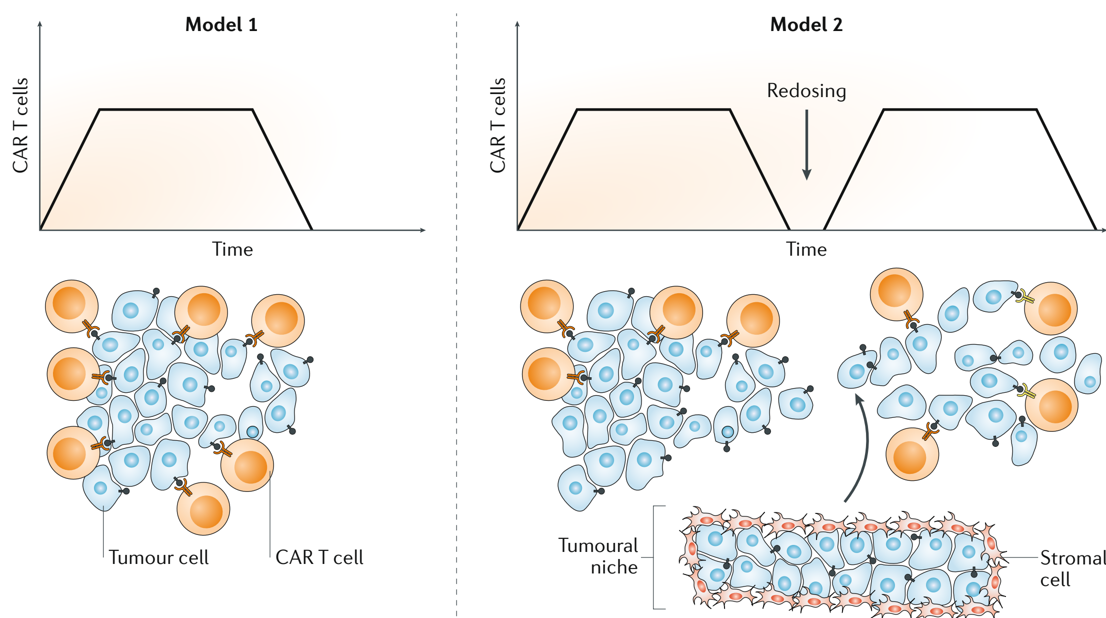

Fig. 2 | **Persistence of CAR T cells and tumour evolution.** Two tumour models can theoretically be considered in terms of chimeric antigen receptor (CAR) T cell persistence requirements. In tumour model 1, most of the tumour cells express the target antigen (represented as a bar on tumour cells) and are recognized by CAR T cells. If the ratio of effectors (such as CAR T cells) to targets (in this case, tumour cells) is sufficient after initial expansion of CAR T cells, efficient killing of tumour cells allows eradication of the tumour. In tumour model 2, some tumour cells do not express the target antigen and/or are protected from CAR T cell killing owing to their location in a niche (represented here as tumour cells surrounded by stromal cells) that confers resistance to cancer treatments. These protected tumour cells will persist and repopulate the tumour bulk. In this case, long-term persistence of CAR T cells is required to eliminate the remaining tumour cells, or a strategy of CAR T cell redosing should be applied. A major advantage of the allogeneic approach is also the possibility to administer sequentially CAR T cells with different target specificities to avoid the emergence of clones that do not express the initial target antigen.

infections and viral reactivation. This approach is therefore limited to the treatment of well-selected patients with haematological malignancies and is probably not applicable in solid tumours, in which only lower levels of toxicity are accepted in standard practice. Furthermore, it is difficult to repeat intense lymphodepletion for the readministration of allogeneic CAR T cells.

Because HLA class I molecules are key mediators of immune rejection, a third approach is to delete MHC class I molecules on allogeneic CAR T cells[92]. This can be achieved by disrupting β_2-microglobulin, which is essential for forming functional HLA class I molecules on the cell surface. As HLA molecules serve as major ligand inhibitors of NK cells, the complete absence of HLA class I molecules may nevertheless render allogeneic CAR T cells more sensitive to NK cell recognition and destruction. However, this concept of 'missing self' may be less valuable in the allogeneic setting, especially in case of mismatches between killer cell immunoglobulin-like receptors (KIRs) on the recipient NK cells and their ligands (HLA class I molecules) on the CAR T cells, as in such cases NK cell reactivity may already be present before deletion of HLA class I molecules[93,94]. Furthermore, NK cells may have poor cytolytic functions in some malignancies[95]. For instance, deficiencies in the expression of activation molecules, such as natural cytotoxic receptors, have been reported in acute myeloid leukaemia (AML)[96,97]. Therefore, the risk of destruction of HLA class I-negative allogeneic CAR T cells should be appreciated in the clinical context. One appealing strategy to prevent NK cell-dependent lysis is to insert on the CAR T cells the non-classical HLA molecule HLA-E or HLA-G (which bind inhibitory receptors on NK cells)[98] or overexpress siglec 7 and siglec 9 ligands (to inhibit NK cells that express the inhibitory receptors siglec 7 and siglec 9)[99]. Finally, activated T cells express high levels of HLA class II molecules[100] as well, which may also be involved in the rejection of allogeneic CAR T cells. Inhibition of HLA class II expression can be achieved by the disruption of regulatory factors that control the transcription of MHC class II genes such as those encoding MHC class II transactivator (*CIITA*) and *RFXANK*[101]. Translational data will be needed to determine the most efficient strategies of gene editing to avoid rejection of allogeneic CAR T cells. This is crucial as the development of allogeneic CAR T cells made invisible to the human system may allow the indications to be extended in particular in the field of solid tumours.

Killer cell immunoglobulin-like receptors
(KIRs). These receptors on natural killer (NK) cells recognize groups of for human leukocyte antigen class I alleles. The interaction between a KIR and a class I allele inhibits reactivity of the NK cell. The absence of recognition of the appropriate KIR ligand on a mismatched cell triggers NK cell reactivity.

Determination of the optimal T cell subpopulations to select during CAR-T cell manufacturing. T cells are a heterogeneous population characterized by different phenotypes and functions (BOX 2). Different studies have been conducted using adoptive T cell transfer in mice and non-human primates to delineate the role of the different T cell subtypes. Although $CD8^+$ effector memory T cells (T_{EM} cells) have strong cytotoxic properties, only central memory T cells (T_{CM} cells) and

Box 2 | **Memory T cell subtypes**

Naive T cells (T_N cells) continuously recirculate between secondary lymphoid organs and blood via the lymphatic system by expressing the lymphoid homing receptors CC-chemokine receptor 7 (CCR7) and CD62 ligand (CD62L). In response to antigens in the periphery, T_N cells will proliferate and differentiate into different types of effector and memory T cells, which then migrate to different tissues[158].

Central memory T cells (T_{CM} cells) are localized in secondary lymphoid organs and are characterized by the expression of CD45RO, CCR7 and CD62L on their surface. T_{CM} cells display a capacity for self-renewal but have a low level of effector functions[158,159].

Effector memory T cells (T_{EM} cells) are found in non-lymphoid peripheral tissue types, including lung, liver and intestine[159], and can recirculate between blood and tissue[160]. They express CD45RO on their surface but not CD62L or CCR7 (REF.[108]). They secrete effector cytokines, such as IL-4 and interferon-γ, as well as perforin and granzyme B, in line with their cytotoxic properties[161]. T_{EM} cells play an important part in bacterial, viral and parasitic infections.

Terminally differentiated effector memory T cells (T_{EMRA} cells) re-express CD45RA, which is a marker usually found on T_N cells (CD45RA is lost in T_{CM} and T_{EM} cells, in which it is replaced by the CD45RO isoform)[162].

Stem cell-like memory T cells (T_{SCM} cells) have stem cell-like properties of self-renewal, can persist for decades and have increased proliferative capacity compared with other memory T cells. They are characterized by the expression of CD45RA, CCR7, CD95, CD122, CXC-chemokine receptor 3 (CXCR3) and the integrin CD11a (the β2-subunit)[103,163]. IL-7 and IL-15 are involved in the generation and expansion of T_{SCM} cells[164].

Resident memory T cells (T_{RM} cells) are a long-lasting population mainly localized in peripheral lymphoid and non-lymphoid tissues such as lung, skin and the gastrointestinal and genitourinary tracts. They are characterized by the expression of surface markers such as CD103, CD69 and CD49a, and by the absence of the lymph node homing receptors CD62L and CCR7. Their differentiation is regulated by transforming growth factor-β and IL-15 (REFS[165,166]).

other less differentiated T cell subsets, such as T_N cells and stem cell-like memory T cells (T_{SCM} cells), are critical for in vivo expansion, survival and long-term persistence[102,103]. Preclinical results have shown that CAR T cells generated from $CD8^+$ and $CD4^+$ T_N cell and T_{CM} cell subsets are more potent than those derived from the T_{EM} cell subset, and underlined the interest in using $CD8^+$ and $CD4^+$ T cell subsets in defined ratios[104]. In the clinical context, the frequency of a T cell subset expressing CD8, protein tyrosine phosphatase receptor type C (PTPRC; also known as CD45RA) and CC-chemokine receptor 7 (CCR7), corresponding to T_N or T_{SCM} cells, was found to correlate with the overall in vivo expansion of CAR T cells in patients with lymphoma[105]. Data obtained from autologous CD19 CAR T cells in patients with chronic lymphocytic leukaemia suggest that the quantity of T_{SCM} cells and T_{CM} cells and the lack of exhaustion markers — such as programmed cell death 1 (PD-1), T cell immunoglobulin mucin receptor 3 (TIM3; also known as HAVCR2) or lymphocyte activation gene 3 protein (LAG3) — are major parameters for persistence and activity[106]. The persistence of CAR-T cell therapy was also shown to be dependent on the number of $CD4^+$ T cells[107].

A potential problem in the autologous approach to CAR-T cell therapy is that patients who have previously received lymphocytotoxic chemotherapy are often lymphopenic and have higher frequencies of T_{EM} cells compared with T_{SCM} and T_{CM} cells[104,108]. A significant advantage of the allogeneic approach is therefore the ability to select donors with higher frequencies of T_N, T_{SCM} and T_{CM} cells and to control the process of CAR-T cell manufacturing to determine specific characteristics of the infused T cells, such as the optimal ratio of the subpopulations of T_{SCM} cells, T_{CM} cells, T_{EM} cells, and terminally differentiated effector memory T cells (T_{EMRA} cells) in both $CD4^+$ and $CD8^+$ subsets in the final cell product. Immunomagnetic separation of distinct T cell subsets before CAR engineering may render the infused product more homogeneous and may allow selection of less differentiated T cell subsets before transduction. However, such an approach adds significant complexity in the generation of CAR T cells[108]. Culture conditions may also be adapted to amplify some specific subsets. For instance, IL-7 and IL-15 increase the frequency of $CD8^+CD45RA^+CCR7^+$ cells during the ex vivo expansion of CAR T cells[105,109]. The culture medium may also strongly influence cell metabolism and the maintenance of a non-terminally differentiated memory T cell phenotype[110]. Finally, the method of transduction itself may favour the selection of a specific T cell subtype. CAR T cells generated using *piggyBac* transposition have been shown to be predominantly of a T_{SCM} phenotype[111].

Translational research is required to better understand the links between initial expansion, persistence and the characteristics of the administered CAR T cells in an allogeneic approach. This will provide clues to optimize the immunological composition of cell products. We can nevertheless speculate that a controlled T_{SCM} cell + T_{CM} cell/ T_{EM} cell + T_{EMRA} cell ratio will be a significant advantage in terms of persistence and efficacy.

Immunomagnetic separation
A technique for separating cells by means of their antigens bound to antibodies coating microscopic paramagnetic beads, which can then be separated by magnetic attraction.

PiggyBac transposition
The *piggyBac* transposon is a movable genetic element that efficiently transposes between vectors and chromosomes through a 'cut-and-paste' mechanism.

Clinical perspectives

Haematological malignancies. Allogeneic CAR T cells offer the possibility of treating relapsed or refractory malignancies with a readily available product. This may be a major advantage in diseases that progress rapidly, such as AML and ALL. Trials have already begun in ALL and AML, and are planned in lymphomas and multiple myeloma (TABLE 2). The targets are similar to those for autologous approaches, including CD19 and CD22 in ALL and B cell lymphomas, respectively, CD30 in Hodgkin lymphoma and anaplastic large cell lymphoma, B cell maturation protein (BCMA; also known as TNFRSF17), CS1 (also known as SLAMF7) and CD38 in multiple myeloma, and CD123, CD33 and CLL1 in AML (for a review, see REF.[112]). The shorter persistence of allogeneic CAR T cells may be an advantage in the case of targets such as CD123 that are also expressed by normal cells. CD123 is a promising target for AML that is also expressed on some normal haematopoietic stem cells and progenitors and on endothelial cells from small-calibre blood vessels[113–115]. Similar concerns exist for CD38 and CS1 in multiple myeloma[86,116]. In addition to the relapsed and refractory settings, allogeneic CAR T cells may be used at an earlier stage to eradicate persisting residual disease. Frontline treatment may be also envisioned in the case of malignancies with poor prognosis in which conventional therapeutics have insufficient efficacy (such as newly diagnosed AML in the European LeukemiaNet adverse genetic risk group)[117].

Owing to the limited persistence of allogeneic CAR T cells, allogeneic CAR-T cell therapy may be a bridge to a

definitive therapy such as allogeneic SCT in some circumstances[2]. However, one can also postulate that allogeneic CAR-T cell therapy will be a curative approach through consolidation cycles. A systematic strategy of redosing may indeed eradicate persisting cells that still express the target antigen (FIG. 2). An attractive approach is also to combine CAR T cells of different specificities to eliminate potential clones that would not express or would have lost the initial target (such as combination of CD19 and CD22 CAR T cells in ALL or B cell lymphomas)[118]. A limitation for the number of cycles of redosing is the repeated use of a lymphodepleting conditioning before CAR T cell administration owing to the associated toxicity. Because only moderate-intensity conditioning regimens can be envisioned in this setting to allow homeostatic expansion of the CAR T cells, this approach will probably require the use of next-generation allogeneic cells that have been made invisible to the host immune system, as described earlier. Another appealing approach would be to follow allogeneic CAR-T cell therapy with use of some therapeutics able to boost adaptive immune responses (such as immune checkpoint modulators) or with a cancer vaccine to control the residual disease[119].

In solid tumours. To date, CAR T cells have shown much less satisfactory results in solid tumours than in haematological malignancies, with some stabilization and very few objective responses[82]. This relative ineffectiveness of 'traditional' CAR T cells is due to several factors, including a lack of tumour-specific targets, the immunosuppressive tumour microenvironment, the problem of homing and access to the tumour site and a lack of CAR-T cell expansion[120–124]. As described previously, the shorter-term persistence of allogeneic CAR T cells may be an advantage for less tumour-specific targets so as to decrease the risk of 'on-target–off-tumour' chronic toxicity. Tumour-associated immunosuppression can be overcome in different ways. Optimizing expansion protocols is still of interest to induce more robust CAR T cells that can resist the harsh tumour microenvironment and oxidative stress[125]. In this context, several strategies can be proposed to make CAR T cells resistant to the tumour microenvironment. CAR T cells expressing catalase maintain their antitumour activity under H_2O_2-induced oxidative stress[126], and CAR T cells that are responsive to a hypoxic environment have also been generated[127]. Furthermore, the off-the-shelf approach allows the use of multiple steps of gene editing to optimize CAR-T cell function. A few strategies have been proposed to decrease the sensitivity of T cells to negative immune checkpoints and immunosuppression, such as CAR T cells with PD-1 disruption or expressing a PD-1–CD28 chimeric construct that uses the cytoplasmic part of CD28 to transform an inhibitor signal into an activator signal[128,129]. A CAR T cell modified to secrete a single-chain variable fragment blocking PD-1 with increased antitumour activity has recently been reported[130]. Transforming growth factor-β (TGFβ) inhibits the function of T cells and seems to play a major part in the immune exclusion phenomena of T cells[131]. CAR T cells expressing dominant-negative TGFβ receptor type 2 are associated with resistance to exhaustion and long-term persistence in in vivo mouse models[132]. One elegant approach consists in combining multiple signals from the tumour microenvironment to activate T cells specifically in the tumour. T cells engineered with a three-component split CAR system — which recognizes prostate stem cell antigen (PSCA), TGFβ and IL-4 — whose endodomains recapitulate a physiological T cell signalling by delivering signal 1 (activation through CD3ζ), signal 2 (costimulation through 4-1BB) and signal 3 (cytokine release (IL-7)) are selectively activated in a tumour microenvironment that is characteristic of pancreatic cancer[133].

Several chemokine receptors have been evaluated preclinically to promote tumour access by T cells. Overexpression of CXC-chemokine receptor 2 (CXCR2) increases the migration of T cells via recognition of

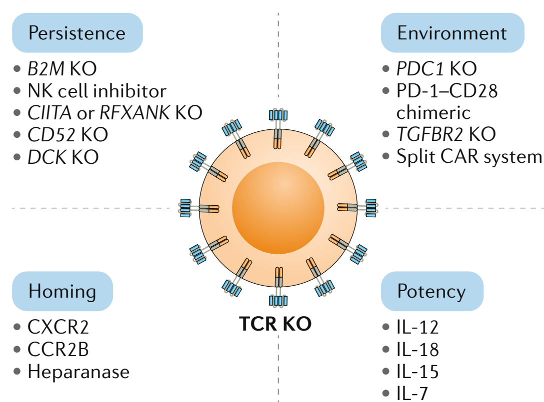

Fig. 3 | **Examples of gene editing strategies to optimize CAR-T cell functions.** Persistence of gene-edited allogeneic chimeric antigen receptor (CAR) T cells, in which T cell receptor (TCR) is eliminated, can be increased by disruption of major histocompatibility complex class I molecules (by knocking out the β_2-microglobulin gene (*B2M*)), possibly major histocompatibility complex class II molecules (through deletion of *CIITA* or *RFXANK*) and addition of a natural killer (NK) cell inhibitor (such as HLA-E). Persistence can also be achieved by increasing resistance to optimized lymphodepletion by deleting *CD52* (for resistance to an anti-CD52 antibody) or by deleting the deoxycytidine kinase gene (*DCK*) for resistance to purine nucleotide analogues. Allogeneic CAR T cells can be modified to counteract some mechanisms of immunosuppression in the tumour microenvironment, such as disrupting programmed cell death protein 1 (PD-1) or expressing a PD-1–CD28 chimeric construct using the cytoplasmic part of CD28 to transform an inhibitor signal into an activator signal[128,129]. Downregulation of transforming growth factor-β (TGFβ) signalling by expression of dominant-negative TGFβ receptor type 2 (*TGFBR2*) or activation of multiple signals from the tumour microenvironment specifically at the tumour site by engineering split CAR systems with different chimeric receptors that convert an immunosuppressive signal into an activating signal[133] has also been reported. Homing of the CAR T cells can be improved by expression of adequate chemokine receptors such as CXC-chemokine receptor 2 (CXCR2) or CC-chemokine receptor 2B (CCR2B) and also by increasing tumour penetration by expression of enzymes such as heparanase. Finally, CAR T cells can be modified to secrete cytokines (such as IL-7, IL-12, IL-15 and IL-18) that promote their survival and/or greater antitumour activity. KO, knockout.

CXC-chemokine ligand 1 (CXCL1) produced by the tumour[134]. Moon et al. evaluated an anti-mesothelin CAR overexpressing CCR2B in a mesothelioma model and found that increased migration of CAR T cells into the tumour was associated with increased antitumour activity[135]. A similar observation was made with CAR T cells specific for the tumour antigen GD2 and co-expressing CCR2B in a CC-chemokine ligand 2 (CCL2)-secreting neuroblastoma model[136]. Regarding approaches to enhance tumour penetration of T cells, CAR T cells that express heparanase, a heparan sulfate-degrading enzyme, have increased antitumour activity as a result of greater penetration into the tumour[137].

Finally, some researchers have modified CAR T cells so that they secrete cytokines to promote their survival and/or greater activity. CAR T cells that secrete IL-12 (constitutively or after activation) have an increased cytotoxic activity and are capable of inducing differentiation of T_N cells into type 1 T helper cells that will attract endogenous T cells and innate immune cells[138]. Similarly, IL-18-secreting CAR T cells have increased expansion and persistence[139], and co-expression of IL-15 is likely to promote CAR-T cell proliferation and persistence[140].

Several approaches would therefore allow the development of optimized next-generation CAR T cells, with better tumour selectivity, better tumour access capabilities and increased activity in an immunosuppressive context (FIG. 3). The gene editing approaches available today allow multiple modifications to be combined in an allogeneic CAR-T cell strategy. However, they must meet a rigorous quality control and regulatory qualification process that accounts for the risk of increased off-target genome editing after multiple genetic modifications. Furthermore, in light of the large number of potential modifications (as discussed earlier), it will be necessary to select the optimal modifications to implement according to each tumour context. In the long term, allogeneic gene-edited CAR-T cell approaches could be a 'supra-physiological' treatment of choice, capable of modifying the natural history of immunologically cold tumours, regardless of the MHC class I expression by the tumour.

Conclusion

CAR-T cell therapy has already changed the therapeutic landscape of some haematological malignancies and remains one of the most promising approaches in the treatment of cancer. The development of off-the-shelf universal CAR T cells readily available for patient treatment, potentially at a reduced cost, would significantly increase access to this class of therapeutics. Gene editing technologies have already resulted in strategies to control the risk of GVHD by efficiently eliminating TCR expression and have unlocked new techniques to make allogeneic CAR T cells invisible (or at least minimally visible) to the host immune system. Even though many challenges remain in enhancing the efficacy of CAR T cells, especially in solid tumours, there are many approaches that will allow optimization, as we have discussed. This novel family of therapeutics may revolutionize cancer treatment, which justifies the intense efforts of academic groups and of biotechnology and pharmaceutical companies in this field.

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Author contributions
S.D. contributed to all aspects of the article. P.D and L.P. researched data, provided substantial contribution to the content and wrote the article. S.A.G. contributed substantially to discussion of the content, wrote the article and edited and reviewed the manuscript before submission. G.M. and S.D. edited and reviewed the article before submission.

Competing interests
S.D. has been an employee of Cellectis and has served as a consultant for or on the scientific advisory boards of Servier, Celyad, PDC*line Pharma, Erytech, AstraZeneca, Elsalys and Netris Pharma. L.P. and P.D. are employees of Cellectis. S.A.G. has received research and/or clinical trial support from Novartis, Servier and Kite and has served as a consultant for or on study steering committees or scientific advisory boards of Novartis, Cellectis, Adaptimmune, Eureka, TCR2, Juno, GlaxoSmithKline, Vertex, Cure Genetics, Humanigen and Roche.

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