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Review article

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CAR-X cell engineering

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Abstract

Chimeric antigen receptor (CAR)-T cell therapy has revolutionized the treatment of haematological malignancies and continues to transform the treatment of various other diseases. Nonetheless, current CAR-T cell therapy has limitations that hinder its therapeutic efficacy for expanded applications, some of which might be attributed to intrinsic T cell features. Using immune cells beyond conventional T cells has therefore emerged as a promising strategy to compensate for such limitations. In this Review, we discuss different CAR-engineered immune cells for therapeutic applications by highlighting how unique immune attributes, along with cell-specific manufacturing strategies and CAR designs, address challenges faced by conventional CAR-T cell therapy.

Sections

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Key points

- Limitations of conventional chimeric antigen receptor (CAR)-T cells, including exhaustion, antigen heterogeneity and poor solid tumour infiltration, have driven the development of alternative CAR-engineered immune cell (CAR-X cell) platforms.
- CAR-X cell platforms leverage lineage-specific immunobiology, such as innate cytotoxicity, tissue tropism, immune regulation or reduced alloreactivity, to overcome functional constraints inherent to conventional T cells.
- CAR-X cell therapy efficacy requires lineage-tailored CAR designs and manufacturing strategies, including cell sourcing, expansion protocols and gene editing compatibility, which collectively determine scalability and translational robustness.
- Early clinical evaluations of CAR-natural killer cell, CAR-macrophage, CAR-regulatory T cell and CAR-natural killer T cell therapies demonstrate feasibility and distinct therapeutic benefits, including reduced inflammatory toxicity, reduced alloreactive risks or targeted immune suppression.
- The therapeutic efficacy of CAR-X cell therapies depends on persistence, scalable manufacturing and safety control, alongside deeper mechanistic understanding to enable lineage-tailored next-generation CAR engineering.

Introduction

Chimeric antigen receptor (CAR)-T cell therapy is a major advance in cancer immunotherapy that has transformed treatment for patients with otherwise refractory B cell malignancies[1–3]. Its remarkable clinical success in haematological malignancies has established CAR-T cell therapy as a powerful therapeutic platform, which is now being rapidly extended to a wider range of diseases such as autoimmune conditions[4]. These advances are grounded, in part, by the intrinsic immune attributes of T cells that support the functional efficacy of CAR-T cell therapy. T cells possess potent, antigen-specific cytotoxicity, which is naturally triggered through T cell receptor (TCR) and co-stimulatory signalling pathways. Key components of these pathways, such as CD3ζ and CD28, can be incorporated into CAR constructs to activate cytotoxicity independently of the native TCR. Such activation promotes rapid proliferation and differentiation into effector and memory subsets, enabling both robust tumour elimination and long-term immune surveillance. The efficient mobility in response to chemokine cues further enables T cells to traffic to pathological tissues or cells[5]. Moreover, T cells can be genetically modified using viral or non-viral methods, supporting CAR expression and additional engineering strategies to boost function and safety. Collectively, these properties have contributed to the clinical success of CAR-T cell therapy.

Despite these advantages, CAR-T cell therapy has revealed limitations rooted in its intrinsic immune properties. The poor tumour-infiltrating capacity and vulnerability to the hostile tumour microenvironment (TME) restrict efficacy against solid tumours. Solid tumours also have high antigen heterogeneity, whereas CAR-T cell designs often aim for restricted expression of target antigens on tumour cells. Prolonged ex vivo expansion during manufacture and extensive in vivo antigen stimulation further drive CAR-T cell exhaustion, necessitating additional engineering interventions that increase engineering burden and cost[6]. Moreover, in the context of high antigen density, CAR-T cells can acquire targeted antigens from tumour cells through a process termed trogocytosis, in which cells physically transfer membrane proteins to another cell. This process can lead to fractricide, a phenomenon where CAR-T cells mistakenly recognize and kill each other instead of tumour cells[7].

Thus, there has been growing interest in developing alternative immune cell platforms for CAR-based immunotherapy. Incorporating CAR constructs onto immune cell types beyond conventional T (T_{conv}) cells, collectively referred to as CAR-X cells here, might offer distinct immunological advantages that help overcome key challenges and enable broader clinical applications across diseases. For instance, natural killer (NK) cells exhibit potent cytotoxicity in a major histocompatibility complex class II (MHC-II)-independent manner, indicating a lower risk of severe adverse events and intrinsic allogeneic potential[8]. Macrophages demonstrate robust tissue infiltration and inflammation regulation, offering potential in overcoming the barriers of an immunosuppressive TME[9]. By contrast, regulatory T (T_{reg}) cells are naturally immunosuppressive and can be repurposed to target autoimmune or hyperinflammatory disorders[10]. Beyond these well-characterized immune cell types, new CAR constructs are also being introduced into less-appreciated immune cells, including unconventional T cells and neutrophils[11,12] (Box 1). Collectively, there is an ongoing strategic shift in cellular immunotherapy: exploring and applying alternative CAR-X cell platforms to align beneficial immunobiology with disease-specific needs.

In this Review, we examine CAR engineering strategies across various immune cell types (NK cells, macrophages, T_{reg} cells and unconventional T cells). For each, we illustrate the therapeutic promise from beneficial immunobiology, manufacturing, and CAR designs to disease indications. Finally, specific challenges faced by each CAR-X cell in terms of translational hurdles and safety profiles are emphasized.

The immunobiology of CAR-X cells

Different immune cell platforms possess distinct effector functions and recognition mechanisms, which can be harnessed by CAR engineering to expand the therapeutic scope and address barriers that constrain CAR-T_{conv} cell therapy (Table 1).

NK cells

NK cells are crucial components of the innate immune response, capable of rapidly responding to pathogenic cells without prior sensitization. Most circulating NK cells are of the $CD16^{+}CD56^{dim}$ phenotype, possessing strong cytotoxic abilities against tumour cells and virus-infected cells. This cytotoxicity is mediated by multiple mechanisms, mainly including the release of various cytokines, production of cytotoxic granules (perforins and granzymes), and engagement of death receptors such as Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL)[13,14] (Fig. 1). Compared to T cells with an effector phenotype, NK cells have less pro-inflammatory cytokine release and therefore have a lower risk of inducing inflammatory side effects such as cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (Table 1). Their shorter lifespan also reduces the risk of prolonged activation or excessive cytotoxicity but poses a challenge for adoptive transfer therapy[15]. These cytotoxic mechanisms, combined with favourable safety profiles, position NK cells as a robust and versatile platform for CAR engineering.

The activation of NK cells is mediated by both activating and inhibitory signals. NK-activating receptors (such as NKG2D, NKp46 and CD16α) trigger cytotoxicity against cells expressing stress-induced ligands such as MICA and MICB[16,17]. By contrast, inhibitory receptors on NK cells (such as KIR and NKG2A) bind to MHC-I molecules on healthy cells, recognizing 'self' signals and suppressing NK cell activation[18,19]. This process enables NK cells to detect tumour cells with absent or downregulated MHC-I, which is a common immune evasion strategy harnessed by some solid tumours[19]. Through CD16α, NK cells also recognize IgG-coated pathogenic cells and mediate antibody-dependent cellular cytotoxicity (ADCC), contributing to antibody-dependent elimination[20]. These recognition mechanisms confer NK cells with a robust and broad targeting ability, making them suitable for cellular immunotherapies in tumours with heterogeneous antigens or downregulated MHC-I expression (Fig. 1).

Despite their innate cytotoxicity and favourable safety profile, NK cells have limited in vivo functional persistence (approximately 1–2 weeks in the absence of exogenous cytokine support) with limited antigen-driven clonal expansion potential, thereby restricting sustained antitumour activity[21]. Compared with T_{conv} cells, NK cells possess innate antiviral defence mechanisms, leading to lower transduction efficiencies by viral vectors[22]. Furthermore, NK cells are also susceptible to immunosuppressive signals within the TME (Table 1).

Macrophages

Macrophages are indispensable components of the innate immune system, maintaining immune homeostasis by phagocytosing pathogens and cellular debris, secreting pro-inflammatory cytokines and chemokines, and presenting antigens to activate adaptive immunity (Fig. 1). Macrophages exhibit high chemotaxis towards tumour-derived chemokines and can produce matrix metalloproteinases (MMPs) (particularly MMP9 and MMP12), which can degrade extracellular matrix (ECM) components to facilitate strong tumour infiltration[23,24] (Table 1). Antitumour macrophages mostly adopt an M1-polarized phenotype, which can be induced by interferon-γ (IFNγ) or bacterial lipopolysaccharides. M1-polarized macrophages perform effector functions including phagocytosis, production of cytotoxic reactive oxygen and nitrogen species, and secretion of pro-inflammatory cytokines (for example, IL-1β, IL-6, TNF and IL-12)[25]. This pro-inflammatory cytokine and chemokine release has a key role in driving systemic inflammation and recruiting cytotoxic T cells and NK cells, further expanding immune activation against pathological insults.

By contrast, M2 macrophages, induced by anti-inflammatory signals, such as IL-4 and TGFβ, promote tissue repair but also contribute to tumour progression[26]. Within the TME, hypoxia and immunosuppressive factors (such as TGFβ) skew tumour-associated macrophages (TAMs) towards an M2-like phenotype, which mediates immunosuppression, secretes pro-angiogenic factors such as vascular endothelial growth factor (VEGF), and remodels the ECM via MMP2 and MMP9 (refs. 27,28). The ECM remodelling process promotes tumour invasion and metastasis and the formation of immunosuppressive niches. Thus, although the effector functions of M1 macrophages offer promising therapeutic potential against solid tumours, functional plasticity marks a major challenge for clinical translation of macrophage-based immunotherapies (Table 1).

Extensive efforts have focused on modulating macrophage functional plasticity towards the M1-polarized phenotype. Macrophages, particularly TAMs, receive not only activating signals but also inhibitory ones within the TME[29] such as CD47–SIRPα axis signalling (Fig. 1). CD47 is ubiquitously overexpressed on various tumour cells, and binds to SIRPα on TAMs, triggering intracellular signalling that promotes M2 polarization, thereby facilitating immune evasion[30]. Multiple approaches have been developed to block the CD47–SIRPα axis to suppress M2 polarization of macrophages, including anti-CD47–SIRPα antibodies, SIRPα–Fc fusion proteins and recombinant SIRPα variants[31]. By disrupting SIRPα-mediated inhibitory signalling in TAMs, these strategies restore macrophage phagocytosis and promote repolarization towards an M1-like phenotype, thereby enhancing antitumour capacity[31].

Other approaches to reprogramme TAMs towards an M1-like phenotype include targeting the NF-κB pathway; inhibiting IKKβ (a major intracellular activator of the NF-κB pathway) reprogrammes TAMs to a pro-inflammatory state, enhances tumour phagocytosis and promotes IL-12-dependent NK cell recruitment in a mouse model of ovarian cancer[32]. Similarly, nanoparticle-mediated delivery of mRNAs encoding M1-polarizing transcription factors (such as IRF5) can also skew immunosuppressive macrophages towards an antitumour phenotype[33]. This method achieved tumour regression in animal models of various solid tumours and avoided systemic inflammatory toxicities, highlighting its translational potential[33].

Collectively, these findings indicate the controllability of macrophage functional plasticity, which can be further combined with CAR engineering to boost target specificity and amplify their therapeutic potential.

T_{reg} cells

T_{reg} cells are a specialized subtype of $CD4^+$ T cells that mediate immune tolerance and homeostasis. The TCRs of T_{reg} cells specifically recognize

Box 1 | Neutrophils as a potential chassis for CAR-based immunotherapy

Neutrophils are key innate immune cells that constitute 40–70% of white blood cells and have critical roles in defending against pathogens through antigen presentation to adaptive immunity, direct phagocytosis and the release of antimicrobial agents[225]. For example, they can form neutrophil extracellular traps, which can entrap and eliminate pathogens and prevent their dissemination[226]. Additionally, neutrophils secrete cytokines and reactive oxygen species that contribute to their potent antimicrobial and cytotoxic capabilities. Certain neutrophil subsets have also been implicated in antitumour activities. For example, immature activated neutrophils isolated from lipopolysaccharide-treated mice promoted immune activation and tumour control in a mouse model of non-alcoholic steatohepatitis-associated hepatocellular carcinoma[227,228].

Chimeric antigen receptor (CAR) engineering has been explored in neutrophils; for example, human pluripotent stem-cell-derived CAR-neutrophils reduced tumour burden in a mouse model of glioblastoma, probably owing to their high mobility and ability to cross the blood–brain barrier[12,229,230]. However, they had limited effect on other tumour models, for example, in prostate tumour cells in vitro[229,230]. Nonetheless, neutrophils have a very short half-life in the peripheral blood, which makes them unsuitable for adoptive transfer therapy but might benefit drug delivery approaches. For example, engineered CAR-neutrophils carrying tumour microenvironment-responsive nanodrugs crossed the blood–brain barrier and delivered the antitumour drugs, resulting in reduced tumour load in a mouse model of glioblastoma[231].

Table 1 | Immunobiology, manufacturing and translational aspects of CAR-X cells

	Functional properties	Manufacture and scalability	Advantages over CAR-T_{conv} cells	Potential disadvantages/ translational hurdles	Refs.
NK cells	Cytotoxicity through cytokine release and cytotoxic granules (for example, perforins, granzymes) Death receptor-mediated killing via FasL and TRAIL, triggering apoptosis CD16α-mediated ADCC	PBMC-derived and UCB-derived NK cells are clinically validated sources Resistant to viral transduction, often requiring feeder-based expansion and exogenous cytokines Ex vivo with IL-2, IL-15, membrane-bound IL-21	Comparable cytotoxicity with lower risk of inflammatory toxicities Readily obtainable and expandable from multiple sources Reduced alloreactivity, better potential for off-the-shelf, allogeneic products	Short in vivo persistence, limited antigen-driven expansion, modest cytotoxicity Resistant to viral transduction, necessitating feeder-based protocols or non-viral delivery systems Susceptibility to immunosuppressive TME	13,14,20,81, 83,90,184
Macrophages	Direct phagocytosis of tumour cells Antitumour M1 phenotype: ROS/RNS production, pro-inflammatory cytokine release, and ECM degradation via MMPs	PBMC-derived monocytes and iPS cells are clinically suitable sources Viral transduction is widely used In vivo engineering is promising for CAR-macrophages Sequential ex vivo exposure to bFGF, VEGF, SCF, IGF1, IL-3, M-CSF and GM-CSF	Strong solid tumour infiltration M1 macrophages mediate phagocytosis, pro-inflammatory cytokine release, ECM remodelling and immune cell recruitment	Functional plasticity Potentially adopt pro-tumoural M2 phenotype in TME M1 CAR-macrophages might induce severe systemic inflammation	9,25,100–102,105
T_{reg} cells	Immunosuppressive cytokine secretion (for example, IL-10, TGFβ) IL-2 deprivation from effector T cells Co-stimulatory blockade by CTLA4 binding to CD80/86 on APCs Suppressive metabolic effects through adenosine production	nT_{reg} cells are isolated from PBMCs but with limited expansion potential IL-2 and mTOR inhibitors, such as rapamycin, are essential for lineage stability iT_{reg} cells can be generated by overexpressing *FOXP3* in $CD4^+$ T_{conv} cells	Immunosuppressive abilities to precisely target diseases with over-activated immune responses Can promote the restoration of immune homeostasis	Risk of expanded over-immunosuppression, increasing susceptibility to infections and malignancies Potentially lose suppressive phenotype and convert into pro-inflammatory effector T cells	35,36,121,128,202
iNKT cells	Secretion of both pro-inflammatory (for example, IFNγ, TNF) and anti-inflammatory (for example, IL-4, IL-10) cytokines Cytotoxicity similar to that of NK and effector T cells: cytokine release, cytotoxic granules and death receptor engagement	Can be sorted from PBMCs or generated from UCB-derived HSCs IL-2 and IL-21 can increase the proportion of $CD62L^+$ iNKT cells αGC-PBMCs, αCD3/αCD28 antibodies, or aAPCs are used for ex vivo expansion	Recognize immunosuppressive compartments via CD1d engagement MHC-independent recognition High chemotactic responses to tumour-derived chemokines Better adaptability and infiltration to solid tumours	Low abundance (0.01–0.1% of total T cells), requiring complex strategies for expansion Elevated exhaustion markers during consistent tumour antigen stimulation	51,138,143,215
γδ T cells	Intrinsic cytotoxicity through the secretion of effector cytokines and cytotoxic granules Antigen presentation to effector T cells and NK cells	Vδ2 cells can be selectively enriched from PBMCs via zoledronate Vδ1 cells can be enriched via special TCR antibodies IL-2, IL-15, IL-21 and IFNγ used for efficient ex vivo expansion	Vδ2 cells have potent cyto-toxicity and can act as APCs to amplify antitumour effects Vδ1 cells are memory-like, preferentially reside in mucosal tissues and can infiltrate solid tumours γδ T cells express NK-activating receptors to use NK cytotoxic programmes such as ADCC	CAR-Vδ2 cells can lose cytotoxicity under extensive tumour antigen stimulation No robust protocol for CAR-Vδ1 cell manufacture has been established yet Low abundance (around 4% of total T cells)	60,68,149,151,153
MAIT cells	Cytotoxicity via effector cytokines and cytotoxic granules Neutrophil recruitment and DC activation	Isolated from PBMCs Transduced with a lentivector delivering CAR genes Cultured with various cytokines: IL-2, IL-7 and IL-15	Recognize immunosuppressive compartments via MR1 engagement Low risks for alloreactivity	Low abundance (around 3.1% of total T cells) Potentially lead to tissue inflammation Limited understanding due to scarce preclinical and clinical studies	52,68,71,220

αGC, α-galactosylceramide; aAPCs, artificial antigen-presenting cells; ADCC, antibody-dependent cellular cytotoxicity; APCs, antigen-presenting cells; CAR, chimeric antigen receptor; DC, dendritic cell; ECM, extracellular matrix; FasL, Fas ligand; HSCs, haematopoietic stem cells; IFNγ, interferon-γ; iNKT, invariant natural killer T; iPS, induced pluripotent stem; iT_{reg}, induced regulatory T; MAIT, mucosal-associated invariant T; MHC, major histocompatibility complex; MMPs, matrix metalloproteinases; MR1, MHC class I-related protein 1; NK, natural killer; nT_{reg}, natural regulatory T; PBMCs, peripheral blood mononuclear cells; RNS, reactive nitrogen species; ROS, reactive oxygen species; T_{conv}, conventional T; TCR, T cell receptor; TME, tumour microenvironment; TRAIL, TNF-related apoptosis-inducing ligand; T_{reg}, regulatory T; UCB, umbilical cord blood; Vδ, variable δ-chain; VEGF, vascular endothelial growth factor.

self-peptides and MHC molecules, enabling T_{reg} cells to modulate activities of antigen-presenting cells (APCs) and effector lymphocytes. The immunosuppressive phenotype of T_{reg} cells is coordinated by a FOXP3-centred transcriptional network and a distinct T_{reg} cell-specific epigenetic landscape. During T_{reg} cell development, FOXP3 suppresses the expression of *IL2* and genes encoding pro-inflammatory cytokines while upregulating *IL2RA*, *CTLA4* and *FOXP3* itself[34]. The sustained expression of these T_{reg} cell signature genes is ensured by the T_{reg} cell-specific epigenome, which is characterized by hypomethylation in the enhancer region of these signature genes[35,36].

FOXP3-mediated signature gene expression is responsible for the major suppressive mechanisms of T_{reg} cells. The constitutively high expression of CD25 (α-chain of IL-2 receptor, encoded by *IL2RA*) enables T_{reg} cells to consume soluble IL-2, thereby limiting its availability to effector T cells[37,38]. This IL-2 deprivation suppresses effector T cell activation and proliferation[39,40]. Meanwhile, CTLA4 on T_{reg} cells binds CD80 and CD86 on APCs, competitively reducing their engagement with the co-stimulatory receptor CD28 on effector T cells[41,42]. The combination of IL-2 deprivation and co-stimulatory blockade drives effector T cells towards functional anergy or even apoptosis (Fig. 1).

In addition to contact-dependent suppression, T_{reg} cells also secrete immunoregulatory cytokines (notably TGFβ and IL-10) to inhibit the activation, cytokine production and proliferation of effector

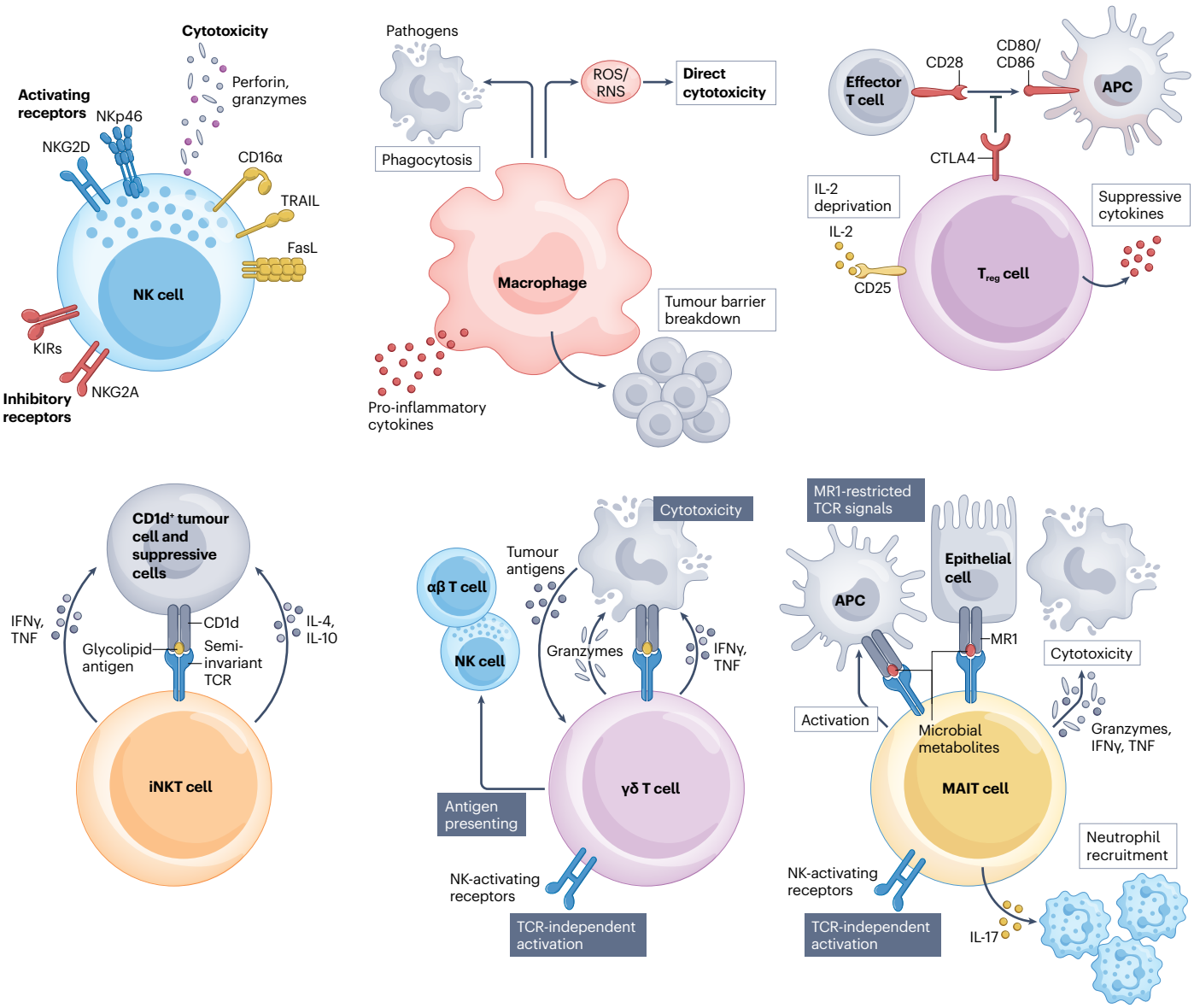

Fig. 1 | Generic functional properties of distinct CAR-X cells. Natural killer (NK) cells, macrophages, regulatory T (T_{reg}) cells and unconventional T cells (including NKT cells, γδ T cells and mucosal-associated invariant T (MAIT) cells) possess distinct mechanisms of cytotoxicity and target recognition and modulatory properties, offering diverse functional advantages that can be leveraged by chimeric antigen receptor (CAR)-based cellular immunotherapy. APC, antigen-presenting cell; FasL, Fas ligand; IFNγ, interferon-γ; iNKT, invariant natural killer T; MR1, major histocompatibility complex class I-related protein 1; RNS, reactive nitrogen species; ROS, reactive oxygen species; TCR, T cell receptor; TRAIL, TNF-related apoptosis-inducing ligand.

T cells and APCs[43,44]. T_{reg} cells can sense the metabolic changes under hyperinflammation; for example, FOXP3 promotes the expression of CD39 and CD73, two surface ectonucleotidases that hydrolyse extracellular ATP into adenosine, which can induce intracellular suppressive signalling pathways in targeted cells. Under hyperinflammatory conditions, CD39 and CD73 detect the increased extracellular ATP release by over-activated immune cells and produce adenosine to suppress their activities[45,46].

Collectively, T_{reg} cells possess unique immunosuppressive functions and are thereby suitable for the treatment of diseases characterized by over-activated immune responses such as autoimmune diseases and graft-versus-host disease (GvHD). However, natural T_{reg} cells constitute approximately 5% of circulating $CD4^+$ T cells, which poses a practical limitation for large-scale expansion and adoptive transfer[47]. Combining intrinsic immunosuppressive capacity with CAR-mediated antigen recognition, along with tailored expansion and engineering strategies, might enhance target specificity, improve functional stability and prevent unwanted pan-immunosuppression (Table 1).

Unconventional T cells

Unconventional T cells, including NKT cells, γδ T cells and mucosal-associated invariant T (MAIT) cells[48], are specialized T cell subsets that differ from $CD4^+$ and $CD8^+$ αβ T cells in both antigen recognition and functional properties. Unlike T_{conv} cells, unconventional T cells recognize non-peptide antigens presented by non-polymorphic antigen-presenting molecules or through MHC-independent mechanisms[49]. Thus, unconventional T cells have a lower risk of inducing GvHD during adoptive transfer and are therefore promising for 'off-the-shelf' allogeneic cell products. Moreover, these cells can mediate targeted cytotoxicity, exhibiting distinct tissue tropisms and bridging innate and adaptive immune responses[50] (Table 1). Unconventional T cells make up only about 10% of circulating T cells, and their low abundance has contributed to their being less explored in traditional cellular immunotherapy. Still, their unique features might help overcome certain limitations of CAR-T_{conv} cells such as antigen escape and TME suppression (Fig. 1).

NKT cells. NKT cells are a distinct subset of T cells that combine the cytotoxic mechanisms and target recognition of both T cells and NK cells. Invariant NKT (iNKT) cells are the predominant NKT cell subset. They recognize glycolipid antigens presented by the non-polymorphic CD1d molecule through semi-invariant TCRs, which consist of a conserved α-chain paired with a restricted β-chain repertoire, in contrast to T_{conv} cells, which express highly diverse TCRs generated through extensive V(D)J recombination[51]. Notably, iNKT cells exhibit substantial adaptability within hostile TMEs; they can target immunosuppressive compartments (such as TAMs and myeloid-derived suppressor cells), which typically express CD1d on their surface[52]. Through cytotoxic granule release, death receptor engagement and cytokine secretion, iNKT cells can deplete these CD1d-expressing suppressive compartments[53,54]. In addition, iNKT cells display strong chemotactic responses to tumour-derived chemokines, including CCL2 and CCL20, thereby facilitating efficient tumour homing[55,56]. In a preclinical mouse model directly comparing NK cells and iNKT cells, distinct functional states were observed in the TME of late-stage breast cancer. NK cells exhibited features of senescence. By contrast, iNKT cells maintained a hyperactivated phenotype with preserved effector function[57]. This activated state was characterized by reduced expression of exhaustion-associated inhibitory receptors, including CTLA4, TIM3 and PD1, together with increased expression of activating and cytotoxic molecules such as granzyme B, NKG2D and IFNγ[57]. These features support the capacity of iNKT cells to infiltrate solid tumours and sustain functional competence within an immunosuppressive TME (Fig. 1).

γδ T cells. γδ T cells are distinguished by their TCRs, which comprise γ-chains and δ-chains instead of the α-chains and β-chains in T_{conv} cells. Although they only constitute about 4% (0.5–16%) of circulating T cells, γδ T cells have critical and non-redundant antitumour roles alongside αβ T cells[58]. Based on the variable δ-chain (Vδ) composition, γδ T cells are classified into Vδ1, Vδ2, Vδ3 and Vδ5 (ref. 59). Among these, Vδ2 is the predominant group (accounting for 60–95% of γδ T cells), whereas Vδ1 cells constitute up to one-third[60]. Vδ2 cells mostly circulate in peripheral blood and display cytotoxic activity, whereas Vδ1 cells preferentially reside in mucosal tissues[59]. Upon TCR engagement, γδ T cells secrete type I cytokines (including TNF and IFNγ), produce cytotoxic granules and mediate death receptor engagement[61]. They can also act as APCs to cross-present tumour antigens to αβ T cells, thereby amplifying antitumour responses[62,63]. Owing to their potent cytotoxicity, Vδ2 cells have been the focus of γδ T cell immunotherapy in treating various solid tumours[64,65]. Vδ1 cells are also gaining attention because of their memory-like phenotypes, resistance to activation-induced apoptosis and infiltration into solid tumours[66,67]. The presence of tumour-infiltrating Vδ1 cells has been associated with a favourable patient prognosis, suggesting therapeutic value[68]. In addition, γδ T cells also express NK-activating receptors (for example, NKG2D and NKp33) to exert TCR-independent cytotoxicity[69]. For example, Vδ2 cells have a high level of CD16, which can recognize Fc regions of antibodies to mediate ADCC[70].

MAIT cells. MAIT cells preferentially accumulate in mucosal tissues and express a semi-invariant TCR α-chain (Vα7.2-Jα33). MAIT cells account for a mean of 3.1% (0.1–9.2%) of total T cells[71]. Their TCR specifically recognizes microbial-derived metabolites presented by MHC class I-related protein 1 (MR1), a non-polymorphic molecule highly expressed on bone marrow-derived APCs and some epithelial cells[72]. Notably, M2-polarized TAMs can exhibit elevated MR1 expression, rendering $MR1^{high}$ macrophages more susceptible to MAIT cell-mediated targeting, which might facilitate MAIT cell adaptability to the immunosuppressive TME[52]. MR1-restricted activation triggers potent cytotoxicity in MAIT cells through the release of effector molecules such as granzymes and pro-inflammatory cytokines, including IFNγ, TNF and IL-17 (ref. 73) (Fig. 1). IL-17 secretion from tissue-resident MAIT cells promotes neutrophil recruitment, and activated MAIT cells can engage with dendritic cells via CD40L upregulation, thereby transactivating other immune cells[74,75]. Similar to the other two unconventional T cells, MAIT cells also express several NK cell-activating receptors (such as NKG2D, NKp40 and NKp33), enabling MR1-independent activation and broadening their tumour-targeting capacity[76]. Importantly, the MR1-restricted and semi-invariant TCRs on MAIT cells do not mediate alloreactivity during allogeneic bone marrow transplantation, indicating the potential for 'off-the-shelf' cell product[77].

Collectively, these unconventional T cells exhibit unique immune attributes, including a special target spectrum, tissue tropism and adaptability to a hostile TME, which could compensate for the challenges faced by CAR-T_{conv} cell therapy.

Manufacturing and engineering of CAR-X cells

CAR-NK cells

Manufacturing CAR-NK cells. NK cells can be sourced from peripheral blood mononuclear cells (PBMCs) of patients, and expanded with cytokine-supplemented media (mainly IL-2) and irradiated feeder cells[78,79]. However, the low abundance (about 10% of circulating lymphocytes)[80], short functional persistence (typically 1–2 weeks)[21] and resistance to genetic modification of circulating NK cells[22] limit the efficiency of PBMC-derived NK cells. Thus, most preclinical and clinical studies favour non-autologous sources to develop CAR-NK cells, including the NK-92 cell line, umbilical cord blood (UCB), induced pluripotent stem (iPS) cells, $CD34^+$ haematopoietic progenitor cells and human embryonic stem cells[81–84] (Table 2). Among these, UCB-derived NK cells are the most widely used source in clinical studies (NCT00900809 (ref. 85), NCT03056339 (refs. 86,87) and NCT05008575 (ref. 88); Table 3). Clinical UCB is usually obtained from cryobanked cord blood units, which are further purified by CD3, CD19 and CD14 negative selection to sort out NK cells. Exogenous IL-2, feeder cell membrane-bound IL-21 and 4-1BB ligand are crucial for the in vitro maintenance of UCB-derived CAR-NK cells[86]. Human pluripotent stem cells are another important source, which can be differentiated into functional NK cells using feeder-supported culture and stepwise cytokine-driven conditions that promote haematopoietic commitment and NK cell maturation[89]. These induced NK cell sources are favoured because they present a lower risk of alloreactivity, are highly amenable to genetic modification, and can be expanded ex vivo at scale, making them attractive for 'off-the-shelf' product development[90,91].

For CAR transduction, retroviral vectors remain the predominant method, yielding efficiencies of 22.7–91.1% (median ~60%)[86,87]. Non-viral methods, such as electroporation and lipid nanoparticles (LNPs), have been explored, but so far have not matched the efficiency of viral systems[92,93].

CAR engineering of CAR-NK cells. CAR constructs in CAR-NK cells differ from those in CAR-T cells primarily in the selection of co-stimulatory domains. In CAR-T cell designs, CD28 co-stimulation is often associated with stronger cytotoxicity but also with rapid exhaustion, whereas 4-1BB co-stimulation promotes durable antitumour responses and supports memory phenotypes. By contrast, in CAR-NK cells, CD28 co-stimulation can produce potent antitumour activity with persistence and tumour infiltration comparable to 4-1BB co-stimulation ex vivo and in vivo in mouse models of various tumour types[94–96]. Mechanistic studies indicate that CD28 signalling in CAR-NK cells recruits various key kinases, primarily lymphocyte-specific protein tyrosine kinase (LCK) and ζ-chain-associated protein kinase 70 (ZAP70), which could enhance CAR-NK cell functions and mitigate tonic signalling[94,95]. Nonetheless, there is no definitive clinical evidence to determine whether CD28 or 4-1BB is superior in the CAR-NK cell context, as both are frequently applied (Table 2 and Fig. 2).

Other strategies include incorporating NK cell-specific signalling molecules into CAR constructs to harness native NK cell signalling pathways. Such molecules include NKG2D, LFA-1, 2B4, DAP10 and 4-1BB[97,98]. In a comparative evaluation of eight CAR-NK cell designs, a construct combining NKG2D transmembrane domain with 2B4 co-stimulation demonstrated the strongest NK cell-specific cytotoxicity in tumour xenograft models, while exhibiting reduced inflammatory toxicity relative to CAR-T cell counterparts[99]. Moreover, the integration of IL-15 expression is often used to support in vivo CAR-NK cell persistence and function[81]. The iC9 suicide gene switch, which can be pharmacologically activated, was also integrated to prevent excessive cytotoxicity and off-target effects[81,86,87]. Together, these findings emphasize that domain-level customization and extra genetic modifications are effective in optimizing CAR-NK cell therapeutic efficacy and safety.

CAR-macrophages

Manufacturing CAR-macrophages. PBMC-derived monocytes remain the primary source for CAR-macrophage studies in humanized models, whereas the THP-1 monocytic cell line is frequently used as a surrogate in preclinical research (Table 2). In clinical studies, monocytes are mobilized from the peripheral blood with G-CSF before apheresis and subsequently differentiated into macrophages by supplemented GM-CSF (NCT06562647 (ref. 100) and NCT04660929 (ref. 101)) (Tables 3 and 4). Engineered CAR-macrophages are kept in media containing key cytokines, primarily M-CSF and GM-CSF, which support lineage specification and functional maturation[102]. Besides monocytes from PBMCs, iPS cells are another important source for therapeutic CAR-macrophages owing to their superior genetic manipulability and scalability[103]. The major challenge of generating iPS cell-derived CAR-macrophages lies in precisely directing iPS cells into macrophages. A stepwise differentiation protocol used sequential exposure to cytokines, including bFGF, VEGF, SCF, IGF1, IL-3, M-CSF and GM-CSF[103]. Removing IL-3 at the final differentiation stage enables M-CSF and GM-CSF to drive terminal differentiation and functional polarization, yielding CAR-macrophages with consistent phenotype and effector functions.

Viral transduction is the predominant strategy for delivering CAR genes to macrophages; for example, the Ad5f35 adenoviral system achieved robust average CAR transduction efficiency of 79.28%, cell viability of 86.39% and purity of 86.78% (NCT04660929)[101]. Lentiviral vectors have also been used to transduce CAR-macrophages; transduction can be facilitated by optimizing infection conditions, including removal of polybrene, extending viral exposure to overnight incubation, incorporating a codon-optimized VSV-G envelope, and delaying infection until 7 days after monocyte isolation[104].

Nonetheless, as macrophages have unique nucleic acid-sensing pathways, which trigger antiviral defences and restrict gene transfer efficiency, non-viral delivery strategies are gaining increasing attention. For instance, LNPs have been explored to deliver CAR mRNA into macrophages and enable in situ programming[105,106]. Intriguingly, owing to the natural phagocytic ability of macrophages, they are highly effective in internalizing nanoparticles such as LNPs, making macrophages highly amenable for in vivo CAR engineering[107]. Moreover, the abundant tumour-resident TAMs can be reprogrammed into an M1-polarized phenotype through in vivo CAR engineering (such as a DNA nanocarrier), enabling locoregionally antitumour effects and reducing risks of systemic inflammation[108,109].

CAR engineering of CAR-macrophages. Early preclinical CAR-macrophage designs incorporated intracellular domains from macrophage-specific signalling molecules, for example, Megf10 and FcRγ to drive phagocytosis, and CD147 to promote MMP production[110,111] (Table 2 and Fig. 2). Such strategies were later broadened by incorporating domains from pattern recognition receptors and other macrophage-associated receptors. For example, FcγRI, which naturally mediates cytokine release and antibody-dependent cellular phagocytosis in macrophages, has been incorporated into CAR constructs, leading to good antitumour efficacy and phagocytic

Table 2 | Preclinical targets and designs of CAR-X cells

Targeted disease	Targeted antigen	Cell source	Intracellular domains	Extra genetic modifications	Refs.
CAR-NK cells					
B cell malignancies	CD19	NK-92	CD3ζ	None	158
		PBMCs	2B4–CD3ζ	None	159
		UCB	4-1BB–CD3ζ	None	170
		PBMCs	iMC	*IL15* co-expression and iC9 suicide gene	171
		UCB	iCD28–CD3ζ	*IL15* co-expression and iC9 suicide gene	81
	CD20	NKL cells	IgG Fc	MICA extracellular domain in the CAR extracellular region	161
	CD19/CD20	UCB	4-1BB–CD3ζ	None	162
	FLT3	NK-92	CD28–CD3ζ	iC9 suicide gene	163
T cell malignancies	CD5	NK-92	4-1BB–CD28–CD3ζ	None	164
		NK-92	2B4–CD3ζ	None	165
	CD7	PBMCs	CD28–CD3ζ	2-in-1 strategy disrupts the CD7 locus and integrates the CAR gene simultaneously	167
	CD38	PBMCs	CD28–CD3ζ	2-in-1 strategy disrupts the CD7 locus and integrates the CAR gene simultaneously	168
AML	CD33	PBMCs	4-1BB–CD3ζ	None	224
Ovarian cancer	Mesothelin	iPS cells	2B4–CD3ζ	None	99
Breast cancer	HER2	NK-92	CD28–CD3ζ	γ-Irradiation for suicide control	174
	TF	NK-92	CD28–4-1BB–CD3ζ	CD16 co-expression	182
Glioblastoma	HER2	NK-92	CD28–CD3ζ	None	176
	Common epitope of EGFR/EGFRvIII	NK-92	CD28–CD3ζ	None	178,183
Gastric cancer	Mesothelin	NK-92	2B4–CD3ζ	None	181
CAR-macrophages					
$HER2^+$ solid tumours	HER2	Raw264.7 macrophages	CD147	None	111
		THP-1	α1β1 intracellular domain–FcγRI motif	None	112
		THP-1	4-1BB–CD3ζ	None	116
		THP-1	FcγRIIa	shSIRPα in tandem with the CAR gene	189
Neuroblastoma	GD2	hPS cells	CD28–OX40–CD3ζ	None	117
Pancreatic cancer	PSCA	iPS cells	CD28–CD3ζ	*IL15* co-expression and tEGFR suicide gene	118
Glioblastoma	EGFRvIII	iPS cells	CD3ζ–TIR	None	102
HCC	GPC3	iPS cells	CD3ζ–TIR	None	102
Ovarian cancer	Mesothelin	iPS cells	CD3ζ	ACOD1 depletion	191
CAR-T_{reg} cells					
Multiple sclerosis	MOG	Murine naive $CD4^+$ T cells	CD28–CD3ζ	FOXP3 co-expression	128
		PBMCs	CD28–CD3ζ	None	195
T1DM	Insulin	Murine naive $CD4^+$ T cells	CD28–CD3ζ	FOXP3 co-expression	121
	InsB:R3	Murine naive $CD4^+$ T cells	CD28–CD3ζ	None	197
SLE	CD19	Human $CD4^+$ T cells	CD28–CD3ζ	FOXP3 co-expression	202
Crohn's disease	IL-23R	PBMCs	CD28–CD3ζ	None	205

Table 2 (continued) | Preclinical targets and designs of CAR-X cells

Targeted disease	Targeted antigen	Cell source	Intracellular domains	Extra genetic modifications	Refs.
CAR-T_{reg} cells (continued)					
GvHD and transplant rejection	OX40L	PBMCs	CD28–CD3ζ	None	204
	HLA-A2	Human $CD4^+$ T cells	CD28–CD3ζ	FOXP3 co-expression	206
		PBMCs	CD28–CD3ζ	None	207
	tEGFR on transplants	PBMCs	CD28–CD3ζ	None	212
CAR-NKT cells					
Neuroblastoma	GD2	PBMCs	CD28–4-1BB–CD3ζ	None	213
			CD28–4-1BB–CD3ζ	IL-15 co-expression	214
Solid tumours	MUC1	PBMCs	CD28–CD3ζ	None	151
Multiple myeloma	BCMA	$CD34^+$ HSCs	4-1BB–CD3ζ	IL-15 co-expression; HLA-I/II depletion by CRISPR–Cas9	146
CAR-γδ T cells					
$CD19^+$ leukaemia	CD19	PBMCs	CD28–CD3ζ	None	149,150
$GD2^+$ tumours	GD2	PBMCs	CD28–CD3ζ	None	154
mCRPC	PSCA	PBMCs	CD28–CD3ζ	None	218
TNBC	HLA-G	PBMCs	4-1BB–CD3ζ	Secreted PDL1/CD3ε BiTE construct	156
CAR-MAIT cells					
Ovarian cancer	Mesothelin	PBMCs	No details	None	52
$CD19^+$ and $HER2^+$ tumours	CD19 and HER2	PBMCs	4-1BB–CD3ζ	MR1 overexpression	220

AML, acute myeloid leukaemia; BCMA, B cell maturation antigen; BiTE, bispecific T cell engager; CAR, chimeric antigen receptor; GvHD, graft-versus-host disease; HCC, hepatocellular carcinoma; HER2, human epidermal growth factor receptor 2; HLA, human leukocyte antigen; hPS, human pluripotent stem; HSCs, haematopoietic stem cells; IL-23R, IL-23 receptor; InsB:R3, IAg7/InsulinB:9-23 register 3 conformational epitope; iPS, induced pluripotent stem; MAIT, mucosal-associated invariant T; mCRPC, metastatic castration-resistant prostate cancer; MOG, myelin oligodendrocyte glycoprotein; MR1, major histocompatibility complex class I-related protein 1; NK, natural killer; NKL, NK-like; OX40L, OX40 ligand; PBMCs, peripheral blood mononuclear cells; PSCA, prostate stem cell antigen; shSIRPα, short hairpin RNA targeting SIRPα; SLE, systemic lupus erythematosus; T1DM, type 1 diabetes mellitus; tEGFR, truncated EGFR; TNBC, triple-negative breast cancer; T_{reg}, regulatory T; UCB, umbilical cord blood.

activity in vivo[112]. Other functional receptors, such as MerTK[113], Megf10 and FcRγ[114], have similarly reinforced antigen-dependent phagocytosis, improved antigen presentation and reduced tumour burden across solid tumour models. However, these CAR constructs also lead to safety concerns about macrophage-mediated systemic inflammation, and therefore no further clinical evaluation has been conducted. By contrast, the classical CD3ζ-CAR construct has clear safety profiles. Although CD3ζ is not typically expressed in native macrophages, CD3ζ-CAR can promote phagocytosis and cytokine production of CAR-macrophages in vitro, and markedly reduced tumour burden with tolerable side effects in mouse models of ovarian cancer and GD2-expressing neuroblastoma[115–117]. This outcome might be due to the structural homology between CD3ζ and FcRγ[9]. This functional robustness has made CD3ζ-CAR the first CAR-macrophage design to enter a clinical trial[101] (NCT04660929; Table 3).

One key functional consideration of CAR-macrophages is to promote and maintain the M1-like phenotype after infusion. The tandem fusion of CD3ζ with a TIR domain from TLR4 increases NF-κB nuclear translocation and sustains pro-inflammatory cytokine expression, which are strong signs of M1-like polarization[102]. However, although no apparent body weight loss was observed in murine models treated with CD3ζ-TIR-CAR-macrophages, the potent pro-inflammatory cytokine production and effector functions associated with M1 polarization raise potential concerns regarding systemic inflammatory side effects. Further modifications improving both functionality and safety are needed before migrating to clinical applications such as incorporating membrane-bound IL-15 and a truncated EGFR (tEGFR) safety switch[118].

CAR-T_{reg} cells

Manufacturing CAR-T_{reg} cells. In current CAR-T_{reg} cell studies, $CD4^+CD25^{high}CD127^-$ natural T_{reg} (nT_{reg}) cells sorted from $CD4^+$-enriched PBMCs are preferred owing to their functional stability[119]. CAR constructs are typically delivered into nT_{reg} cells via retroviral vectors, followed by stimulation with anti-CD3/CD28 beads to induce activation and expansion[120,121]. Engineered CAR-T_{reg} cells are then cultured in X-VIVO15 medium supplemented with recombinant IL-2, which is essential for their survival and maintenance of *FOXP3* expression[122]. To further sustain the stability of the *FOXP3*-dependent immunosuppressive phenotype and selectively limit the proliferation of effector T_{conv} cells, mTOR inhibitors, such as rapamycin or everolimus, are commonly included alongside IL-2 (refs. 123,124). Although these nT_{reg}-based protocols have enabled ex vivo generation of CAR-T_{reg} cells in preclinical CAR-T_{reg} cell studies, the inherently low abundance of nT_{reg} cells (approximately 5% of circulating $CD4^+$ T cells) remains a key constraint for clinical translation.

By contrast, induced T_{reg} (iT_{reg}) cells are generated by converting $CD4^+$ T_{conv} cells, offering a more scalable source compared with nT_{reg} cells. iT_{reg} cells have been less appreciated in current CAR-T_{reg} cell studies due to the technical barrier of producing iT_{reg} cells with a stable in vivo immunosuppressive phenotype[35]. iT_{reg} cells are prone to losing

Table 3 | Clinical reports of CAR-X cells

	Targeted disease	Stage	Antigen	Cell source	Performance	Trial identifiers/ Refs.
CAR-NK cells	R/R AML	Phase I	CD33	NK-92	3 patients with R/R AML were treated but no sustained clinical responses were observed in all patients 1 patient exhibited a temporary decrease in minimal residual disease, and all patients experienced disease progression in 4 months 1 patient developed grade 1 CRS, and no severe toxicities were observed	NCT02944162 (ref. 169)
				UCB	10 patients with R/R AML were treated with anti-CD33 CAR-NK cells and soluble IL-15 CR was observed in 60% of patients (6/10) by day 28, median PFS was 71.5 days and median OS was 137 days Only 1 patient bridged to allogeneic HSCT achieved long-term remission, while others relapsed 1 patient experienced grade 2 CRS, and no grade ≥3 CRS was observed	NCT05008575 (ref. 88)
	$CD19^+$ lymphoid tumours (NHL and CLL)	Phase I–II	CD19	Allogeneic UCB	11 patients were treated (6 with NHL and 5 with CLL) ORR was 73% (8/11 patients), and CR was 64% (7/11 patients, 4 with NHL and 3 with CLL) No inflammatory toxicities linked to CAR-NK cells were observed, and haematological toxicities were linked to chemotherapy	NCT03056339 (ref. 87)
	$CD19^+$ B cell malignancies	Phase I–II	CD19	Allogeneic UCB	37 patients with R/R $CD19^+$ B cell malignancies were treated Day 30 complete response rate was 27% (10/37), 1-year complete response rate was 37.8% (14/37), 1-year OS was 68%, and 1-year PFS was 32% No severe toxicities; 1 patient experienced grade 1 CRS, and haematological toxicities were linked to chemotherapy	NCT03056339 (ref. 86)
	R/R B cell lymphoma	Phase I	CD19	iPS cells	86 patients with R/R B cell lymphoma were treated with two regimens: regimen A (monotherapy, $n=18$) and regimen B (with rituximab, $n=68$) ORR was 54% (37/68, B), complete response rate was 37% (25/68, B) 1-year PFS was 3.5 months and 1-year OS was 8.1 months (both in median) Grade 1–2 CRS occurred in 10% of patients, no grade ≥3 CRS or neurotoxicity were observed, and no treatment-related deaths occurred	NCT04245722 (ref. 160)
	Colorectal cancer	Phase I	NKG2D ligands	PBMCs	3 patients with metastatic colorectal cancer were treated 2 patients showed a significant reduction in tumour cells in ascite fluid and stable disease in peritoneal target lesions Another patient showed a complete metabolic response in liver lesions	NCT03415100 (ref. 173)
	Glioblastoma	Phase I	HER2	NK-92	9 patients with recurrent $HER2^+$ glioblastoma were treated 5 patients displayed stable disease and 4 showed progressive disease; median PFS was 7 weeks and median OS was 31 weeks 3 patients experienced severe adverse events: 1 with postoperative meningitis, 1 with localized brain oedema, and 1 with intracranial haemorrhage	NCT03383978 (ref. 177)
	dcSSc	Case report	CD19/ BCMA	iPS cells	1 patient with severe dcSSc was treated with dual-targeting CAR-NK cells Treatment led to significant B cell depletion with minimal toxicity The patient had remarkable clinical improvements during the 6-month follow-up, showing reduced autoantibodies and reversed fibrosis	NR[185]
	SLE	Phase I	CD19	PBMCs	3 patients with refractory SLE were treated All 3 patients achieved SRI-4 response, with decreased SLEDI-2000 score and improvement in cutaneous symptoms Most adverse events were manageable No CRS or ICANS were observed	NCT06208280 (ref. 186)
CAR-macrophages	$HER2^+$ solid tumours	Phase I	HER2	Autologous monocytes	14 patients with advanced $HER2^+$ solid tumours were treated with CT-0508, an anti-HER2 CAR-macrophage therapy derived from autologous monocytes 44% of patients with a HER2 3+ tumour had stable disease at 8 weeks, no meaningful activity in patients with HER2 2+ tumours; median PFS was 1.47 months Tumour burden was reduced by 20% in a patient with breast cancer and by 14% in a patient with salivary gland cancer Grade 1–2 CRS in 64% (9/14), all resolved in 1–4 days Other toxicities, such as infusion reactions, haematological changes and mild gastrointestinal symptoms, were observed	NCT04660929 (ref. 101)

Table 3 (continued) | Clinical reports of CAR-X cells

	Targeted disease	Stage	Antigen	Cell source	Performance	Trial identifiers/ Refs.
CAR-NKT cells	Neuroblastoma	Phase I	GD2	PBMCs	12 children with R/R neuroblastoma were treated ORR was 25% (3/12) with 1 CR and 2 PR Stable disease in 2 patients and CAR-NKT cell persistence was correlated with responses 1 patient experienced grade 2 CRS and frequent grade 3–4 haematological toxicities due to lymphodepletion	NCT03294954 (ref. 216)

AML, acute myeloid leukaemia; BCMA, B cell maturation antigen; CAR, chimeric antigen receptor; CLL, chronic lymphocytic leukaemia; CR, complete remission; CRS, cytokine release syndrome; dcSSc, diffuse cutaneous systemic sclerosis; HER2, human epidermal growth factor receptor 2; HSCT, hematopoietic stem-cell transplantation; ICANS, immune effector cell-associated neurotoxicity syndrome; iPS, induced pluripotent stem; NHL, non-Hodgkin lymphoma; NK, natural killer; NR, not reported; ORR, overall response rate; OS, overall survival; PBMCs, peripheral blood mononuclear cells; PFS, progression-free survival; PR, partial response; R/R, relapsed or refractory; SLE, systemic lupus erythematosus; SLEDAI-2000, SLE disease activity index 2000; SRI-4, SLE responder index 4; UCB, umbilical cord blood.

FOXP3 expression and acquire effector T cell phenotypes, especially under inflammatory conditions[125–127]. CAR-iT$_{reg}$ cells have been generated by simultaneously introducing exogenous *FOXP3* and CAR genes into CD4$^+$ T$_{conv}$ cells, but functional evaluations were largely limited to in vitro assays[121,128]. Although in vivo lineage stability was not assessed, the modest in vivo results might reflect the instability of these CAR-iT$_{reg}$ cells[121,128]. Along these lines, *FOXP3* induction alone is insufficient to establish T$_{reg}$ cell-specific DNA hypomethylation at the signature gene locus, which is essential for a stable T$_{reg}$ cell phenotype[35,36,129]. Thus, ensuring both *FOXP3* expression and a T$_{reg}$ cell-specific epigenetic landscape is critical for generating CAR-iT$_{reg}$ cells that are functionally stable and clinically scalable.

For example, for more effective *FOXP3* induction, TGFβ and cyclin-dependent kinase 8 (CDK8) and CDK19 inhibitors can be added in combination with IL-2, as not only can they induce *FOXP3* expression but also restrict effector T cell differentiation[130]. Moreover, deprivation of CD28 co-stimulatory signalling during the in vitro conversion process can facilitate the establishment of T$_{reg}$ cell-specific DNA hypomethylation at enhancer regions of T$_{reg}$ cell signature genes, independently of *FOXP3* (ref. 131). Combining these strategies yields stable and functional iT$_{reg}$ cells with suppressive potency in mouse models of inflammatory bowel disease and GvHD[132]. Integrating such stable and functional iT$_{reg}$ cell strategies with CAR engineering is a promising direction in developing CAR-T$_{reg}$ cells with enhanced lineage stability, suppressive potency and translational scalability.

CAR engineering of CAR-T$_{reg}$ cells. CAR constructs in CAR-T$_{reg}$ cells have not incorporated many new or tailored designs, mostly adopting the second-generation CAR structure. However, CAR-T$_{reg}$ cells exhibit distinct preferences for co-stimulatory domains compared with T$_{conv}$ cells. In a comparative in vivo evaluation of ten CAR-T$_{reg}$ cell constructs incorporating different co-stimulatory domains, the wild-type CD28 design showed superior suppressive functions over all other constructs, including those containing 4-1BB[133]. Other in vivo studies in autoimmune disease and GvHD models also confirmed that CD28 co-stimulation confers markedly enhanced functional stability and therapeutic efficacy compared with 4-1BB[134,135]. This difference reflects the unique immunobiology of T$_{reg}$ cells; CD28 signalling is essential for maintaining T$_{reg}$ cell proliferation and CTLA4 expression[136]. By contrast, 4-1BB co-stimulation in CAR-T$_{reg}$ cells is prone to triggering tonic activation, which reduces lineage stability[137]. The transient exposure of 4-1BB CAR-T$_{reg}$ cells to mTOR inhibitors and vitamin C during ex vivo expansion can mitigate tonic signalling-induced dysfunction probably owing to epigenetic modulations at the signature gene locus[129,137]. Nonetheless, CD28 remains the most functionally stable co-stimulatory domain for CAR-T$_{reg}$ cells.

Unconventional CAR-T cells

Manufacturing unconventional CAR-T cells. Clinical iNKT cells are extracted from autologous PBMCs with specialized microbeads that target the invariant TCR α-chain, Vα24-Jα18 (ref. 138). Isolated iNKT cells are stimulated and cultured using a feeder-dependent system featuring α-galactosylceramide (αGC)-pulsed PBMCs. αGC is a synthetic glycolipid antigen that can be presented by CD1d on APCs in PBMCs; therefore, αGC-pulsed PBMCs can stimulate the activation and expansion of iNKT cells[139]. In addition, αGC-pulsed artificial APCs (aAPCs) combined with IL-2 enabled the generation of clinical-scale CAR-iNKT cell doses (≥1×10^7 cells per infusion)[140]. Supplementation with recombinant IL-2 and IL-21 during ex vivo expansion further enhanced the enrichment of CD62L$^+$ iNKT cells, increasing their abundance to 80.4% after primary expansion compared with 56.9% using IL-2 alone. These expanded CAR-iNKT cells demonstrated antitumour activity in vitro and in lymphoma mouse models[141]. Despite the efficiency of this PBMC-derived, feeder-dependent protocol, iNKT cells only account for 0.01–0.1% of circulating T cells, which poses challenges to scalable clinical application[58].

Developing off-the-shelf CAR-iNKT cells using scalable allogeneic sources is a promising alternative. Haematopoietic stem cells (HSCs) from cord blood sources can be guided to differentiate into various types of immune cells, including iNKT cells[142]. To generate cord blood-derived allogeneic CAR-iNKT cells, CD34$^+$ HSCs are co-edited by CAR lentivirus and CRISPR–Cas9 (B2M–CIITA guide RNAs)[142,143]. B2M and CIITA guide RNAs guide the knockout of human leukocyte antigen class I (HLA-I) and HLA-II molecules, resulting in HLA$^-$ CAR-HSCs[144]. Allogeneic CAR-HSCs are then differentiated into mature allogeneic CAR-iNKT cells with a stepwise procedure involving lymphoid progenitor expansion and maturation supplements, CD3–CD28–CD2 T cell activator, and IL-15 (refs. 145,146). For in vitro expansion, various approaches are available with comparable efficiency, including αCD3 and αCD28 expansion, αGC and PBMC expansion, and artificial APC expansion[145,146]. This protocol can generate 1×10^{12} allogeneic CAR-iNKT cells from 5×10^6 CD34$^+$ HSCs, showing the great potential for scalable clinical application[145].

CAR-γδT cell manufacture requires effective lineage enrichment and ex vivo expansion, yet no widely accepted or robust protocols currently exist for either Vδ2 or Vδ1 cells. Vδ2 cells initially received more attention in adoptive transfer studies owing to their higher peripheral abundance and stronger cytotoxic potential compared with Vδ1 cells,

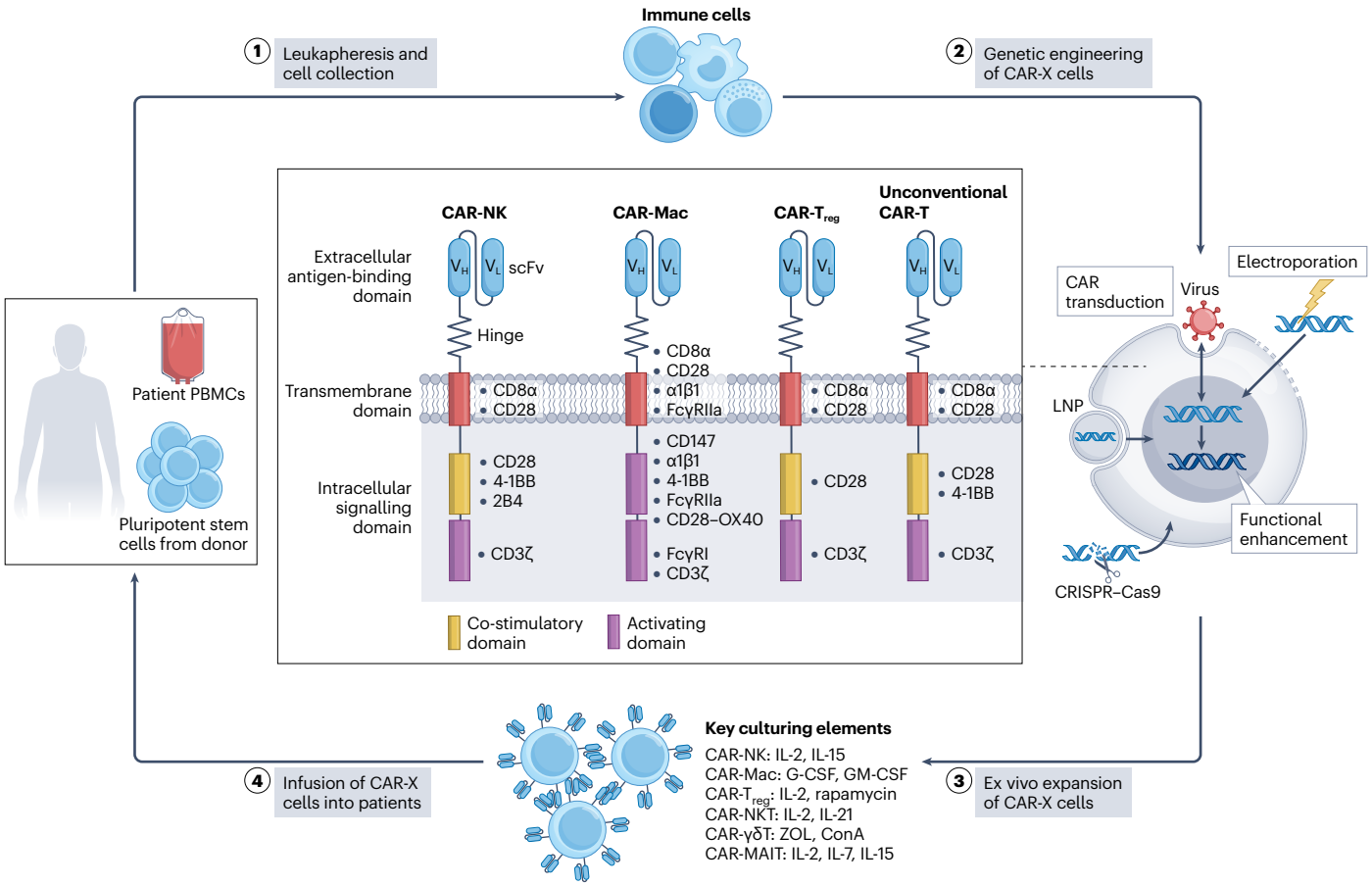

Fig. 2 | Manufacturing and CAR constructs of CAR-X cells. The manufacturing process is generally uniform for each chimeric antigen receptor (CAR)-X cell. Cells are first derived from peripheral blood or non-autologous sources, then engineered to express the CAR and other functional enhancing elements. Major transduction methods in CAR-based immunotherapy include viral vectors, electroporation, lipid nanoparticles (LNPs) and CRISPR–Cas9. Following genetic engineering, CAR-X cells are expanded and maintained ex vivo with specific culturing elements, which are mostly unique cytokine cocktails. Finally, mature and actively functional CAR-X cells are infused into the recipients. CAR constructs across different CAR-X cells differ in the co-stimulatory domain compositions. A second-generation CAR structure is the prevalent backbone for CAR constructs in different CAR-X cells. ConA, concanavalin A; Mac, macrophage; MAIT, mucosal-associated invariant T; NK, natural killer; PBMC, peripheral blood mononuclear cell; scFv, single-chain variable fragment; T_{reg}, regulatory T; ZOL, zoledronate.

which facilitated the development of tailored activation and expansion protocols. The ex vivo activation and expansion of Vδ2 cells leverages their recognition of isopentenyl pyrophosphate, which is produced via the mevalonate pathway in mammalian cells[147]. Treating sorted PBMCs with zoledronate disrupts the mevalonate pathway in APCs, leading to isopentenyl pyrophosphate accumulation and selective activation and enrichment of Vδ2 cells[65,148,149]. Following isolation and viral transduction, supplementation with cytokines (IL-2, IL-15 and IL-21) and aAPCs is required to support in vitro expansion and maintain the in vivo cytotoxicity of CAR-Vδ2 cells[150]. However, CAR-Vδ2 cells generated with these methods rapidly lose cytotoxicity and require extra IL-2 administration upon consistent tumour antigen stimulation[151].

Vδ1 cells are interesting for CAR engineering owing to their memory-like phenotype, resistance to activation-induced apoptosis and infiltration into solid tumours[66,67]. A clinical-grade expansion and differentiation protocol of Vδ1 cells was developed involving a cocktail of cytokines (such as IL-15, IL-2 and IFNγ) and TCR stimulation via the OKT3 antibody[67,150]. In the CAR context, current CAR-Vδ1 cell studies mostly adopt an antibody-based enriching strategy to generate and expand Vδ1 cells using similar cytokine cocktails (soluble IL-2, IL-15 and IL-21) and aAPCs to those in CAR-Vδ2 cells[152,153]. The T cell mitogen concanavalin A (ConA) selectively expands Vδ1 cells from PBMCs, but the overall yield and gene-modification efficiency remain limited. ConA-activated Vδ1 cells exhibit low CAR transduction efficiencies (approximately 20–40%), compared with a mean of 61.57% in CD3-activated and CD28-activated αβ T cells[154]. Moreover, even after expansion, αβ T cells remain the dominant population in culture, thereby constraining the generation of clinically sufficient CAR-Vδ1 cell products[154]. A robust and effective ex vivo expansion protocol is still needed.

Given the scarcity of CAR-MAIT cell or MAIT cell adoptive transfer studies, a robust protocol for ex vivo CAR-MAIT cell manufacture is still lacking. MAIT cells can be isolated from PBMCs by targeting TCR Vα7.2 and transduced with a CAR-expressing lentivector, then expanded in vitro with a cytokine cocktail including IL-2, IL-7 and IL-15 (ref. 52).

CAR engineering of unconventional T cells. Most current unconventional CAR-T cell studies adopt CAR constructs from conventional CARs, and tailored CAR designs are scarce (Fig. 2). Exploratory efforts have incorporated cytokine co-expression to enhance persistence and cytotoxic functions; for example, inclusion of IL-15 in CAR-iNKT cells improves functionality, yielding durable persistence both in vitro and in vivo without evident toxicity[138,145]. Co-expression of IL-12 can further increase CD62L expression in CD62L$^+$ CAR-iNKT cells, induce a prolonged memory phenotype, and sustain an in vivo antitumour response[155]. In CAR-Vδ1T cells, co-expression of IL-2 enhances in vitro cytotoxicity and amplifies the secretion of cytokines, including TNF and IFNγ[153]. Besides cytokine co-expression, strategies such as incorporating bispecific T cell engager (BiTE) secretion have also been explored in unconventional CAR-T cells. For instance, HLA-G-targeting CAR-γδT cells

Table 4 | Ongoing clinical trials of CAR-X cells

	Targeted disease	Antigen	Stage	Status	Trial identifiers
CAR-NK cells[a]	B cell malignancies	CD19			NCT03690310, NCT04639739, NCT04887012, NCT04796675
		CD22	Phase I	Not yet recruiting	NCT06743503
		CD19/CD22	Early phase I	Not yet recruiting	NCT03824964
	T cell malignancies	CD5	Phase I–II	Recruiting	NCT05110742
	R/R AML	CLL-1	Phase I	Recruiting	NCT06307054
	Multiple myeloma	BCMA	Phase I–II	Not yet recruiting	NCT06242249
		BCMA/GPRC5D	Not applicable[b]	Not yet recruiting	NCT06594211
	SLE	CD19/BCMA	Early phase I	Recruiting	NCT06792799
	Autoimmune diseases	CD19	Phase I	Recruiting	NCT06208280
	Recurrent ovarian, fallopian tube and primary peritoneal cancer	CD38	Phase I	Recruiting	NCT06342986
	Pancreatic cancer	NKG2D	Early phase I	Recruiting	NCT06503497
	Glioblastoma	HER2	Phase I	Active, not recruiting	NCT03383978
	Ovarian cancer	Claudin6, GPC3, mesothelin and AXL	Phase I	Recruiting	NCT05410717
CAR-macrophages	Gastric cancer	HER2	Early phase I	Not yet recruiting	NCT06224738
	Mesothelin$^+$ solid tumours	Mesothelin	Not applicable[b]	Recruiting	NCT06562647
CAR-T_{reg} cells	GvHD after AHCT	CD6	Phase I	Recruiting	NCT05993611
	Transplant rejection	HLA-A2	Phase I–II	Active, not recruiting	NCT04817774
			Phase I–II	Recruiting	NCT05234190
	Hidradenitis suppurativa	NR	Phase I	Recruiting	NCT06361836
CAR-NKT cells	B cell malignancies	CD19	Phase I	Recruiting	NCT05487651, NCT03774654
	Renal cell carcinoma	CD70	Phase I	Recruiting	NCT06182735
	CD70$^+$ solid tumours	CD70	Phase I	Recruiting	NCT06394622, NCT06728189
CAR-γδ T cells	B cell malignancies	CD20	Phase I	Active, not recruiting	NCT04735471
	Autoimmune diseases	CD20	Phase I	Recruiting	NCT06375993
	SLE	CD19	Phase I–II	Recruiting	NCT06106893
	R/R solid tumour	NKG2DL	Phase I	Unknown status	NCT04107142
	Advanced cancers	NKG2DL	Phase I	Recruiting	NCT05302037
	R/R solid tumours	PDL1 and/or HLA-G	Phase I–II	Recruiting	NCT06150885
	R/R ccRCC	CD70	Phase I–II	Recruiting	NCT06480565
	Meningeal metastases of B7H3$^+$ solid tumours	B7H3	Not applicable[b]	Recruiting	NCT06592092

AHCT, allogeneic haematopoietic stem cell transplantation; AML, acute myeloid leukaemia; BCMA, B cell maturation antigen; CAR, chimeric antigen receptor; ccRCC, clear cell renal cell carcinoma; GvHD, graft-versus-host disease; HER2, human epidermal growth factor receptor 2; HLA, human leukocyte antigen; NK, natural killer; NR, not reported; R/R, relapsed or refractory; SLE, systemic lupus erythematosus; T_{reg}, regulatory T. [a]Only representative ongoing CAR-NK cell trials are displayed to show major targeted diseases and antigens, as there are currently over 100 ongoing CAR-NK cell trials registered on ClinicalTrials.gov. [b]Without FDA-defined phases.

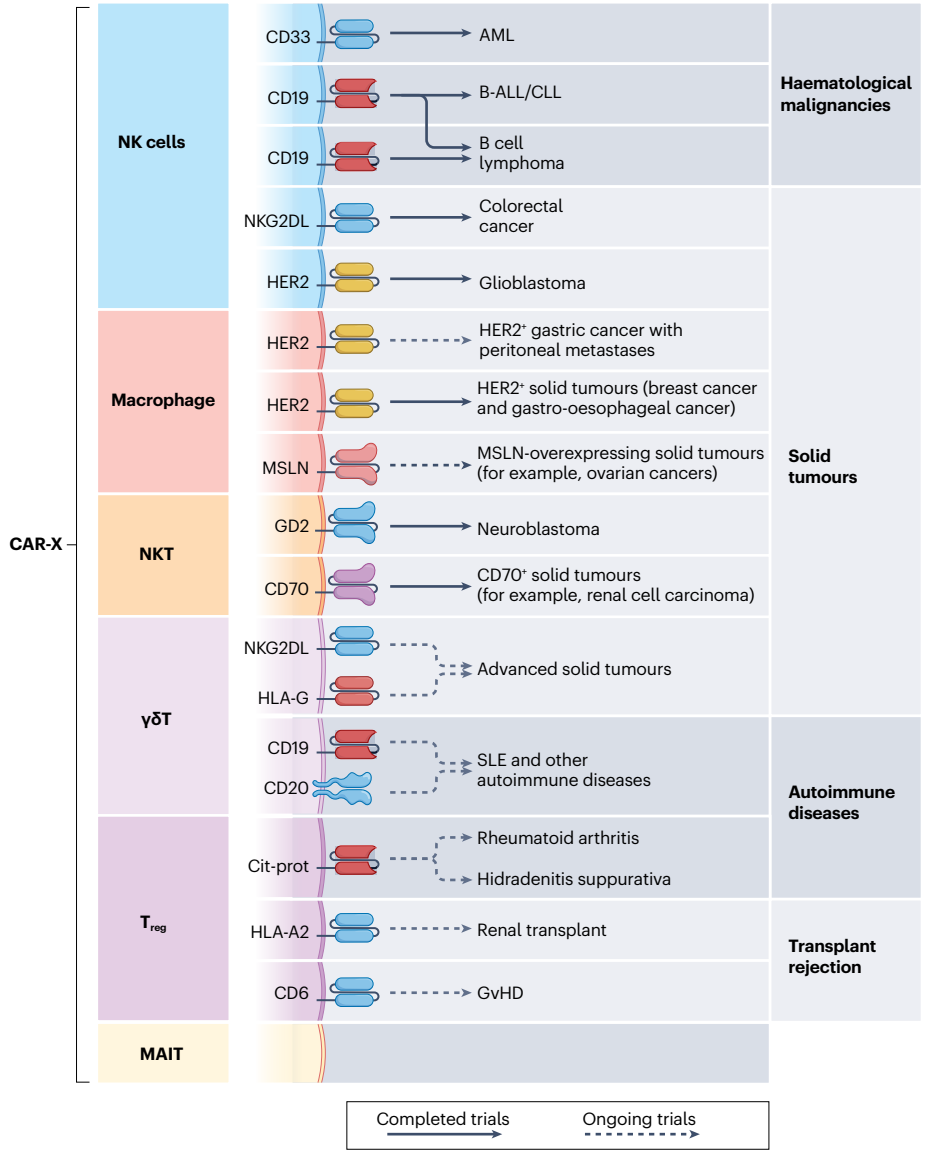

Fig. 3 | Therapeutic applications of different CAR-X cells. CAR-X cell therapies strategically align the unique immunological attributes of different immune cells with specific disease contexts or therapeutic needs by incorporating chimeric antigen receptor (CAR) constructs tailored to each cell type and target. This approach enables rational therapeutic applications across haematological malignancies, solid tumours, autoimmune diseases and graft-versus-host disease (GvHD). AML, acute myeloid leukaemia; B-ALL, B cell acute lymphocytic leukaemia; CLL, chronic lymphocytic leukaemia; HER2, human epidermal growth factor receptor 2; HLA, human leukocyte antigen; MAIT, mucosal-associated invariant T; MSLN, mesothelin; NK, natural killer; SLE, systemic lupus erythematosus; T_{reg}, regulatory T.

secreting PDL1–CD3ε BiTEs counteracted PDL1 upregulation on primary tumour cells and strengthened cytotoxic responses in vitro and in vivo[156].

Collectively, current unconventional CAR-T cell designs mostly rely on the established CAR constructs from CAR-T_{conv} cells. Defining the functional immunobiology of unconventional CAR-T cells is crucial to guide tailored CAR construct designs.

Therapeutic applications of CAR-X cells

CAR-NK cells

NK cells are the second most extensively studied immune cell platform for CAR-based immunotherapies and are widely regarded as a promising alternative to CAR-T_{conv} cells as they can mitigate complications such as CRS and GvHD. The therapeutic potential of CAR-NK cells has been explored in a wide range of tumours, with various clinical trials demonstrating promising efficacy, potential challenges and safety profiles (Tables 2, 3, and 4 and Fig. 3).

Clinical progress of CAR-NK cells against B cell-lineage and T cell-lineage haematological malignancies. Pilot CAR-NK cell studies primarily target CD19, an extensively validated target against B cell-lineage haematological malignancies[157–159]. The combination of IL-15 and the iC9 suicide gene into CAR-NK cells led to 68% overall survival and 32% progression-free survival at 1 year in 37 patients with B cell malignancies[86] (NCT03056339; Table 3). Notably, 31 of these patients had undergone at least three prior lines of therapy but had still relapsed, highlighting the efficacy of this approach compared to the standard of care. Similarly, iPS cell-derived CAR-NK cells showed favourable safety and preliminary activity in 86 patients with B cell lymphoma, incorporating the Fc receptor CD16 in combination with anti-CD19-CAR to enhance ADCC by NK cells. This approach achieved a CRS rate of 11.6% in all patients, alongside an objective response rate of 54.4% and median response duration of 16.9 months[160] (NCT04245722; Table 3). CAR-NK cells have also been developed against other B cell

antigens, including CD20 and FLT3, showing positive antitumour activity in preclinical models[161–163] (Tables 2 and 4). For instance, bifunctional CAR-NK cells killed $CD20^+$ tumour cells in vitro through dual recognition of CD20 and NKG2D[161,162]. Similarly, FLT3-CAR-NK cells exhibited cytotoxicity against $FLT3^+$ B cell acute lymphocytic leukaemia cells in vitro and inhibited B cell acute lymphocytic leukaemia progression in xenograft mouse models with controllable safety[163].

T cell malignancies, such as T cell acute lymphoblastic leukaemia (T-ALL), remain refractory haematological disorders. Because T-ALL cells frequently express surface markers (such as CD5 and CD7) that overlap with those on engineered CAR-T cells, developing CAR-T cell therapies for T-ALL is highly challenging. However, NK cells and T cells do not share many of these markers, making CAR-NK cells a more promising option for treating T-ALL. For example, anti-CD5 CAR-NK cells exert potent antitumour activity in T-ALL xenograft mouse models without inducing fratricide (self-killing due to shared antigen expression among effector cells) or immunodeficiency, with a clinical trial currently under way[164–166] (NCT05110742; Table 4). A more recent study introduced a '2-in-1' strategy to construct fratricide-free CAR-NK cells; this approach involves inserting the EF1α-driven anti-CD7 CAR gene into the CD7 locus using CRISPR–Cas9, enabling simultaneous knockdown and knock-in at the same locus[167]. The generated anti-CD7-CAR-NK cells eliminated residual $CD7^+$ NK cells and showed stronger cytotoxicity against $CD7^+$ T cells in vitro than unmodified NK cells. This fratricide-free locus-specific CAR-NK cell strategy also showed promising efficacy in $CD7^+$ and $CD38^+$ models, indicating the broad potential of this strategy[167,168] (Table 2). In summary, CAR-NK cells, with their reduced fratricide risk and innovative strategies such as locus-specific engineering, offer a promising solution for treating T cell malignancies.

Clinical progress of CAR-NK cells against myeloid haematological malignancies. CAR-NK cells have also been applied in acute myeloid leukaemia (AML) treatment. The first clinical trial for CAR-NK cells in AML constructed NK-92-derived anti-CD33-CAR-NK cells to treat three patients with AML (NCT02944162)[169]. Administered CAR-NK cells displayed sufficient in vivo expansion (at doses up to 5×10^9 cells per patient) and no severe toxicities, but treating efficacy was limited and relapse occurred[169]. The insufficient in vivo persistence and modest cytotoxicity of NK cells might explain the suboptimal performance[15]. To overcome these limitations, cytokine expressions (primarily IL-15) have been incorporated alongside CAR engineering, as well as applying UCB or iPS cells as cell sources, which are more amenable to extra genetic modifications[99,170,171]. These advances improved results in a more recent clinical trial, where UCB-derived, IL-15-expressing anti-CD33-CAR-NK cells were administered to 10 patients with relapsed/refractory AML (NCT05008575)[88]. This trial reported a 60% complete response rate, with one patient achieving long-term remission, indicating the substantially improved in vivo performance of CAR-NK cells against AML[88] (Table 3).

Clinical progress of CAR-NK cells against solid tumours. CAR-NK cells are also being investigated for a range of solid tumours, targeting several well-established markers (Table 2). Upregulated stress-induced ligands on the surface are common in various solid tumours, and these ligands can be recognized by the NK cell-activating receptor NKG2D[172]. Replacing the single-chain variable fragment (scFv) domain in a conventional CAR construct with the NKG2D extracellular domain enabled CAR-NK cells to specifically target metastatic colorectal tumours in the liver of patients[173] (NCT03415100; Table 3). Human epidermal growth factor receptor 2 (HER2), a classical marker expressed in various solid tumours, has been a primary focus for CAR-NK cell solid tumour studies[174–176]. A phase I clinical trial involving intracranial injection of anti-HER2-CAR-NK cells in nine patients with $HER2^+$ glioblastoma reported a median overall survival of 31 weeks, although the treatment showed limited effects on disease progression[177] (NCT03383978; Table 3). Moreover, other targets, such as EGFR, B7-H6 and mesothelin, have also demonstrated preclinical promise but lack assessments in the hostile TME[178–181] (Table 2). For example, EGFR-CAR-NK cells exhibited cytolytic capacity against glioblastoma cells in vitro and suppressed tumour growth with prolonged survival upon intracranial delivery in orthotopic glioblastoma mouse models. B7-H6-CAR-NK cells induced cell death in fulvestrant-resistant breast cancer cells in vitro, whereas mesothelin-CAR-NK cells demonstrated antigen-specific antitumour activity both in vitro and in vivo, supporting their preclinical potential in ovarian and gastric cancer. Nevertheless, limited in vivo persistence and modest cytotoxicity remain the major functional hurdle of CAR-NK cells.

Several strategies have been developed to improve CAR-NK cell performance in solid tumours, including additional genetic modifications (*IL15* and suicide genes)[182]. Moreover, dual-targeting CAR-NK cells, which target both EGFR and EGFRvIII, resulted in early prevention of tumour growth and prolonged survival in a mouse model of xenografted glioblastoma compared to mono-targeting CAR-NK cells[183]. Functional assessments of tumour-infiltrating NK cells identified *CALHM2* as a key gene limiting NK cell efficacy in solid tumours. *CALHM2*-knockout CAR-NK cells improved in vivo antitumour efficacy in a solid tumour model that was refractory to normal CAR-NK cells, indicating the promise of genetically modifying CAR-NK cells to bypass natural immune checkpoints[184]. The feasibility and safety profiles of incorporating additional genetic editing in CAR-NK cells for patients require further assessments from ongoing clinical studies (Table 4).

Clinical progress of CAR-NK cells against autoimmune disorders. Recent clinical studies have provided early evidence for the therapeutic potential of CAR-NK cells in autoimmune disorders. For example, QN-139b, an iPS cell-derived dual CAR-NK cell product targeting CD19 and B cell maturation antigen was administered to a patient with refractory diffuse cutaneous systemic sclerosis[185] (Table 3). Four infusions were well tolerated without substantial toxicities over 6 months, leading to the disappearance of autoantibodies, normalization of complement, and marked improvements in skin and lung function. Multi-omics analyses revealed elimination of pathogenic B cells, suppression of fibrosis and vascular remodelling, suggesting that QN-139b might induce an immune reset, although confirmation in larger trials is required[185]. Another recent clinical trial assessed CD19-CAR-NK cells engineered with membrane-bound IL-15 in three patients with refractory systemic lupus erythematosus (SLE; NCT06208280)[186]. Infusions were completed without preconditioning, were well tolerated, and led to rapid B cell clearance, SLE responder index (SRI-4) responses, tapering or discontinuation of corticosteroids and immunosuppressants, and eventually to polyclonal B cell reconstitution with preserved vaccine-specific recall[186]. Together, these findings highlight CAR-NK cell therapies as a scalable, lower-toxicity platform with the capacity to safely reset autoreactive B cell immunity across distinct autoimmune settings.

CAR-macrophages

The superior tumour-infiltrating and phagocytotic abilities of macrophages make CAR-macrophages a promising candidate for treating

solid tumours, which are often refractory to CAR-T_{conv} cell therapy. Currently, CAR-macrophage research is mostly in the preclinical stage, aiming to achieve stable M1 polarization (Table 2 and Fig. 3).

Clinical progress of CAR-macrophages. The first report of CAR-macrophage administration in patients involved treatment of two patients with advanced ovarian cancer with anti-mesothelin-CAR-macrophages (SY001, NCT06562647)[100]. This CAR-macrophage product induced near-complete tumour regression in xenograft mouse models of ovarian cancer. However, the two patients treated only had stable disease over a 28-day follow-up period without clear therapeutic benefits[100]. A more recent clinical trial treated 14 patients with $HER2^+$ solid tumours with CD3ζ-based anti-HER2-CAR-macrophages[101] (NCT04660929; Table 3). The administered CAR-macrophages migrated to the solid tumour TME and cross-activated $CD8^+$ T cells. However, therapeutic efficacy was limited as only 44% of patients with HER2 3+ (high HER2 expression level) tumours had stable disease at 8 weeks, with no meaningful activity in patients with HER2 2+ tumours[101]. The median progression-free survival was only 1.47 months. Different treatment-related adverse events occurred in all patients, with grade 1 and 2 CRS in 64% (9/14) of patients, all of which resolved within 1–4 days. These results suggest that applying CAR-macrophages to patients with solid tumours is technically feasible but therapeutic efficacy is limited with current designs[101]. Notably, although modest in this trial, the high proportion of inflammatory toxicities still requires caution for future CAR-macrophage trials.

Enhancing the therapeutic efficacy of CAR-macrophages. Only incorporating effector function-related molecules has a limited effect in promoting and sustaining M1 polarization, which might be the cause of their suboptimal clinical performance. Second-generation CAR-macrophages incorporated the TIR domain into the CAR construct to promote NF-κB-mediated pro-inflammatory cytokine production and M1 polarization[102,187]. These CD3ζ-TIR dual-signalling CAR-macrophages exhibited superior phagocytosis, sustained M1 polarization and enhanced TME modulation compared to their first-generation counterparts, with no apparent inflammatory toxicities in mice[102]. Administration of M1-activating factors, such as IFNγ and lipopolysaccharides, promoted M1 polarization in vivo, but the clinical translation is challenging due to safety concerns[188]. A more targeted approach involves disrupting the CD47–SIRPα immune checkpoint axis using CAR-integrated anti-SIRPα short hairpin RNA, which boosts the antitumour functions of CAR-macrophages without influencing non-tumour cells[189]. Metabolic remodelling has emerged as another strategy to modulate macrophage polarization. Itaconate, a key metabolite regulating M1 polarization, is synthesized by the enzyme ACOD1 (ref. 190). Depletion of ACOD1 in CAR-macrophages promoted their pro-inflammatory state and tumour regression in mouse models of ovarian and pancreatic cancers[191]. Collectively, these strategies enhance the stability of M1 polarization in CAR-macrophages, potentially improving their antitumour function and translational value in future clinical evaluation.

CAR-T_{reg} cells

Adoptive transfer of polyclonal T_{reg} cells has been therapeutically effective in a range of preclinical and clinical studies for conditions characterized by excessive immune activity such as type 1 diabetes mellitus and GvHD[192,193]. However, concerns regarding off-target over-immunosuppression and potential pro-tumorigenic effects have limited further clinical exploration[194]. The incorporation of CARs can provide antigen-specific targeting, which improves safety and reduces cell doses for treatment, thereby addressing key translational challenges (Table 2 and Fig. 3).

CAR-T_{reg} cells against autoimmune diseases. CAR-T_{reg} cell therapy for autoimmune diseases often leverages two main strategies: niche-directed and effector-directed strategies. Niche-directed CAR-T_{reg} cells target antigens specific to affected tissues or cells, conferring localized immunosuppression against pathogenic immune activities. For instance, myelin oligodendrocyte glycoprotein (MOG)-CAR-T_{reg} cells preferentially accumulate in the central nervous system, suppressing local pathogenic immune cells and delaying autoimmune encephalomyelitis onset in mice[128,195]. Similarly, CAR-T_{reg} cells targeting carcinoembryonic antigen (CEA) in inflamed colons or insulin in pancreatic β-cells showed high tissue specificity and local suppression, although insulin-CAR-T_{reg} cells had minimal impact on diabetes progression in mice[121,196]. This variability of effects on disease progression highlights the challenge of identifying suitable niche antigens that direct CAR-T_{reg} cells to pathogenic tissues while ensuring robust immunosuppression. A potential approach involves targeting immune complexes formed between niche antigens and autoreactive immune cells, for example, by constructing insulin–MHC class II-targeting CAR-T_{reg} cells[197]. The approach achieved islet-specific migration of CAR-T_{reg} cells, delayed diabetes onset in 95% of treated mice and maintained diabetes-free status in 35% of treated mice at 20 weeks. However, despite their high tissue specificity and potent immunosuppression, niche-directed CAR-T_{reg} cells might suppress bystander immune cells in the targeted tissue, potentially disrupting local immune homeostasis and promoting tumour growth or immune evasion[198,199].

Effector-directed CAR-T_{reg} cells provide a more straightforward approach to autoimmune diseases by directly targeting the source of immune dysregulation, reducing off-target effects and promoting systemic immune homeostasis. For example, CD19-targeting CAR-T cells are effective against SLE progression, but patients often require extensive extra interventions to prevent opportunistic infections caused by systemic B cell depletion[4,200]. By contrast, CD19-CAR-T_{reg} cells delayed SLE symptom onset while restoring immune compositions in lymphoid organs in a humanized mouse model, highlighting their advantages in balancing immune modulation[201,202]. OX40 ligand (OX40L), presented on APCs such as dendritic cells, is another critical activation molecule contributing to lupus pathogenesis[203] (Table 2). OX40L-CAR-T_{reg} cells reduced the co-stimulatory molecules on and antigen-presenting activities of dendritic cells in vitro[204]. Additionally, IL-23 receptor (IL-23R)-targeting CAR-T_{reg} cells suppressed inflammatory T cells in Crohn's disease, with CAR-specific activation observed in colon biopsies from patients. However, the absence of in vivo efficacy assessments limits the understanding of the therapeutic potential of IL-23R-CAR-T_{reg} cells[205].

CAR-T_{reg} cells against GvHD. CAR-T_{reg} cells also hold great promise in conditions such as GvHD and transplant rejection (Table 2). In GvHD following haematopoietic stem cell transplantation, HLA mismatches, particularly HLA-A2, have a critical role in triggering immune alloreactivity[206]. HLA-A2 is expressed by various cells (including APCs); therefore, HLA-A2-targeting CAR-T_{reg} cells (A2-CAR-T_{reg} cells) can suppress antigen presentation to alloreactive T cells from the donor, thereby preventing xenogeneic GvHD in mice[120,207]. In non-human primates, A2-CAR-T_{reg} cells migrated to transplant sites with persistent local immune suppression[208]. Additionally, A2-CAR-T_{reg} cells suppressed

HLA-induced immune rejection against skin transplants, preserving skin integrity and improving transplantation outcomes in mice[209]. These promising results have promoted two ongoing clinical trials of A2-CAR-T_{reg} cells for preventing transplant rejection (NCT04817774 and NCT05234190; Table 4).

However, targeting widespread MHC proteins such as HLA-A2 raises concerns about T_{reg} cells potentially converting to cytotoxic phenotypes or undergoing exhaustion[210,211]. Alternative targets, such as OX40L (which is specifically expressed by APCs), have mitigated xenogeneic GvHD in mice[204]. Another approach is to engineer tEGFR-expressing stem cells and tEGFR-targeting CAR-T_{reg} cells together[212]. When co-introduced into the recipient mice, these CAR-T_{reg} cells provided specific and efficient protection of the transplanted stem-cell-derived tissues from immune rejection.

Unconventional CAR-T cells

Engineering unconventional CAR-T cell approaches have been largely exploratory, aiming to harness their cytotoxic potential, broader target recognition and reduced MHC dependency (Table 2). However, most of these studies have simply transplanted CAR constructs and design strategies from CAR-T_{conv} cells onto unconventional T cells, and primarily focus on evaluating cytotoxic efficacy (Fig. 2). Nonetheless, most of these strategies overlook the distinct functional properties and tissue tropism of unconventional T cells. Further research should move beyond direct adaptation and explore CAR designs tailored to the intrinsic biology of these cells. A deeper mechanistic understanding of their activation, persistence and functions in the TME will be essential for optimizing their clinical applications.

Therapeutic CAR-iNKT cells. CAR-iNKT cells have been developed for targeting solid tumours in preclinical models (Table 2). CAR-iNKT cells targeting GD2 (a marker highly expressed on neuroblastoma cells) demonstrated site-specific trafficking, tumour infiltration and antitumour activity[213]. Co-expression of IL-15 further enhanced in vitro expansion and reduced exhaustion markers, resulting in prolonged in vivo persistence and improved tumour surveillance in a xenogeneic mouse model of neuroblastoma[214]. CAR-iNKT cells also recognize CD1d-expressing TAMs and other immunosuppressive elements (mainly myeloid-derived suppressor cells) while targeting tumour cells expressing the CAR antigen in the TME of ovarian cancer and multiple myeloma animal models[52,143,215]. Combined with the NK-specific recognition molecules, CAR-iNKT cells can deplete tumour cells expressing the CAR antigen and NK-specific stress receptors as well as CD1d-expressing immunosuppressive cells in the TME, preventing tumour resistance and evasion in xenografted ovarian cancer mouse models[215].

Currently, there is one published phase I clinical trial targeting GD2 in patients with neuroblastoma[138,216]. Sufficient in vivo expansion and tumour trafficking were observed: a median of 4.5×10^8 (range 2.9×10^8–9.1×10^8) CAR-NKT cells were generated per infusion product, and the abundance of CAR-NKT cells in peripheral blood was positively associated with their accumulation in tumour tissue. However, initial clinical results were suboptimal, with an only 25% objective response rate (3 out of 12) and only one complete response[138,216] (NCT03294954; Table 3). The elevated level of exhaustion markers in circulating CAR-iNKT cells might have been the reason for these suboptimal clinical effects. Transcriptomic analysis revealed that *BTG1* expression was associated with those exhaustion markers, and the knockdown of *BTG1* improved tumour regression and prolonged survival in neuroblastoma mouse models[216]. Thus, fully realizing the therapeutic potential of CAR-iNKT cells still needs further immunobiology studies and more tailored CAR engineering strategies.

Therapeutic CAR-γδ T cells. The first CAR-γδ T cell study was conducted in 2004, and it transduced Vδ2 cells with a first-generation CAR specific for CD19 or GD2 (ref. 217). A later study introduced the second-generation anti-CD19-CAR into γδ T (both Vδ1 and Vδ2) cells, showing that CAR-γδ T cells could be expanded on $CD19^+$ aAPCs[150]. Anti-GD2-CAR-γδ T (both Vδ1 and Vδ2) cells revealed targeted killing of $GD2^+$ tumour cells in vitro, antigen cross-presentation to responder T cells and migration towards tumour cells in vitro[154]. CAR-Vδ2 T cells targeting prostate stem cell antigen (PSCA) could also infiltrate and exert antitumour effects in metastatic castrate-resistant prostate cancer in vivo. Anti-PSCA-CAR-γδ T cells eliminated tumours in mice, prolonged survival and reduced metastatic castrate-resistant prostate cancer-specific symptoms[218]. To improve homing, another approach is to replace the CAR scFv domain with the external portion of NKG2D, enabling the recognition of tumour-associated NKG2D ligands[219]. NKG2D-CAR-Vδ2 cells prolonged survival in a mouse model of ovarian cancer, and a clinical trial has been conducted to verify its therapeutic efficacy in patients with relapsed and/or refractory metastatic solid tumours[219] (NCT04107142; Table 4). Another strategy is to incorporate a PDL1–CD3ε BiTE[156]. The BiTE binds PDL1 on tumour cells and CD3ε on bystander T cells, promoting CAR-γδ T cell infiltration and amplifying cytotoxic effects through recruited T cells. Engineered to be secreted by anti-HLA-G-CAR-γδ T cells within the TME, this strategy reduced tumour burden and improved survival in mice, leading to a clinical trial evaluating its therapeutic potential[156] (NCT06150885; Table 4). These findings underscore the therapeutic potential of CAR-γδ T cells, but challenges such as expansion efficiency, CAR-Vδ1 cell utility and toxicity profiles must be addressed to proceed to clinical translation.

Therapeutic CAR-MAIT cells. CAR-MAIT cells have also been explored for therapeutic purposes: for example, anti-mesothelin-CAR-MAIT cells were constructed and co-cultured with immunosuppressive M2-polarized macrophages in a 3D-organoid culture, mimicking the immunosuppressive TME of solid tumours[52]. CAR-MAIT cells maintained their cytotoxicity while their CAR-T cell counterparts were suppressed, probably because CAR-MAIT cells could recognize TAMs via NK cell-activating receptors and the semi-invariant TCR[52]. Anti-CD19-CAR-MAIT cells and anti-HER2-CAR-MAIT cells also exhibited strong cytotoxicity against tumour cells in vitro[220]. Compared to their $CD8^+$ CAR-T cell counterparts, CAR-MAIT cells released less pro-inflammatory cytokines, potentially lowering the risk of CRS. As both preclinical and clinical studies of MAIT cells are scarce, the therapeutic efficacy of CAR-MAIT cells still needs to be confirmed.

Outlook

Each CAR-X cell platform leverages unique immune attributes that might complement or even surpass CAR-T_{conv} cells, particularly in achieving improved safety profiles, enhanced tumour infiltration and broader therapeutic applicability. However, key challenges in cell-type-specific functionality, scalable manufacturing and safety profiles remain to be addressed before clinical translation.

Most alternative CAR platforms are innate-like or regulatory immune cells, which are not naturally optimized for sustained, intensive cytotoxicity against tumour cells or pathogens. Thus, cell-type-specific CAR designs need to be developed to achieve optimized therapeutic

outcomes. Moreover, the clinical translation of CAR-X cell therapies not only depends on functional efficacy but also on a scalable and reliable manufacturing process for cell sourcing and ex vivo expansion.

In vivo CAR engineering has emerged as a promising solution for streamlining production, reducing costs and achieving 'off-the-shelf' therapy, notably eliminating the need for lymphodepletion preconditioning[221]. Technically, this is achieved using viral vectors (such as lentivirus) or non-viral nanocarriers (such as LNPs). Although viral vectors offer durable CAR expression, they pose risks of insertional mutagenesis and immunogenicity[222]. Conversely, LNP-mRNA platforms ensure safety through transient, non-integrating expression but require serial dosing for sustained efficacy[221,223]. Beyond extensive investigation in T cells, in vivo CAR engineering has been tested in CAR-NK cells[93] and CAR-macrophages[105,106,109] in preclinical models. Their phagocytic nature makes macrophages highly effective at internalizing nanoparticles, and tumour-resident TAMs represent an abundant source for generating tumour-infiltrating CAR-macrophages via in vivo CAR delivery[107,108]. Such intrinsic uptake capacity partially mitigates delivery barriers, but heterogeneity of target cell populations and the immunosuppressive TME might still limit functional reprogramming efficiency. For other cell types (T_{reg} cells and unconventional T cells), applying in vivo CAR engineering is more challenging because of their low abundance and distinct tissue tropism.

Besides expansion, limited in vivo persistence is another challenge as these immune cells generally lack the long-lived memory phenotype in T_{conv} cells. Strategies such as supplemented cytokine expression, metabolic reprogramming or tailored co-stimulatory domains could improve persistence but this requires a deeper understanding of their immunobiology, especially for the rare unconventional T cells.

Despite providing unique advantages over CAR-T_{conv} cells, such as reduced inflammatory toxicities and lower alloreactivity, CAR-X cell platforms also introduce unique safety issues. For example, CAR-macrophages are often engineered to polarize towards an M1-like phenotype, which produces high levels of pro-inflammatory cytokines and drives systemic inflammation[25]. In the CAR-macrophage clinical trial (NCT04660929; Table 3), antigen stimulation substantially upregulated TNF, IL-6, IL-1β and other cytokines[101]. Although no severe CRS (≥grade 3) or immune effector cell-associated neurotoxicity syndrome were observed, systemic inflammation remains a challenge for CAR-macrophage clinical translation.

Despite these limitations, the field of CAR-based cellular immunotherapy is rapidly evolving to fulfil unmet therapeutic needs and to extend applications from haematological malignancies to a much broader range of diseases, including patients with refractory tumours, autoimmune disorders, solid tumours and beyond.

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