CAR immune cells: design principles, resistance and the next generation

https://doi.org/10.1038/s41586-023-05707-3

Received: 5 March 2022

Accepted: 4 January 2023

Published online: 22 February 2023

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Louai Labanieh[1,2,3] & Crystal L. Mackall[2,3,4,5] ✉

The remarkable clinical activity of chimeric antigen receptor (CAR) therapies in B cell and plasma cell malignancies has validated the use of this therapeutic class for liquid cancers, but resistance and limited access remain as barriers to broader application. Here we review the immunobiology and design principles of current prototype CARs and present emerging platforms that are anticipated to drive future clinical advances. The field is witnessing a rapid expansion of next-generation CAR immune cell technologies designed to enhance efficacy, safety and access. Substantial progress has been made in augmenting immune cell fitness, activating endogenous immunity, arming cells to resist suppression via the tumour microenvironment and developing approaches to modulate antigen density thresholds. Increasingly sophisticated multispecific, logic-gated and regulatable CARs display the potential to overcome resistance and increase safety. Early signs of progress with stealth, virus-free and in vivo gene delivery platforms provide potential paths for reduced costs and increased access of cell therapies in the future. The continuing clinical success of CAR T cells in liquid cancers is driving the development of increasingly sophisticated immune cell therapies that are poised to translate to treatments for solid cancers and non-malignant diseases in the coming years.

CARs are synthetic modular proteins that redirect immune cell reactivity toward a target of interest. This versatile platform has demonstrated substantial clinical effects in the treatment of B cell and plasma cell malignancies, and the potential to expand its application is driving rapid technological developments and large investments from academia and the biopharmaceutical industry. Six CAR T cell products have been approved by the US Food and Drug Administration (FDA) for 12 indications, including large B cell lymphoma[1–4] (LBCL), B cell acute lymphoblastic leukaemia[5–7] (B-ALL), mantle cell lymphoma[8] and follicular lymphoma[9]. In pivotal trials, CD19-CAR therapy outperformed standard of care (SOC) as second line therapy for LBCL[10,11], and was highly effective as a first line therapy[12], paving the way for its application in earlier-stage disease. The generalizability of the CAR platform beyond CD19 targeting is now established, with two BCMA-CAR T cell therapies (BCMA is also known as TNF receptor superfamily member 17) having been approved by the FDA for treatment of multiple myeloma[13,14], and high response rates with CD22-CARs in B-ALL[15,16] and LBCL[17], CD30-CARs in Hodgkin lymphoma[18], CD7-CARs in T cell acute lymphoblastic leukaemia[19–22] (T-ALL), CD20-CARs in LBCL[23], and GPRC5D-CARs in multiple myeloma[24] (Table 1). Standardized toxicity grading and management has resulted in low treatment-related mortality with current commercial CAR T cells[1–11].

Despite this progress, many challenges remain. Fewer than 50% of patients treated with commercial CAR T cells for B cell malignancies experience durable disease control[1–7]. CAR T cells have shown signs of activity in solid tumours[25–29], but high rates of consistent durable responses have not been demonstrated (Table 1). Autologous cell manufacturing is labour-intensive and expensive and commercial scaling is not yet adequate to meet clinical needs. This Perspective synthesizes current understanding of the immunobiology of CAR T cells, emphasizing resistance mechanisms in cancer, design principles and emerging approaches to enhance efficacy. We focus primarily on developing CAR T cells for cancer treatment, but many of the principles are relevant to other immune cell therapies for cancer and to nascent efforts to develop cell therapies for non-malignant diseases. Owing to space constraints, we focus primarily on the most recent literature and on the emerging efforts to enhance efficacy, and refer the reader to recent authoritative reports for additional information on CAR-related toxicities[30] and clinical outcomes[31].

CAR T immunobiology and mechanisms of resistance

Sustained broad-based advances by many groups focused on developing immune cell therapies for cancer have been essential for the success of CAR T therapy (Fig. 1). CARs were invented by Eshhar and colleagues with the goal of harnessing the expansion, killing and persistence of natural T cells while overcoming major histocompatibility complex (MHC) restriction of the T cell receptor (TCR), to enable broader therapeutic applicability[32,33]. After iterative optimization by many groups[34,35], a receptor incorporating a scFv as the antigen-binding domain, a hinge/transmembrane domain, TCRζ and a CD28 or 4-1BB costimulatory endodomain emerged as the CAR prototype (Fig. 2). This architecture

[1]Department of Bioengineering, Stanford University, Stanford, CA, USA. [2]Center for Cancer Cell Therapy, Stanford Cancer Institute, Stanford University, Stanford, CA, USA. [3]Parker Institute for Cancer Immunotherapy, San Francisco, CA, USA. [4]Division of Hematology, Oncology, Stem Cell Transplantation and Regenerative Medicine, Department of Pediatrics, Stanford University, Stanford, CA, USA. [5]Division of Blood and Marrow Transplantation and Cell Therapy, Department of Medicine, Stanford University, Stanford, CA, USA. ✉e-mail: cmackall@stanford.edu

Table 1 | Targets of CAR T cell therapies with clinical evidence of efficacy

Target	Disease	Response rate[a]	Survival	Comments	Date of FDA approval	Refs.
Haematological malignancies						
CD19	B-ALL	CR or CRi: 81%,	EFS: 50% OS: 76% at 12 months	Tis-cel approved for R/R B-ALL (≤25 yr of age)	Aug 2017	5
CD19	LBCL	CR: 58%,	PFS: 44% at 12 months OS: 52% at 18 months	Axi-cel approved as 3rd line treatment for LBCL (>18 yr of age)	Oct 2017	3
CD19	LBCL	CR: 40%	RFS: 65% OS: 49% at 12 months	Tis-cel approved as 3rd line treatment for LBCL (>18 yr of age)	May 2018	4
CD19	MCL	CR: 67%	PFS: 61% OS: 83% at 12 months	Brex-cel approved for R/R MCL (>18 yr of age)	July 2020	8
CD19	FL	CR: 74%	PFS: 65% OS: 87% at 18 months	Axi-cel approved as 3rd line treatment for R/R FL (>18 yr of age)	Mar 2021	222
CD19	LBCL	CR: 53%	PFS: 44% OS: 58% at 12 months	Liso-cel approved for 3rd line LBCL (>18 yr of age)	Feb 2021	1
BCMA	MM	CR: 33%	Median PFS: 8.8 months OS: 78% at 12 months	Ide-cel approved for 5th line treatment for MM (>18 yr of age)	Mar 2021	13
CD19	B-ALL	CR: 56%	RFS: 58% at 6 months OS: 71% at 12 months	Brex-cel approved for R/R B-ALL (>18 yr of age)	Oct 2021	223
BCMA	MM	sCR: 67%	PFS: 77% OS: 89% at 12months	Cilta-cel approved for 5th line MM (>18 yr of age)	Feb 2022	14
CD19	FL	CR: 69%	PFS: 67% at 12 months	Tis-cel approved for 3rd line treatment of FL (>18 yr of age)	May 2022	9
CD19	LBCL	(Axi-cel vs SOC) CR: 65% vs 32%	(Axi-cel vs SOC) EFS: 41% vs 16% OS: 61% vs 52% at 24 months	Axi-cel approved as 2nd line treatment for LBCL (>18 yr of age)	April 2022	10
CD19	LBCL	(Liso-cel vs SOC) CR: 66% vs 39%	(Liso-cel vs SOC) EFS: 45% vs 24% OS: 79% vs 64% at 12 months	Liso-cel approved as 2nd line treatment for LBCL (>18 yr of age)	June 2022	11
CD19	LBCL	CR: 78%	PFS: 75% OS: 91% at 12 months	Front line therapy for high-risk LBCL		12
CD22	B-ALL	CR: 70%	Median RFS: 6 months Median OS: 13.4 months	CD19-CAR T cell therapy had failed in 88% of these patients		15,16
CD22	LBCL	ORR: 86% CR: 67%	Median PFS: not reached	CD19-CAR T cell therapy had failed in 95% of these patients		17,224
CD30	HL	CR: 59%	PFS: 36% OS: 94% at 12 months	Greater CD30 CAR T persistence and higher PFS with fludarabine-based LD		18
CD7	T-ALL	CR: 90%	Not available	Allogeneic donor-derived CD7-CAR T cells; GVHD grade 1–2 in 60% of patients		20
CD7	T-ALL or TLBL	CR: 7/8	Not available	Autologous CD7-CAR T cells rendered fratricide-resistant using a CD7 PEBL		19
CD38	AML	CR or CRi: 4/6	50% relapse rate at 6 months	Allo-HSCT refractory patient population; no off-target effects on monocytes or lymphocytes		225
κ light chain	NHL, CLL, or MM	CR: 2/9	Not available	No or limited pre-treatment LD. One CR sustained for at least 3 yr		226
CD20	LBCL	CR: 54.5%	PFS 41.7% at 24 months	All patients had prior rituximab; longest CR at least 57 months		23
Solid tumours						
GD2	NB	CR: 27% of patients with active disease	Median OS: 31 months	1st generation CAR expressed by EBV-reactive T cells; one patient had sustained CR for at least 60 months		227
GD2	DMG	9/10 patients with radiographic or clinical benefit	Not available	Initial IV infusion followed by multiple ICV infusions; one patient had >95% reduction in tumour volume		28,228
HER2	Sarcomas	CR: 27%	Not available	No on-target, off-tumour toxicity of HER2-CARs; patient with RMS metastatic to bone marrow had a CR for >12 months		229
IL-13Rα2	GBM	CR: 1/1	Not available	CR sustained for 7.5 months with 16 locoregional administrations over 220 days		27

Continued

Target	Disease	Response rate[a]	Survival	Comments	Date of FDA approval	Refs.
EGFR	BTC	CR: 6%	Median PFS: 4 months	One out of 17 patients achieved a CR for at least 22 months. Manageable mucosal toxicities.		230
Mesothelin	MPD	11% complete metabolic response by PET	OS: 83% at 1 yr Median OS: 23.9 months	Regionally delivered intrapleural CAR T cell administration plus PD-1 blockade		98
Claudin-18.2	GC or PC	ORR: 48.6% Disease control rate: 73.0%	Median PFS: 3.7 months OS: 81% at 6 months	83% of patients showed tumour regression; 11% showed reversible grade 3/4 gastrointestinal toxicities		29
PSMA	MCRPC	5/13 patients had >30% reduction in PSA	Median PFS: 4.4 months Median OS: 15.9 months	PSMA CAR T cells expressing a dominant-negative TGFβRII. 5 out of 13 patients with high-grade CRS, one fatal		172
Non-malignant disease						
CD19	SLE	1/1 clinical remission	Not available	Rapid decrease of autoantibodies from >5,000 U ml^{-1} to 4 U ml^{-1}		213

[a]If fewer than ten patients were treated, absolute response numbers are provided as a fraction; otherwise, they are provided as the percentage response rate.
Allo-HSCT, allogeneic haematopoietic stem cell transplant; AML, acute myeloid leukaemia; BTC, biliary tract cancer; CLL, chronic lymphocyte leukaemia; CR, complete response; CRi, complete response with incomplete haematologic recovery; CRS, cytokine release syndrome; DMG, diffuse midline glioma; EBV, Epstein–Barr virus; EFS, event-free survival; FL, follicular lymphoma; GBM, glioblastoma; GC, gastric cancer; GVHD, graft-versus-host disease; HL, Hodgkin lymphoma; ICV, intracerebroventricular; IV, intravenous; LD, lymphodepletion; MCL, mantle cell lymphoma; MCRPC, metastatic castration-resistant prostate cancer; MM, multiple myeloma; MPD, malignant pleural disease; NB, neuroblastoma; NHL, non-Hodgkin lymphoma; ORR, overall response rate; OS, overall survival; PC, pancreatic cancer; PEBL, protein expression blocker; PET, positron emission tomography; PFS, progression-free survival; PSA, prostate-specific antigen; PSMA, prostate-specific membrane antigen; RFS, relapse-free survival; RMS, rhabdomyosarcoma; R/R, relapsed or refractory; sCR, stringent complete response; SLE, systemic lupus erythematosus; TLBL, T cell lymphoblastic lymphoma. Axi-cel, axicabtagene ciloleucel; brex-cel, brexucabtagene autoleucel; cilta-cel, ciltacabtagene autoleucel; ide-cel, idecabtagene vicleucel; liso-cel, lisocabtagene maraleucel; tis-cel, tisagenlecleucel.

is utilized by five out of the six FDA-approved agents, with the sixth incorporating the same architecture with two nanobody heavy chains (VHH) as the antigen-binding domains[14]. Antigen engagement of the prototype 1.5–2.2 kilobase (kb) receptor largely replicates antigen specific activation and killing mediated by the TCR–CD3 complex in natural T cells; however, significant distinctions exist between the biology of CAR T cells and natural T cells that provide opportunities and challenges for application of these therapeutic agents, as discussed below.

Resistance due to antigen modulation

A major distinction between CAR and TCR signalling is that CARs require higher antigen density for full T cell activation[36–38]. Despite the higher affinity of single-chain variable fragments (scFvs) compared with TCRs and the generally higher density of CAR expression compared with TCR–CD3 complexes[36], TCRs induce full activation in response to less than 100 peptides per antigen presenting cell[39,40], whereas CARs require more than 1,000 target molecules per target cell[15,41–45]. The basis for the difference includes diminished proximal kinase recruitment by CARs[38,44,46,47], a less developed immune synapse[46], reduced engagement of co-receptors and greater induction of negative downstream regulators[36]—in part related to tonic signalling, in which CAR aggregation, often driven by the scFv, induces antigen-independent activation[48]. Modifications to the design of CAR prototypes can tune the antigen density threshold to some extent, with

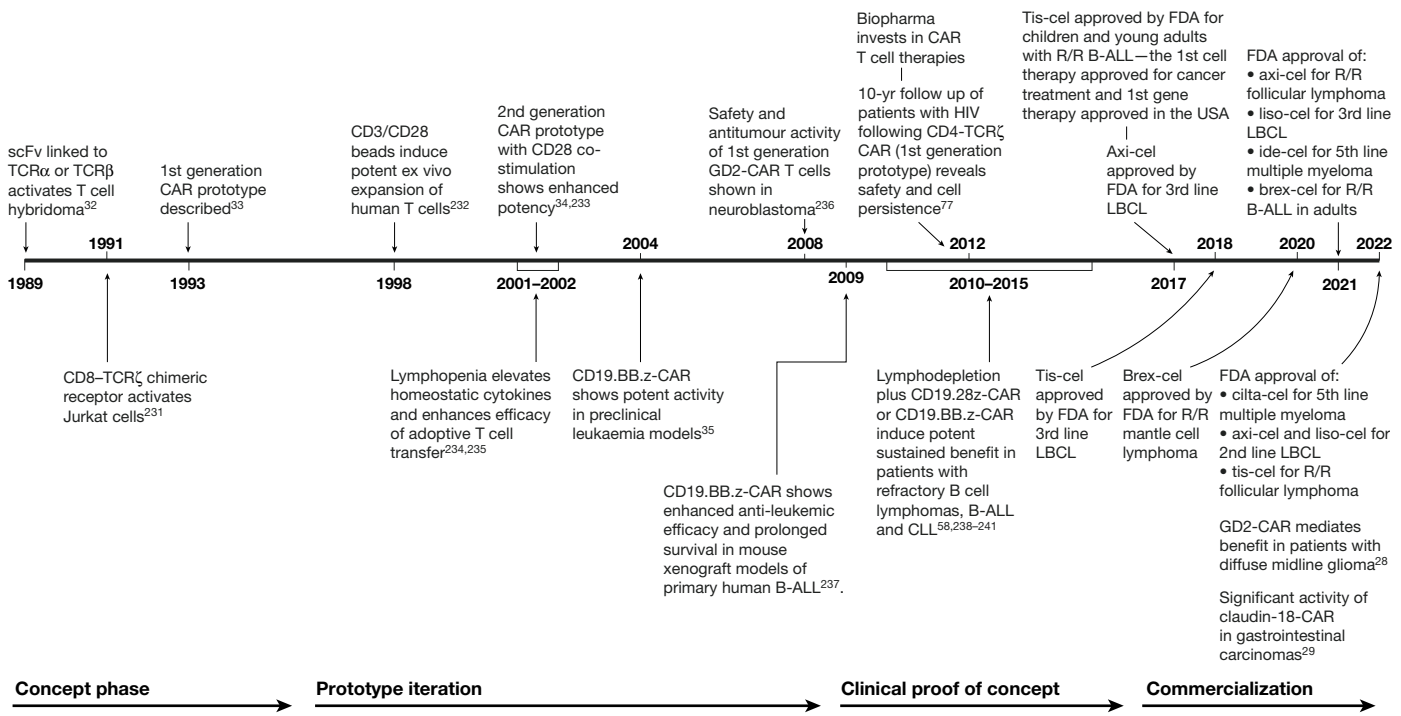

Fig. 1 | Timeline of key milestones in CAR T cell development. A timeline of developments in CAR T cell therapies[231–241].

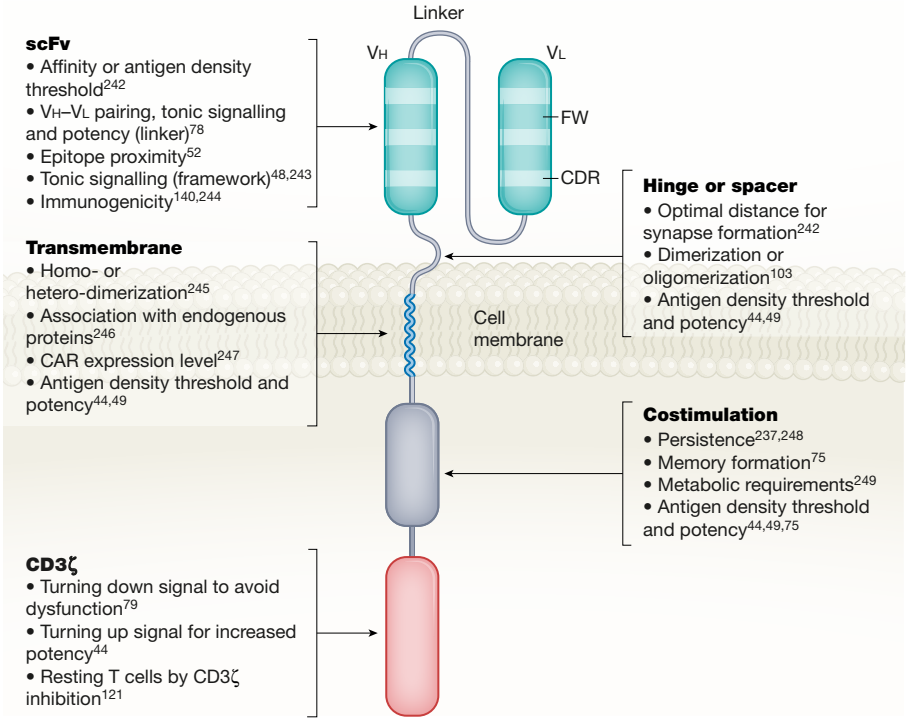

Fig. 2 | CAR structure–function relationships. Prototype CARs comprise a target-binding domain such as a scFv, a hinge or spacer domain that projects the binder away from the cell surface and provides conformational flexibility, a transmembrane domain that anchors the receptor in the cell surface, and costimulatory and CD3ζ signalling domains that provide activation signals. Small modifications in CAR structure can have profound effects on CAR T cell function, including modulation of the antigen density threshold for activation, persistence, potency, tonic signalling, CAR expression level and the propensity for dimerization[237,242–249]. CDR, complementarity-determining region; FW, framework region.

features that modulate signal strength, scFv affinity, CAR expression density, hinge/transmembrane architecture and synapse spacing, having significant effects[44,49–52] (Fig. 2). Because greater signal strength lowers the antigen density threshold, features that enhance T cell fitness independently of CAR design also reduce the CAR antigen density threshold[44,53]. These insights are foundational for developing safe and effective CAR T cell therapies, since toxicity and efficacy are intimately related to the expression characteristics of the targeted antigen. CARs targeting molecules that are absent from vital tissues, such as B cell lineage antigens, should be engineered for activation at low antigen density to diminish the risk of low antigen recurrence, whereas CARs targeting molecules that are highly expressed in cancer but with low expression in vital tissues should be engineered with higher antigen density thresholds to exploit a therapeutic window on the basis of differential antigen density[49].

Antigen modulation is a major cause of CAR T cell resistance in B cell malignancies, and is likely to pose an even greater challenge in solid tumours, where most targetable antigens show significant heterogeneity[54–56]. In B-ALL in children and young adults, approximately 50% of relapses are associated with CD19 loss[5,57,58], and in LBCL, approximately 30% of relapses are CD19-negative and an additional 30% express CD19 at levels below the antigen density threshold for commercial CARs[45,59]. The role of antigen modulation in resistance to BCMA-CAR T therapy in multiple myeloma is less well defined. Baseline BCMA expression levels are heterogeneous among patients and have not been associated with clinical responses[60–62]. BCMA loss—associated with genetic mutation and deletion—is a rare cause of resistance[13,63,64] (less than 5% of cases), however, antigen modulation is observed following BCMA-CAR treatment[61,62]. Antigen density can be modulated through a variety of mechanisms, including genetic mutation[63,65,66], alternative RNA splicing[65], cell lineage switching[67], epigenetic and/or posttranscriptional mechanisms[15], trogocytosis[50], hyperglycosylation[68] and antigen shedding[69]. Downregulation of some targets is amenable to therapeutic intervention with small molecules, such as CD22 upregulation by bryostatin[70], CD70 upregulation by azacitidine[71] and BCMA upregulation by γ-secretase inhibitors, which inhibit antigen shedding[69,70].

Resistance due to inadequate T cell function

A second major cause of CAR T cell resistance is related to inadequate T cell potency, persistence, functional persistence and/or dysfunction, and is typically associated with disease recurrence in the absence of antigen modulation. Dysfunction often results from T cell exhaustion, characterized by global transcriptional and epigenetic reprogramming that converges on terminal differentiation[53,72]. T cells in the apheresis and/or manufactured CAR T cell product sometimes manifest exhaustion[73,74], and high tumour burdens induce exhaustion following adoptive transfer. CAR-intrinsic factors also contribute to exhaustion, with the costimulatory domain having a major role. CD28-costimulated CARs manifest more rapid and greater expansion, secrete more inflammatory cytokines, and show limited persistence owing to T cell exhaustion when compared with 4-1BB and first-generation CARs, which in some cases, may persist for years[5,58,75–77]. The pro-exhaustion effect of the CD28 costimulatory domain is magnified in CARs with tonic signalling[48,53]. The effect of tonic signalling depends on the magnitude of the signal and is context-dependent, with some CARs demonstrating enhanced function and persistence in the presence of tonic signalling[78]. Tuning down the signalling strength of CD28-based CARs through mutations in CD3ζ or CD28 domains can attenuate their propensity for exhaustion and improve persistence[79,80], as can mutations that interfere with downregulation and ubiquitination of 4-1BB-costimulated CARs[81]. Of interest, a recent long-term follow-up study demonstrated that long-lived CARs were CD4-positive, raising the prospect that this subset may be less susceptible to exhaustion and thereby exhibit greater persistence[76,82].

Box 1

Next-generation CAR enhancements

Enhancements in development that are in clinical testing are listed in bold; those at proof-of-concept stage are underlined.

Multispecificity and/or logic gating

OR gates: **bivalent**[45,100,104,105], **bicistronic**[103], **two vectors**[101,102], **co-infusion**[250], higher order[251]

NOT gates: PD-1[179,180], CTLA-4[179], TIGIT[180], LIR-1[181]

IF–THEN gates: synNotch[173,174]

AND gates: split CAR[252], LINK CAR[176]

TME-localized: masked CAR[253], hypoxia sensing[254], conditional granzyme[255], EGFR BiTE[114]

Fitness enhancements

Gene overexpression: **c-Jun**[53], *BATF*[117], *PGC1A* (also known as *PPARGC1A*)[256]

Gene knockout: ***PD1* (also known as *PDCD1*)**[123], ***TGFBR2***[142], ***HPK1* (also known as *MAP4K1*)**[132], *NR4A2*[126], *RASA2*[127,131], *TET2*[115], *DNMT3A*[116], *TOX*[257], *CBLB*[127], *CD5*[127], *SOCS1*[127], *TCEB2* (also known as *ELOB*)[127], REGNASE-1 (also known as *ZC3H12A*)[128], *DHX37*[129], *PTPN2*[130], *IKZF2*[124], *TLE4*[124], *ID3*[72], *SOX4*[72], *ACAT1*[258], adenosine A_{2A} receptor[133], diacylglycerol kinase[259], *LAG3*[260], *GM-CSF* (also known as *CSF2*)[261], mediator kinase module[125]

Small molecule: **dasatinib**[28,121], AKT inhibitor[119], ibrutinib[120]

Suicide gene: **EGFRt**[153], **CD20 epitope**[262], **HER2t**[263], **HSV-tk**[152], **iCasp9**[138,151]

Regulatable platforms

AP1903-inducible costimulation[264], **antibody-coupled**[265], **fluorescein-CAR**[266], **switchable CAR T cells**[267], **antigen receptor complex (ARC) T cells**[268], SNIP[162], SUPRA[177], co-LOCKR[178], drug-regulated degrons[121,157,161], drug-induced dimerization[156,157], drug-activated binders[159], CAR disruption[158], protease-cleavable CARs[162,163], Tet-inducible[155], PROTACs[160], ultrasound[269], light[270]

Armouring

Dominant negative: **TGFβR**[171,172], **PD-1**[143], FAS[145]

Checkpoint: **PD-1-Fc**, **anti-PD-1 scFv**[146]

Cytokines: **IL-12**[109,110,271], **IL-18**[111,272,273], **NFAT-induced IL-12**[274], **IL-15 and IL-21**[275]

Switch receptors: **PD-1–CD28**[276], **IL-4R–IL-2Rβ**[277], IL-4R–IL-7R[147], FAS–41BB[148], GM–IL-18[85]

Other: **FAP-CAR**[278], heparanase overexpression[279], catalase overexpression[280], solHVEM[281], PKA disruptor[282]

Engaging the endogenous immune system

Chemokine overexpression: **CXCR5**[283], **CCR4**[284], **CCL19**[99], CCR2[285], CX3CR1[286]

Other: **CD40L overexpression**[112], RN7SL1 extracellular vesicles[108], FLT3L overexpression[113]

Expansion and persistence

IL-15: **secretion**[138,196], **sushi domain**[287], **tethered**[288]

IL-7: **secretion**[99], **mutant constitutive**[136], tethered[289]

Other: JAK–STAT CAR[137], ortho IL-2[290], IL-2–IL-9 chimera[291], chimeric costimulatory receptor[107,292]

Alternative signalling

TRuC[185], **Ab–TCR**[184], STAR[183], HIT[182]

Stealth or fratricide resistant

Knockout: **TRAC**[150,190,262], **B2M**[293], **CIITA**[191], **CD52**[190], **CD7**[134], **CD5**[135]

Overexpression: **HLA-E**[192], CD47[193]

Endoplasmic reticulum retention: **CD7 PEBL**[19], CD3 PEBL[294]

The clinical effect of shorter persistence of CD28- versus 4-1BB-costimulated CARs varies by disease. In LBCL, tumour eradication occurs rapidly, and CD28 and 4-1BB costimulated CAR T cells demonstrate similar efficacies[1,3,4,83]. By contrast, in B-ALL, persistence of CAR T cells beyond 6 months is associated with increased rates of relapse—thus, CD28 costimulated CAR T cells are less effective unless patients receive a post-CAR bone marrow transplant to consolidate remission[57,84]. In multiple myeloma, functional persistence of anti-BCMA-CAR T cells is associated with a longer duration of response[13]. It remains unclear whether CD28 or 4-1BB costimulation is preferred for solid tumours, where both strong signalling strength and persistence are desirable. Prototype CARs incorporating both CD28 and 4-1BB costimulatory domains have not demonstrated superiority, leading investigators to integrate novel[85,86] or synthetic costimulatory domains[87] with the goal of endowing maximal signalling power alongside durable persistence. Pooled CAR screening has been used to identify optimal CAR signalling domains and designs and elucidate CAR design principles[87–89]. Genome-wide CRISPR screens have identified the CD2–CD58 axis as a mediator of T cell potency[90] and IFNγR signalling has been demonstrated to be required for productive CAR T cell adhesion and cytotoxicity in solid but not liquid tumours[91]. CAR T cell potency is also limited by immunosuppressive molecules (TGFβ, IL-10, IL-6 and checkpoint molecules) in the tumour microenvironment (TME), and work is underway to combine CAR T cell therapies with immunomodulators designed to activate immunity within the TME and/or to arm immune cells to resist specific immunosuppressive mediators (Box 1).

Impaired trafficking and locoregional delivery

Impaired trafficking to the tumour site may also limit CAR T cell efficacy, especially in solid tumours. In preclinical models of central nervous system tumours, intratumoral or intracerebroventricular (ICV) administration has improved therapeutic benefit, with an approximate tenfold lower regional dose being required to achieve the same efficacy as intravenous administration[92–94]. Several clinical trials have demonstrated the safety of locoregional delivery of CAR T cells into the central nervous system[27,28,95] and in a patient with glioblastoma multiforme, ICV delivery of IL-13Rα2 CAR T cells induced a complete

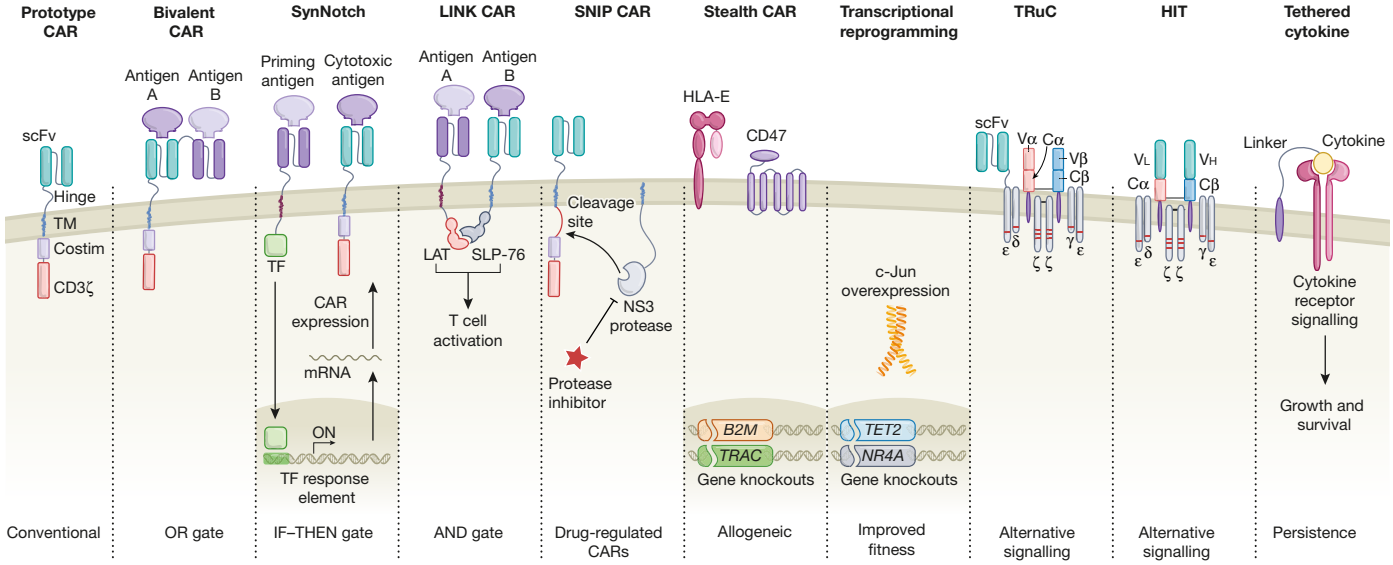

Fig. 3 | Next-generation platforms. Investigators have leveraged numerous bioengineering strategies to develop advanced CAR platforms to improve the safety and efficacy of immune cell therapies. Multispecific (bivalent) CARs, which operate as an OR gate, may be able to overcome obstacles related to tumour heterogeneity and antigen loss, whereas combinatorial antigen sensing systems such as SynNotch or LINK CAR increase the specificity of CAR T cells by requiring two antigens for activity. Safety switches such as drug-regulated and adapter CARs could mitigate CAR-induced toxicities and enhance efficacy by tuning signalling. Knockout of *TRAC*, *B2M* and *CIITA* genes ablates TCR, MHC class I and MHC class II expression, respectively, and human leukocyte antigen (HLA)-E and CD47 overexpression shield stealth CAR T cells from natural killer cell- and macrophage-mediated rejection. Deletion of *NR4A* and *TET2* genes or ectopic overexpression of c-Jun results in transcriptional rewiring that renders T cells exhaustion resistant. By fusing a scFv domain to TCR subunit, TRuC and HIT receptors redirect the specificity of the endogenous receptor and exhibit increased antigen sensitivity compared with CARs, while secreting lower levels of cytokine. Integration of a cytokine gene in the CAR vector provides a growth and survival signal to improve the persistence of T cells. Costim, costimulatory domain; TF, transcription factor; TM, transmembrane domain.

response, whereas intratumoral administration was not effective[27]. In a study of patients with diffuse midline gliomas, ICV delivery of GD2-CAR T cells induced antitumour effects and clinical responses, and repeated dosing was associated with sustained benefit, raising the prospect that delivery to the central nervous system may abrogate immune sensitization, which has probably limited the effectiveness of multidose intravenous CAR T cell regimens[28,96,97]. In patients with lung cancer involving the pleura, regional delivery of mesothelin-CAR T cells in combination with PD-1 blockade mediated stable disease and metabolic responses[98]. Cell-intrinsic strategies to improve T cell homing to and persistence in the TME, such as secretion of IL-7 and CCL19[99], are also being explored.

The next generation of CAR T cells

The various next-generation platforms being used to overcome tumour resistance mechanisms, augment immune cell fitness, improve specificity, tune CAR signalling, enhance safety, and increase antigen sensitivity are discussed in this section.

Platforms to diminish antigen escape

Bispecific CAR targeting may be achieved by administration of a mixed cell product, bicistronic expression of two receptors, two scFvs incorporated into a single receptor[100], or co-transduction of multiple CARs, with each approach presenting opportunities and challenges. Co-infusion is financially, labour- and cell-intensive and co-infusion and co-transduction generate heterogeneous products, risking the emergence of a subpopulation that dominates the pool of cells after infusion[101,102]. Bicistronic vectors may result in reduced protein expression, and in one clinical trial, a bicistronic construct demonstrated limited persistence[103]. Several trials with bispecific receptors targeting CD19 plus CD20 or CD22 have been reported[45,104,105], and in one, the receptor mediated diminished potency toward CD22 and tumour cell variants exhibiting low or no surface expression of CD19 emerged[45]. Cilta-cel, a BCMA-CAR recently approved by the FDA, incorporates two tandem VHH binders in one receptor, which binds two different epitopes on BCMA. Clinical results with cilta-cel demonstrate a sCR of 83% and 55% PFS at 27 months, the highest reported so far using CARs for multiple myeloma[14,106] (Table 1). In summary, clinical data with multispecific CARs is nascent but demonstrates safety and promise for improved efficacy by diminishing antigen escape.

Novel receptors designed to lower the antigen density threshold are also being developed. Katsarou and colleagues have expressed a chimeric costimulatory receptor (CCR), which lacks a CD3ζ domain, in *trans* with a prototype CAR, and reported that CCR engagement activated the prototype CAR at very low antigen density, preventing antigen low escape in preclinical models[107]. Induction of antitumour responses toward non-CAR T cell antigens—as reported following CAR T cell therapy in a patient with rhabdomyosarcoma—could diminish resistance due to antigen modulation[54]. Several approaches are under development to augment innate and adaptive immunity (Box 1), including CAR-mediated delivery of the immunostimulatory RNA RN7SL1[108], coexpression of ligands or cytokines that reshape the TME such as IL-12[109,110], IL-18[111], CD40L[112] or Flt3L[113], engineering CAR T cells to secrete bispecific T cell engagers (BiTEs), taking advantage of CAR T cell accumulation within the tumour site and avoiding systemic toxicity of the BiTE[114], or using non-traditional immune cells that may mediate more potent endogenous antitumour activity.

Enhancing T cell potency

Extensive work is underway to enhance immune cell fitness (Fig. 3 and Box 1). Significant effort is focused on epigenetic modulation, in part on the basis of an exceptional responder in a clinical trial of CD19-CAR for CLL—in which lentiviral integration disrupted the *TET2* gene, a mediator

of DNA methylation, resulting in substantial clonal T cell proliferation and a sustained antitumour response[115]. Similarly, knockout of the *DNMT3A* gene enhances the antitumour activity of CAR T cells in preclinical models[116]. Overexpression of transcription factors to prevent exhaustion has also shown promise, including overexpression of the AP-1 factor JUN, which enhances T cell expansion and persistence, diminishes terminal differentiation and lowers the antigen density threshold, presumably owing to increased signal strength[53]. Similarly, overexpression of BATF transcription factors has been reported to enhance T cell potency[117]. Manufacturing strategies are being developed to optimize CAR T cell phenotype towards stem-like and central memory subsets, including shorter culture duration[118], inhibition of PI3K–mTOR–AKT[119], BTK[120] or tyrosine kinase[121] signalling, and culture in memory-promoting cytokines[122].

CRISPR-mediated gene editing was first applied clinically in the setting of adoptive T cell therapy, in which PD-1 was deleted from cells engineered to express NY-ESO-1, a cancer-specific TCR transgene[123]. The engineered cells did not demonstrate enhanced persistence or potency, but the study demonstrated the feasibility and safety of the approach, and accelerated efforts to apply gene editing technologies to enhance immune cell therapies. Several genes have been identified as candidates for editing to enhance T cell fitness[72,124–133] (Box 1), and CRISPR-mediated disruption of T cell markers such as CD7 and CD5 has enabled CAR T cell therapies for T cell malignancies, while avoiding CAR T cell lysis[134,135] (termed 'fratricide'). We anticipate increasing clinical trial activity incorporating gene-edited immune cells into adoptive immune cell therapy platforms to enhance their potency, expand the landscape of targetable antigens and avoid immune sensitization.

To enhance persistence, some investigators have sought to integrate cytokine signals into the CAR receptor or express cytokines in trans[136,137], including a clinical trial in which CAR-expressing natural killer cells transgenically expressing IL-15 demonstrated prolonged persistence[138]. Immune rejection may also limit CAR T cell persistence as anti-CAR immune responses—often targeting mouse, humanized or fully human scFvs—can be measured in many patients[29,97,139,140]. Consistently, clinical experience demonstrates the limited utility of second and subsequent intravenous CAR T cell doses, which can be improved using enhanced lymphodepleting regimens[96]. These findings raise the prospect that stealth platforms—which are currently being developed to enable off-the-shelf allogeneic products (discussed in 'Platforms to enhance access and efficacy')—could enhance CAR T cell efficacy by enhancing persistence or enabling multiple CAR T dosing regimens.

Diverse efforts are underway to address the suppressive TME (Box 1), including genetic ablation or expression of dominant-negative TBGβ[141,142], PD-1[143,144] or Fas receptors[145], and engineering CAR T cells to secrete checkpoint-blocking scFvs[146]. Some investigators have engineered switch receptors, fusion proteins that convert a suppressive signal within the TME to an activating signal in the CAR T cells[147,148]. Whether tonic activating signals induced by such receptors result in long-term CAR T cell enhancement or predispose them to exhaustion and terminal differentiation remains to be determined. Biomaterials-based approaches for enhancing the expansion and persistence are also being explored[149].

CAR tuning and regulatable platforms

Substantial efforts are underway to enhance safety and potency by tuning or dampening CAR signalling to diminish toxicity and exhaustion. This concept was first proposed by Eyquem and colleagues, who used CRISPR to knock-in CAR receptors into the *TRAC* locus and observed improved potency and diminished exhaustion due to antigen-induced CAR downregulation mediated by endogenous *TRAC* regulatory elements[150]. Weber and colleagues extended this principle using synthetic biology or small molecules to transiently cease CAR signalling, which enhanced CAR T cell potency when used during manufacturing and improved antitumour effects when applied in vivo after adoptive transfer[121].

Kill switches such as iCasp9[151], HSV tyrosine kinase[152] (HSV-tk) and epitope tags[153] enable the depletion of engineered cells in the event of severe toxicity, and a transgene-free safety switch that renders T cells auxotrophic for uridine has been developed[154]. Regulatable platforms can serve as reversible safety switches and also tune CAR signalling, thereby enhancing T cell potency by providing rest periods that prevent T cell exhaustion[121]. Numerous regulatable platforms have been developed using drug-sensitive promoters[155], induced dimerization[156,157], disruption of split CARs[158], drug-dependent activation of binders[159], proteolysis-targeting chimeras[160] (PROTACs), chemically-dependent degron domains[121,157,161] and drug-regulated CAR proteolysis[162,163]. These systems represent significant advances in synthetic biology, but remain challenged by leaky activity in the OFF state that risks toxicity, diminished CAR expression or activity in the ON state and the use immunosuppressive drugs as regulators[121,155–161]. Labanieh et al. recently developed a protease-regulated grazoprevir-induced 'drug ON' platform, signal neutralization by an inhibitable protease (SNIP), which shows no leaky activity and full functional capacity[162] (Fig. 3). Similar to synNotch[164], SNIP demonstrates superior antitumour efficacy compared with conventional CAR T cells owing to reduced exhaustion, and in an on-target off-tumour ROR1 toxicity model, decreased grazoprevir dosing tuned SNIP CARs to open a therapeutic window in which healthy tissue was spared but ROR1-expressing tumour cells were killed[162]. Similarly, Hernandez-Lopez et al. iterated the synNotch platform to target very highly expressed tumour antigens while avoiding lower levels of the antigens on normal tissues[165]. Thus, regulatable CARs show promise for enhancing efficacy and diminishing toxicity.

Enhancing specificity through Boolean logic

B cell and plasma cell malignancies are especially suited to CAR T cell therapy owing to the high, homogenous expression of lineage antigens that are co-expressed predominantly on B cells and plasma cells, the depletion of which is tolerable. However, a recent case report showed the development of parkinsonism in a patient after BCMA-CAR T cell therapy, with postmortem analysis revealing expression of BCMA on subsets of neurons and astrocytes in the patient's basal ganglia[166]. In another study, single-cell RNA-sequencing analysis showed the expression of CD19 on brain mural cells, raising the prospect that on-target killing may be responsible for neurotoxicity after CD19-CAR T cell therapy. These results highlight the challenge of identifying targets that are not expressed on vital tissue.

So far, the paucity of tumour-specific surface targets on solid tumours has limited the application of the CAR prototype to solid tumours, with unacceptable off-tumour, on-target toxicity having been observed in trials of CARs targeting CAIX[167] and CEACAM5[168]. However, several clinical trials of CAR T cells and other potent antibody-directed therapies have demonstrated good safety profiles in solid tumours (Table 1). The high CAR antigen density threshold is likely to explain the safe targeting of some antigens with known expression on vital tissues—such as GD2, which is expressed at low levels on neural tissues[169,170]. A recent trial demonstrated promising clinical activity of claudin-18.2-CARs was associated with significant but non-dose-limiting toxicity, potentially explained by antigen expression restricted to differentiated epithelial cells buried in gastric mucosa that may be less accessible to CAR T cells[29]. Identifying additional molecules with sufficient differential expression levels for safe targeting, such as oncofetal cell-surface targets is essential for expanding the reach of CAR T cells beyond B cell and plasma cell malignancies. However, the safety of specific targets will need to be continually reassessed as potency and persistence enhancements are deployed, as in recent studies with a PSMA-targeted CAR integrating a dominant-negative TGFβ receptor that was associated with lethal toxicity[171,172].

Next-generation receptors incorporating logic gates could allow better discrimination between tumour and healthy tissue through combinatorial antigen sensing, and expand the repertoire of potential

antigens (Fig. 3). Roybal et al. developed synNotch, an IF–THEN circuit incorporating a synthetic notch receptor against antigen A, which upon engagement, triggers the transcription of a conventional CAR against antigen B[173,174]. The synNotch system has not been tested clinically, but in preclinical models it prevented on-target, off-tumour toxicity when tumours and susceptible vital tissues are not colocalized[175]. Tousley et al. developed an AND gate platform called LINK, which utilizes the proximal TCR signalling molecules LAT and SLP76, each fused to a membrane-bound scFv specific for a unique antigen[176]. Engagement of both antigens colocalizes LAT and SLP76, leading to T cell activation. In an on-target, off-tumour ROR1 toxicity model, LINK CAR T cells cured mice of tumours expressing both antigens without ROR1-mediated toxicity, whereas mice treated with synNotch T cells succumbed to toxicity[175]. Other approaches for combinatorial antigen targeting that are under development include SUPRA[177] and co-LOCKR[178], which redirect CAR T cell specificity through protein switches. Although combinatorial antigen sensing could expand the landscape of targetable tumour antigens, the increased risk of tumour escape owing to loss of either antigen is a potential concern. An alternative approach to enhance specificity is to use an AND NOT gate, in which a prototype activating CAR is expressed in *trans* with an inhibitory CAR (iCAR) targeting an antigen that is expressed on healthy tissue but not on tumour tissue[179–181]. Limited engineering with NOT gates has been undertaken so far and these applications have not been tested clinically.

TCR-like CARs

With the goal of targeting antigens that are expressed at low levels, HLA-independent TCRs (HIT) are designed with the variable domain of the endogenous TCR being altered to target scFvs by gene editing the endogenous *TRAC* locus[182]. When CD80 and 4-1BBL are provided in *trans*, CD19-directed HITs display superior antigen sensitivity compared with prototype CD19-CARs (Fig. 3). Synthetic TCR and antigen receptors (STARs) have a similar design but are not knocked in to the *TRAC* locus; thus, the endogenous TCR specificity is retained[183]. Other approaches for redirecting TCR specificity include the antibody–TCR (AbTCR) platform[184], which replaces the variable domains of TCRγδ with a Fab fragment and TCR fusion constructs[185] (TRuC), which fuse an scFv to a CD3 subunit. A recent comparison of TCR-like chimeric receptors showed that STAR and HIT receptors reproduce TCR antigen sensitivity, whereas TruCs do not[186]. One potential drawback of CAR T cells compared with native T cells is the inability to target intracellular antigens, since most aberrant proteins that drive cancer are intracellular. Yarmarkovich et al. overcame this by developing a prototype CAR with specificity for peptides presented by MHC[187] (pMHC). Using scFv binders specific for a PHOX2B peptide–MHC overexpressed in neuroblastoma, they targeted pMHCs across several HLA allotypes. This strategy could greatly expand the landscape of CAR targets, including key oncogenic drivers.

Platforms to enhance access and efficacy

Diverse approaches are under exploration to increase access of cell therapies, diminish the high manufacturing costs, create stealth immune cells resistant to rejection, and leverage the unique properties of alternative immune cells.

Distributed manufacturing and allogeneic products

Engineering advances have yielded automated closed-system manufacturing, which is providing opportunities for point-of-care manufacturing to diminish the costs, delays and logistical challenges associated with the centralized manufacturing models that are the industry standard. A recent multicentre trial demonstrated the safety and efficacy of cells manufactured at the point of care[188]. Defining the regulatory requirements for point-of-care manufacturing is an area of significant current interest, especially for therapies targeting rare indications, such as paediatric cancers[189].

Allogeneic CAR T cells manufactured from healthy 'super donors' could improve potency by avoiding preexisting T cell dysfunction and decrease the cost and logistical challenges of manufacturing, thereby enhancing access. However, allogeneic T cell therapies must overcome the risk of GVHD mediated by the TCR and rejection of the transferred cells by the host immune system. Gene editing of the endogenous TCR eliminates the risk of GVHD[190], but endowing stealth properties to avoid immune rejection remains a significant challenge, since $CD8^+$, $CD4^+$, natural killer and macrophage cells can reject allogeneic cells and each are regulated by distinct axes, necessitating multiple enhancements (Box 1). Knockout of β_2-microglobulin can eliminate HLA class I surface expression, but paradoxically increases the risk of rejection by natural killer cells. Additional strategies for inducing allogeneic tolerance include knockout of the *CIITA* gene to ablate MHC class II expression[191], and overexpression of HLA-E[192] and CD47[193] to ameliorate natural killer cell- and macrophage-mediated cell rejection.

Many allogeneic approaches use CRISPR–Cas9, and the risks of CRISPR-based mutagenic events could be magnified when producing hundreds or thousands of allogeneic products with a singular manufacturing process. Alternative platforms, such as base editing or prime editing may emerge as preferred alternatives to nuclease-based genome editing since they probably involve lower risk owing to an absence of double strand DNA breaks[194]. CRISPR–Cas systems targeting RNA could also provide opportunities for multiplexed gene knockdowns with greater specificity and efficiency compared with RNA-mediated interference. Although allogeneic donor-derived cells containing multiple gene edits could provide significant advantages, these technologies are nascent and their toxicity profiles remain unknown. Some groups have attempted to prevent immune rejection by augmenting immune suppression of the host using conventional chemotherapy or immunosuppressive antibodies for which the targets are edited from the CAR T cells[190]. Early response rates with this approach are promising, but long-term safety and efficacy have not been demonstrated and concerns remain regarding infectious risks associated with intensive immune-depleting regimens[190].

Alternative immune cells

Several non-T immune cells, including natural killer cells, invariant natural killer T (iNKT) cells, γδ T cells and macrophages exhibit innate antitumour activity and do not induce GVHD, raising the prospect that they could provide an off-the-shelf source of cells with reduced toxicity, enhanced tumour trafficking and/or target antigen-negative variants through innate tumour recognition. However, allogeneic innate immune cells remain susceptible to rejection, raising concerns regarding the durability of their effects if they are not engineered for stealth. Cord blood-derived allogeneic natural killer cells incorporating ectopically expressed IL-15 have shown promise in a phase I trial for NHL and CLL[138]. iNKT-CAR cells mediated activity in mouse models, in part by cross-priming host CD8 cells towards tumour antigens[195], and their safety and feasibility in a phase I trial for neuroblastoma have been demonstrated[196]. γδ T cells engineered to express a CD20-CAR have also shown impressive activity in early studies[197]. Expressing CARs in macrophages requires substantial adaptations of vectors and signalling domains, but antitumour effects associated with augmented phagocytosis, modification of the TME and recruitment of T cells[198,199] have been demonstrated in preclinical models[198], with CD3ζ-based CARs demonstrating equivalent phagocytic activity as Fcγ-based CARs Efforts are also underway to create induced pluripotent stem (iPS) cell-derived CAR T cells[200], natural killer cells[201] and macrophages[202]. The differentiation of iPS cells to natural killer cells has been particularly successful, and clinical testing of these off-the-shelf therapies is currently in progress[203], whereas iPS cell differentiation to fully functional T cells has been more challenging[204]. Given their nearly inexhaustible

expansion potential, iPS cell-derived products could enable mass production of a homogenous cell product integrating numerous enhancements to endow stealth properties, safety switches and potency, and the long-term safety and efficacy results of these emerging platforms are thus eagerly anticipated.

Next-generation gene delivery

Viral vector-based gene delivery has been the gold standard in the field, but vector production and qualification is costly and time consuming. Viral-free platforms for gene delivery are under development, with CRISPR-based gene delivery in human T cells demonstrating proof of principle, although DNA templates are toxic to T cells and the efficiency of this approach remains lower than with viral vectors[205]. Clinical feasibility has been demonstrated with a CD19-CAR site-specifically delivered to the PD-1 locus inducing a high CR rate in NHL, although the manufacturing process did not meet dose requirements for a relatively high proportion of patients[206]. Modifications to DNA templates and small-molecule inhibitor cocktails are improving knock-in efficiencies and cell yields[207]. Transposon-based gene delivery has also been used, although malignant transformation of CAR-engineered T cells was reported in two patients associated with high-copy-number integration using a Piggybac transposon platform[208]. In vivo gene delivery is another emerging approach that could improve accessibility and diminish cost. In this approach, DNA or RNA is delivered systemically using viral vectors[209] or nanoparticles[210] that preferentially target and transduce immune populations in vivo. Immunogenicity could prohibit repeat administration of viral vectors owing to the induction of neutralizing antibodies. Stable expression of a CD19-CAR has been demonstrated using lipid nanoparticles targeting CD3 in mice[210] and T cell-targeted lipid nanoparticles incorporating optimized RNA diminished cardiac fibrosis in a mouse model[211].

CAR therapy for non-malignant diseases

The CAR T platform has been optimized for cancer treatment, but the design principles and expansive synthetic biology toolbox used for CAR T cells are providing opportunities to extend this therapeutic approach to non-malignant diseases, including autoimmunity, senescence, fibrosis and infectious diseases. In preclinical studies, CD19-CAR T cells have demonstrated beneficial effects in systemic lupus erythematosus[212], and a case study reported sustained activity of CD19-CAR therapy in a patient with refractory lupus nephritis[213]. Chimeric autoantibody receptors (CAARs) are prototype CARs that incorporate a scFv targeting the idiotype of an autoreactive B cell clone or use autoantigens as the recognition domain. In preclinical studies, CAARs mediated therapeutic effects against pemphigus vulgaris, and clinical testing is underway. Adoptive transfer of T regulatory (T_{reg}) cells, which mediate suppression rather than cytotoxicity, is an alternative approach for treating autoimmunity. Non-engineered T_{reg} cells have demonstrated activity in mouse models of GVHD, allograft transplantation, type 1 diabetes, systemic lupus erythematosus and multiple sclerosis, and early clinical data demonstrate feasibility of manufacturing and a good safety profile[214]. Compared with non-engineered cells, T_{reg} cells expressing a CAR targeting antigens expressed on the diseased tissues show enhanced specificity and potency[215,216]. Recent data have demonstrated that inadvertent expansion of CAR T_{reg} cells limits the efficacy of commercial CAR T cells, providing proof-of-concept for the utility of CAR-engineered T_{reg} cells[217,218]. Approaches are underway to engineer FOXP3 expression to enforce lineage stability and incorporate safety switches to diminish risk[214]. Recent promising preclinical data were generated in haemophilic mice, in which T_{reg} cells expressing a factor VIII-targeted CAR and FOXP3 prevented the development of neutralizing anti-factor VIII antibodies[219]. Senolytic CAR T cells targeting urokinase-type plasminogen activator receptor have been demonstrated to target senescent cells in vitro and restore tissue homeostasis in models of liver fibrosis[220]. CARs targeting fibroblast activation protein (FAP) have improved cardiac function in a mouse model of cardiac fibrosis[221] and in vivo generation of FAP-CARs using CD5-directed lipid nanoparticles loaded with mRNA also demonstrated benefit[211]. In this model, the non-integrating nature of mRNA ensured that CAR expression was transient, thereby mitigating the risk of toxicity associated with widespread elimination of activated fibroblasts.

Outlook

Adoptive immune cell therapy is established as a transformative therapeutic modality. The past decade has witnessed significant progress in understanding the biology of prototype CAR T cells, identifying antigen modulation and T cell dysfunction as major resistance mechanisms and highlighting the logistical challenges of delivering cell therapies to all patients who could benefit. Modifications to prototype CARs can augment their potency, but increasingly investigators are designing next-generation platforms to create advanced cellular therapies that incorporate a diverse array of enhancements. The fields of immunology, synthetic biology, genetic engineering and cell manufacturing are synergizing to create smarter, safer and more accessible cellular therapies that are poised for increased efficacy and access, diminished risk and cost, and broader utility, for the treatment of cancer as well as non-malignant diseases.

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Acknowledgements C.L.M. and L.L are members of the Parker Institute for Cancer Immunotherapy, which supports the Stanford University Cancer Immunotherapy Program. This work was supported by the St Baldrick's Foundation Empowering Pediatric Immunotherapies for Childhood Cancer (EPICC) Team and NCI 5P30CA124435 (C.L.M.). L.L. was supported by a Siebel Scholars Fellowship, Stanford Graduate Fellowship, National Science Foundation Graduate Research Fellowship (DGE-1656518), and Discovery Innovation Award philanthropically supported through the Biomedical Innovation Initiative.

Author contributions L.L. and C.L.M. conceptualized, wrote and edited the manuscript.

Competing interests L.L. and C.L.M. are inventors on several patents related to CAR T cell therapies. C.L.M. is a cofounder of Lyell Immunopharma, CARGO Therapeutics and Link Cell Therapies, which are developing CAR-based therapies, and consults for Lyell, Syncopation, Link, Apricity, Nektar, Immatics, Ensoma, Mammoth, Glaxo Smith Kline and Bristol Myers Squibb. L.L. is a cofounder of, consults for, and holds equity in CARGO Therapeutics. L.L. is a consultant for and holds equity in Lyell Immunopharma.

Additional information

Correspondence and requests for materials should be addressed to Crystal L. Mackall.

Peer review information *Nature* thanks Justin Eyquem, Kristen Hege, Hans Stauss and Jie Sun for their contribution to the peer review of this work.