Natural killer cells in antitumour adoptive cell immunotherapy

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Abstract | Natural killer (NK) cells comprise a unique population of innate lymphoid cells endowed with intrinsic abilities to identify and eliminate virally infected cells and tumour cells. Possessing multiple cytotoxicity mechanisms and the ability to modulate the immune response through cytokine production, NK cells play a pivotal role in anticancer immunity. This role was elucidated nearly two decades ago, when NK cells, used as immunotherapeutic agents, showed safety and efficacy in the treatment of patients with advanced-stage leukaemia. In recent years, following the paradigm-shifting successes of chimeric antigen receptor (CAR)-engineered adoptive T cell therapy and the advancement in technologies that can turn cells into powerful antitumour weapons, the interest in NK cells as a candidate for immunotherapy has grown exponentially. Strategies for the development of NK cell-based therapies focus on enhancing NK cell potency and persistence through co-stimulatory signalling, checkpoint inhibition and cytokine armouring, and aim to redirect NK cell specificity to the tumour through expression of CAR or the use of engager molecules. In the clinic, the first generation of NK cell therapies have delivered promising results, showing encouraging efficacy and remarkable safety, thus driving great enthusiasm for continued innovation. In this Review, we describe the various approaches to augment NK cell cytotoxicity and longevity, evaluate challenges and opportunities, and reflect on how lessons learned from the clinic will guide the design of next-generation NK cell products that will address the unique complexities of each cancer.

Adoptive cell therapy using engineered immune effectors is a promising new approach to treat haematological and solid malignancies for which treatment options are limited[1–9]. Autologous chimeric antigen receptor (CAR) T cell therapy, the first to enter clinical translation and commercialization, has led to remarkable improvements in patients with aggressive B cell malignancies, producing long-term sustained remissions in many cases. However, despite these successes, challenges remain. The complex manufacturing process of CAR T cell therapy increases costs and lengthens the vein to vein time, thus representing an obstacle for patients who, owing to rapidly progressing disease, are in critical need of treatment. Furthermore, the requirement for the patient's own cells as the source material restricts eligibility, as many patients are often heavily pretreated and lymphopenic, and may not have sufficient cells to yield a viable product. In the clinic, CAR T cell-related cytokine-release syndrome (CRS) and neurotoxicities represent an additional concern, requiring inpatient monitoring. Given these limitations, there is growing interest in exploring cell sources that are off the shelf and could be applied universally. Because T cells recognize and mediate a response against non-self via the T cell receptor (TCR), use of CAR T cells in the allogeneic setting requires additional genetic editing steps to remove the TCR to mitigate the risk of graft-versus-host disease (GvHD). Natural killer (NK) cells, on the other hand, recognize their targets in a human leukocyte antigen (HLA)-unrestricted manner and thus do not present these same risks, making them attractive candidates for universal cellular immunotherapy[10]. NK cell effector function is controlled by a complex array of activating and inhibitory receptors that can distinguish between healthy and 'stressed' cells[11]. The cumulative cues resulting from receptor–ligand engagements determine whether NK cells deliver a 'kill' or 'not kill' signal. Healthy cells are spared via recognition of self-major histocompatibility complex (MHC) class I molecules which bind inhibitory killer cell immunoglobulin-like receptors (KIRs) that signal to stop NK cell function. By contrast, through the mechanism of missing-self recognition, NK cells attack abnormal self-cells, such as tumour cells, which downregulate expression of MHC class I molecules in an attempt to evade T cell responses, and upregulate activating ligands that are induced by stress such as DNA damage or malignant transformation[12–16].

Autologous chimeric antigen receptor (CAR) T cell therapy
A patient-specific cellular therapy in which the patient's own T cells are genetically modified to express a chimeric antigen receptor.

Lymphopenic
A lower than normal number of lymphocytes in the blood.

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These various attributes provide NK cells with unique advantages for allogeneic therapeutic applications. With the accelerated development of innovative strategies and the emergence of next-generation technologies that allow for deeper biological investigations, various NK cell products can be designed for cancer treatment. In this Review, we describe how these unique properties of NK cells are leveraged for adoptive NK cell immunotherapy, and provide an overview of the evolving engineering strategies to augment NK cell potency and persistence, as well as the efforts to redirect NK cell specificity to tumours through next-generation CAR molecules, engineered TCR and pre-complexing with cell engagers. Lastly, we conclude with a perspective on the challenges and opportunities ahead as we confront solid tumours and safeguard immune effector cells from the suppressive pressures within the tumour microenvironment, while also devising strategies to monitor and mitigate inadvertent safety concerns.

Biological properties of NK cells

Despite the successes of engineered T cell immunotherapies[1–8], the clinical benefit has been limited to a fraction of patients and a few indications, thus highlighting the need for new strategies. Leveraging innate immunity to broaden the scope of antitumour responses is an attractive option. Within the innate immune system, NK cells are specialized immune effector cells, and are suspected to have a role in tumour immunosurveillance[10], as suggested by the correlation of low NK cell activity with increased cancer susceptibility and higher risk of metastasis observed in both preclinical and clinical studies[17–19]. NK cells develop from $CD34^+$ progenitor cells in the bone marrow, although it is as yet unclear whether they arise from a unique set of precursor cells or from multipotent progenitors that also give rise to T lymphocytes, B lymphocytes and myeloid cells[20]. Unlike T cells and NKT cells, NK cells lack expression of the clonotypic TCR and the associated CD3 complex responsible for signal transduction. NK cells are generally classified under a dichotomous distribution based on the relative expression of surface proteins CD56 and CD16: $CD56^{bright}CD16^{low/-}$ (immunomodulatory, cytokine-producing) and $CD56^{dim}CD16^+$ (cytotoxic)[21]. Recent advancements in high-parameter cytometry and single-cell proteo-genomics, however, have led to the understanding that NK cells may, in fact, exhibit greater phenotypic heterogeneity that extends beyond these two subsets, giving rise to diverse cell populations endowed with varying functional properties[22].

NK cells possess strong cytotoxicity and, upon forming immunological synapses with targets, elicit a potent response through the release of cytolytic granules and cytotoxic cytokines[23]. Moreover, they can recognize antibody-coated cells through their FcγRIIIA (CD16) receptor and trigger antibody-dependent cellular cytotoxicity (ADCC) and cytokine production[24]. NK cells have also been described as 'immune-regulatory' because of their ability to produce an array of cytokines and chemokines, through which they help shape B cell and T cell responses, and impact the function of dendritic cells, macrophages and neutrophils[11]. This broad range of attributes reveals the sophisticated network of biological mechanisms associated with NK cell function and supports the value of NK cells for immunotherapy.

Memory-like function in NK cells. Early studies reported memory-like responses by NK cells in mouse models of cytomegalovirus infection[25,26], a behaviour not typically associated with innate immune cells. In these studies[26,27], mouse NK cells, when stimulated with a combination of IL-12 and IL-18, acquired a functional phenotype characterized by increased production of IFNγ. Interestingly, after a resting phase, these cells were able to reactivate upon cytokine stimulation or engagement of activating receptors and exhibited an enhanced IFNγ response resembling the memory-like properties of adaptive immune cells. Later, Todd Fehniger's group hypothesized that human NK cells should, likewise, be endowed with memory-like properties. Consistent with this hypothesis, their study demonstrated that human NK cells, preactivated with IL-12, IL-15 and IL-18, followed by 1–3 weeks rest, were able to generate a robust response driven by enhanced IFNγ production upon subsequent exposure to cytokines or to K562 leukaemia cells[28]. Since then, many more groups have described similar memory-like function in various immunological settings, including observations of such responses in humans[29,30].

NK cell source and donor selection

In patients with cancer, NK cells typically display a dysfunctional phenotype marked by altered gene expression profiles and reduced cytotoxic function[31,32], thus diminishing the feasibility of autologous NK cell therapy applications. Moreover, autologous manufacturing platforms are cumbersome and may limit accessibility if patients are not able to provide sufficient cells to undergo downstream processing and engineering.

Because allogeneic NK cells do not cause GvHD, current NK cell therapy programmes rely largely on allogeneic sources to avoid the incumbrances associated with autologous approaches. There are various sources from which NK cells can be derived (FIG. 1), namely peripheral blood mononuclear cells[28–30], cord blood[28–30,33–37], immortalized cell lines[38–40], haematopoietic stem and progenitor cells (HSPCs)[33,34,41] and induced pluripotent stem cells (iPSCs)[42,43]. All sources can provide clinically meaningful cell doses, are amenable to CAR receptor engineering and have transitioned into in-human studies. They, nevertheless, come with unique advantages and challenges, and may possess different underlying transcriptional, phenotypic and functional properties[44].

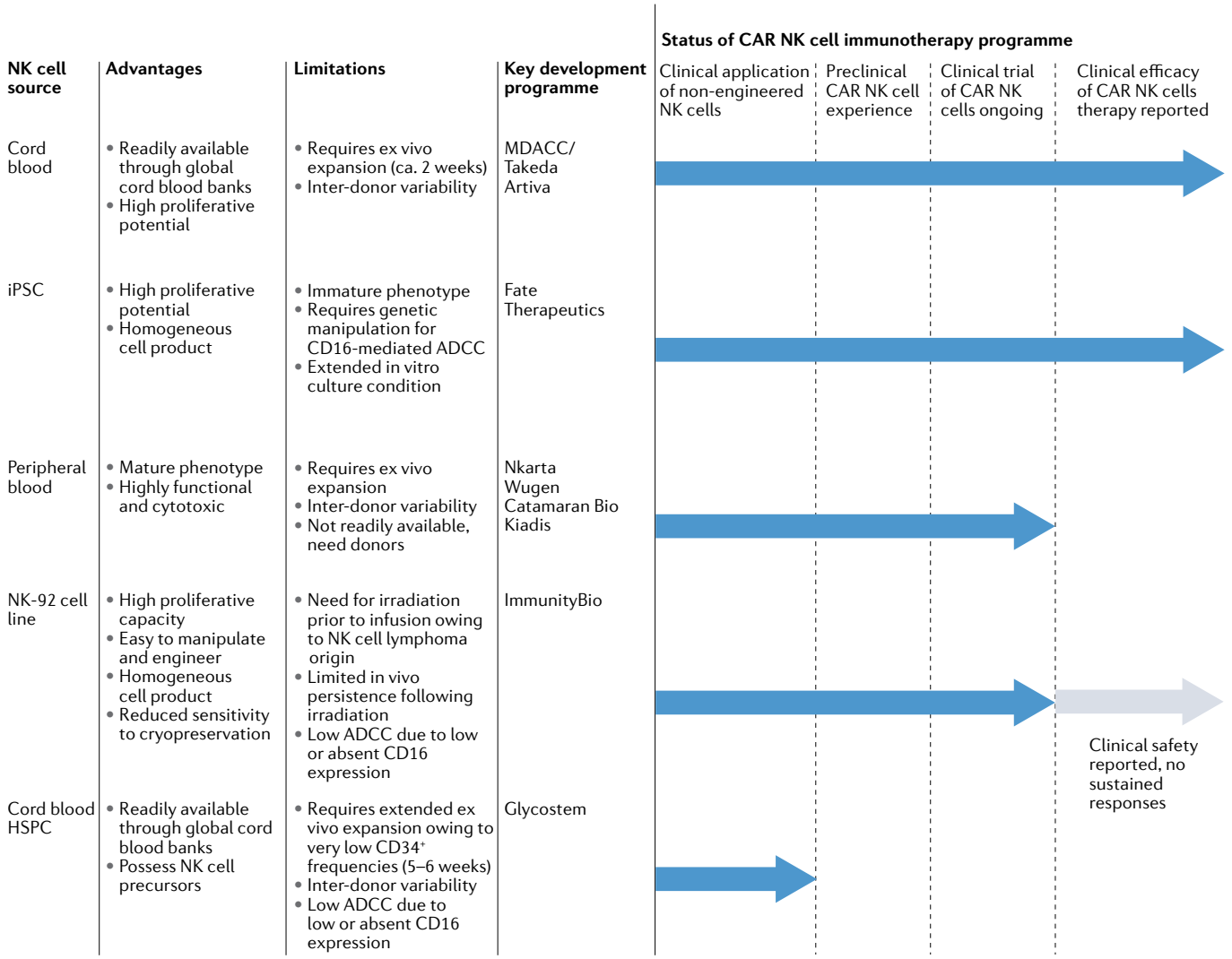
NK-92, the first NK cell-based immunotherapy to receive Investigational New Drug approval by the US Food and Drug Administration (FDA) for clinical testing, is a homogeneous, immortalized NK lymphoma cell line that can be expanded ex vivo to achieve large cell numbers[38] (TABLE 1). NK-92 cells lack expression of most KIRs and are thus less likely to become inhibited, which makes them attractive for cell therapy use[38]. However, their cancerous origin raises safety concerns, and irradiation of NK-92-derived cell products prior to patient administration is required, which, in turn, can

Allogeneic setting
The therapy setting in which adoptive cell therapies are generated from material obtained from a different individual of the same species.

Graft-versus-host disease
(GvHD). A potentially fatal condition that results from the response of allogeneic T cells against the host tissues of recipients who are immunosuppressed, which can occur after adoptive cell therapy or allogeneic haematopoietic stem cell transplantation.

Killer cell immunoglobulin-like receptors
(KIRs). A family of polymorphic activating and inhibitory transmembrane proteins that regulate natural killer cell development and function through interactions with major histocompatibility complex class I molecules.

Induced pluripotent stem cells
(iPSCs). Cells that result from the reprogramming of adult somatic cells into an embryonic-like pluripotent stem cell state, capable of self-renewal and differentiation into tissues originated from the three germ layers (endoderm, mesoderm and ectoderm).



NK cell source	Advantages	Limitations	Key development programme
Cord blood	• Readily available through global cord blood banks • High proliferative potential	• Requires ex vivo expansion (ca. 2 weeks) • Inter-donor variability	MDACC/ Takeda Artiva
iPSC	• High proliferative potential • Homogeneous cell product	• Immature phenotype • Requires genetic manipulation for CD16-mediated ADCC • Extended in vitro culture condition	Fate Therapeutics
Peripheral blood	• Mature phenotype • Highly functional and cytotoxic	• Requires ex vivo expansion • Inter-donor variability • Not readily available, need donors	Nkarta Wugen Catamaran Bio Kiadis
NK-92 cell line	• High proliferative capacity • Easy to manipulate and engineer • Homogeneous cell product • Reduced sensitivity to cryopreservation	• Need for irradiation prior to infusion owing to NK cell lymphoma origin • Limited in vivo persistence following irradiation • Low ADCC due to low or absent CD16 expression	ImmunityBio
Cord blood HSPC	• Readily available through global cord blood banks • Possess NK cell precursors	• Requires extended ex vivo expansion owing to very low $CD34^+$ frequencies (5–6 weeks) • Inter-donor variability • Low ADCC due to low or absent CD16 expression	Glycostem

Fig. 1 | **Advantages and limitations arising from different sources of NK cells.** Natural killer (NK) cells can be derived from several different sources, each of which presents its own advantages and potential challenges. Chimeric antigen receptor (CAR) NK cells have successfully been engineered from different platforms including cord blood[35,37], peripheral blood[52,112,264], NK-92 cells[39,265–274] and induced pluripotent stem cell (iPSC)-derived NK cells[43,48,49]. ADCC, antibody-dependent cellular cytotoxicity; HPSC, haematopoietic stem and progenitor cells; MDACC, University of Texas MD Anderson Cancer Center.

negatively impact their long-term in vivo persistence and overall therapeutic potential[37]. Another disadvantage is that, owing to a lack of CD16 expression, NK-92 cells are devoid of the ability to mediate cell killing via ADCC.

NK cells can also be derived from $CD34^+$ progenitor cells upon in vitro differentiation[33]. In a first-in-human clinical trial, Dolstra et al. showed clinical efficacy in older patients with minimal residual disease-positive acute myeloid leukaemia (AML) who received adoptively transferred HSPC-NK cells[34]. Although preliminary results are encouraging, clinical studies exploring the safety and efficacy of HSPC-NK cells in larger patient populations and various indications will help determine the range of potential clinical applications for these cells. Future work will also need to address whether HSPC-NK cells can be effectively engineered to achieve enhanced tumour specificity.

iPSCs are an appealing source for NK cells given their clonal growth and high expansion capacity, as well as their ability to differentiate in vitro, allowing for the manufacturing of large numbers of homogeneous NK cell products. A potential disadvantage is that iPSC-derived NK cells often express low levels of endogenous CD16, although this can be mitigated through genetic engineering[45]. Another possible concern is that iPSCs may harbour DNA methylation signatures consistent with their somatic tissue of origin[46]. This 'epigenetic memory' could influence the development of specific cell lineages that differ from the donor cell, and should, therefore, be considered when employing iPSC platforms[47]. Nonetheless, a growing number of genetically engineered iPSC-NK cell candidates are emerging in preclinical studies, with a few that have transitioned into clinical trials[48–51]. In a phase I/II trial, iPSC-NK cells expressing CAR showed encouraging results in patients with relapsed or refractory (R/R)

Table 1 | **Clinical studies investigating CAR-engineered NK cell therapy products**

Molecular target	Disease	Construct	NK cell source	Clinical trial identifier and sponsor	First posted	Current status
CD7	CD7$^+$ R/R leukaemia/ lymphoma	CAR.7-CD28-4-1BB-CD3ζ	NK-92	NCT02742727 (REF.[210]); PersonGen BioTherapeutics	2016	Unknown
CD19	R/R B-ALL	CAR.19-41BB-CD3ζ	Peripheral blood from haplo-identical donor	NCT00995137 (REF.[211]); St Jude Children's Research Hospital, Memphis, TN	2009	Completed
CD19	R/R B-NHL	CAR.19-41BB-CD3ζ	Peripheral blood from haplo-identical donor	NCT01974479 (REF.[212]); National University Health System	2013	Suspended for interim review
CD19	R/R CD19$^+$ lymphoid malignancies	CAR.19-CD28-41BB-CD3ζ	NK-92	NCT02892695 (REF.[213]); PersonGen BioTherapeutics	2016	Unknown
CD19	R/R ALL/ CLL/B-NHL	CAR.CD19-CD28-CD3ζ. iCasp9-IL15	Cord blood	NCT03056339 (REF.[214]); MD Anderson Cancer Center	2017	Phase I portion completed; phase II recruiting Interim results reported 2020: ORR = 8/11 (73%) CR = 7/11 (64%) No CRS/ICANS/GvHD CAR NK cells in vivo persistence ≥1 year post infusion[37]
CD19	R/R B-NHL	Not disclosed	iPSC	NCT03824951 (REF.[215]); Allife Medical Science and Technology	2019	Unknown
CD19	R/R B-NHL	Not disclosed	iPSC	NCT03690310 (REF.[216]); Allife Medical Science and Technology	2018	Unknown
CD19	R/R B-NHL/ B-ALL	CAR.19-OX40-CD3ζ (NKX019)	Peripheral blood	NCT05020678 (REF.[217]); Nkarta Therapeutics	2021	Recruiting
CD19	R/R B-NHL/ CLL	CAR.19-NKG2D-2B4-CD3ζ-IL15RF-hnCD16 (FT596 ± rituximab/ obinutuzumab)	iPSC	NCT04245722 (REF.[218]); Fate Therapeutics	2020	Recruiting Interim trial results 2021: ≥90×10^6 cells FT596 ($n = 18$) ± rituximab ORR = 13/18 (72%) CR = 8/18 (44%) including 3/5 (60%) in CAR T cell-naïve patients in the rituximab arm ≥90×10^6 cells FT596 monotherapy ($n = 9$) ORR = 7/9 (78%) CR = 3/9 (33%) No dose-limiting toxicities[49]
CD19	R/R B-NHL	Not disclosed	Not disclosed	NCT04639739 (REF.[219]); Xinqiao Hospital of Chongqing	2020	Not yet recruiting
CD19	R/R B-NHL	Not disclosed	Peripheral blood from HLA-haplo-identical donor	NCT04887012 (REF.[220]); Second Affiliated Hospital, School of Medicine, Zhejiang University	2021	Recruiting
CD19	R/R B-NHL	CAR.19.IL15 (full construct not disclosed)	Cord blood	NCT04796675 (REF.[221]); Wuhan Union Hospital	2021	Recruiting
CD19	R/R B-NHL/ ALL/CLL	Not disclosed	Not disclosed	NCT04796688 (REF.[222]); Wuhan Union Hospital	2021	Recruiting
CD19	B-ALL	Not disclosed (QN-019a ± rituximab)	Not disclosed	NCT05379647 (REF.[223]); Zhejiang University	2022	Recruiting

Table 1 (cont.) | **Clinical studies investigating CAR-engineered NK cell therapy products**

Molecular target	Disease	Construct	NK cell source	Clinical trial identifier and sponsor	First posted	Current status
CD19	R/R B-NHL	CAR.19 HLA-I KO/HLA-E knock-in HLA-II KO/EGFR safety switch knock-in Soluble IL-15 knock-in (full construct not disclosed)	iPSC	NCT05336409 (REF.[224]); Century Therapeutics	2022	Not yet recruiting
CD20	R/R B-NHL	hnCD16 (full construct not disclosed) (FT516 + rituximab)	iPSC	NCT04023071 (REF.[225]); Fate Therapeutics	2019	Recruiting Interim results 2021: $\geq 90 \times 10^6$ cells FT516 + rituximab CAR T cell naive patients ($n = 10$) ORR = 8/10 (80%) CR = 5/10 (50%) Prior CAR T cells ($n = 8$) ORR = 3/8 (38%) CR = 3/8 (38%)[51]
CD22	R/R B-NHL	Not disclosed	iPSC	NCT03692767 (REF.[226]); Allife Medical Science and Technology	2018	Unknown
CD19/CD22	R/R B-NHL	Not disclosed	iPSC	NCT03824964 (REF.[227]); Allife Medical Science and Technology	2019	Unknown
CD33	R/R AML	CAR.33-CD28-4-1BB-CD3ζ	NK-92	NCT02944162 (REF.[228]); PersonGen BioTherapeutics	2016	Unknown Interim results 2018: No durable responses in 3 patients with R/R AML No significant adverse events[39]
CD33	R/R AML	Not disclosed	Not disclosed	NCT05008575 (REF.[229]); Xinqiao Hospital of Chongqing	2021	Recruiting
CD33/CLL1	R/R AML	Not disclosed	Not disclosed	NCT05215015 (REF.[230]); Wuxi People's Hospital	2022	Recruiting
CD70	R/R AML/ MDS/B-NHL	CAR.CD70-IL15 (full construct not disclosed)	Cord blood	NCT05092451 (REF.[231]); MD Anderson Cancer Center	2021	Not yet recruiting
BCMA	R/R B-NHL	Not disclosed	iPSC	NCT03559764 (REF.[232]); Allife Medical Science and Technology	2018	Unknown
BCMA	R/R multiple myeloma	CD8-41BB-CD3ζ	NK-92	NCT03940833 (REF.[233]); Asclepius Technology Company Group	2019	Recruiting
BCMA	R/R multiple myeloma	Not disclosed	Not disclosed	NCT05008536 (REF.[234]); Xinqiao Hospital of Chongqing	2021	Recruiting
BCMA	R/R multiple myeloma	Undisclosed (FT576 ± daratumumab)	iPSC	NCT05182073 (REF.[235]); Fate Therapeutics	2022	Recruiting
CD38/ SLAMF7	R/R multiple myeloma R/R AML	hnCD16A-Il-15RF-CD38$^{-/-}$; full construct not disclosed (FT538 ± daratumumab/ elotuzumab)	iPSC	NCT04614636 (REF.[236]); Fate Therapeutics	2020	Recruiting
NKG2D ligands	R/R AML/ MDS	CAR.NKG2D-OX40-CD3ζ (NKX101)	Peripheral blood	NCT04623944 (REF.[237]); Nkarta Therapeutics	2020	Recruiting

Table 1 (cont.) | **Clinical studies investigating CAR-engineered NK cell therapy products**

Molecular target	Disease	Construct	NK cell source	Clinical trial identifier and sponsor	First posted	Current status
NKG2D ligands	Advanced solid tumours	NKG2D.CD8.DAP12	Peripheral blood (autologous and allogeneic)	NCT03415100 (REF.[238]); The Third Affiliated Hospital of Guangzhou Medical University	2018	Interim results reported 2019: Reduction of ascites generation/decrease in ascites tumour cell counts in 2 patients with colorectal cancer (RECIST: SD); complete metabolic response (PET/CT) of liver lesion in 1 patient with metastatic colorectal cancer (RECIST: SD)[239]
NKG2D ligands	Refractory metastatic colorectal cancer	Not disclosed	Not disclosed	NCT05213195 (REF.[240]); Zhejiang University	2022	Recruiting
NKG2D ligands	R/R AML	Not disclosed	Cord blood	NCT05247957 (REF.[241])	2022	Recruiting
Muc1	Advanced solid tumours	Not disclosed	Not disclosed	NCT02839954 (REF.[242]); PersonGen BioTherapeutics	2016	Unknown
HER2	Recurrent HER2^{+} glioblastoma	CAR.HER2.CD28.CD3ζ	NK-92	NCT03383978 (REF.[243]) (CAR2BRAIN); Johann Wolfgang Goethe University Hospital	2017	Recruiting
PSMA	Castration-resistant prostate cancer	Not disclosed	iPSC	NCT03692663 (REF.[244]); Allife Medical Science and Technology	2018	Not yet recruiting
Mesothelin	Ovarian cancer	Not disclosed	iPSC	NCT03692637 (REF.[245]); Allife Medical Science and Technology	2018	Not yet recruiting
CD276	Ovarian cancer	hnCD16; full construct not disclosed (FT516 + enoblituzumab)	iPSC	NCT04630769 (REF.[246]); Fate Therapeutics	2020	Recruiting
ROBO1	Advanced solid tumours; pancreatic cancer	Not disclosed	Not disclosed	NCT03940820 (REF.[247]); NCT03931720 (REF.[248]); NCT03941457 (REF.[249]); Asclepius Technology Company Group	2019	Recruiting
PDL1	Advanced solid tumours	hnCD16; full construct not disclosed (FT516 + avelumab)	iPSC	NCT04551885 (REF.[250]); Fate Therapeutics	2020	Active
PDL1/PD1	Gastro-oesophageal junction cancer; advanced HNSCC	CAR.PDL1-FcεRIγ (full construct not disclosed) + pembrolizumab	Modified NK-92	NCT04847466 (REF.[251]); National Cancer Institute	2021	Recruiting
Oncofetal trophoblast glycoprotein (5T4)	Advanced solid tumours	Not disclosed	Not disclosed	NCT05194709 (REF.[252]); Wuxi People's Hospital	2022	Recruiting
SARS-CoV-2 S protein; NKG2D ligands	COVID-19	CAR.NKG2D-ACE2-GM-CSF.IL15 (full construct not disclosed)	Cord blood	NCT04324996[253]); Chongqing Public Health Medical Center	2020	Recruiting

ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; BCMA, B cell maturation antigen; CAR, chimeric antigen receptor; CD38$^{-/-}$, CD38 knockout; CLL, chronic lymphocytic leukaemia; CR, complete remission; CRS, cytokine-release syndrome; DAP12, DNAX-activation protein 12; GvHD, graft-versus-host disease; HLA, human leukocyte antigen; hnCD16, high-affinity, non-cleavable CD16; HNSCC, head and neck squamous cell carcinoma; ICANS, immune effector cell-associated neurotoxicity syndrome; IL15RF, IL15 receptor fusion; iPSC, induced pluripotent stem cell; KO, knockout; MDS, myelodysplastic syndrome; NHL, non-Hodgkin lymphoma; NK, natural killer; ORR, objective response rate; PSMA, prostate specific membrane antigen; R/R, relapsed or refractory; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

diffuse large B cell lymphoma, both as monotherapy and in combination with CD20-targeting agents[43,49].

Primary NK cells can be harvested from peripheral blood (PB-NK cells)[28–30,36] or from umbilical cord blood (CB-NK cells)[33–35,37]. CB-NK cells are available frozen off the shelf through blood banks, whereas PB-NK cells require apheresis of healthy donors and donor-specific collection. PB-NK cells served as the platform for

Apheresis
The process that allows for collection of specific components from blood, such as white blood cells, by separating the cellular and soluble fractions, retaining what is of interest and returning the remainder to circulation.

Chimeric antigen receptors
(CARs). Genetically engineered cell surface receptors designed to recognize specific proteins on tumour cells.

the very first successful delivery of a CAR construct into NK cells in work led by Dario Campana in 2005 (REF.[52]) and, today, PB-NK cells provide the basis for various products currently in clinical testing (TABLE 1). Cord blood NK cells have a proven track record for CAR-redirected antitumour cytotoxicity and clinical activity, as pioneered by our group[35,37]. In our study, we demonstrated potent clinical efficacy of genetically engineered cord blood-derived CD19-CAR NK cells against R/R CD19$^+$ lymphoid malignancies in an ongoing phase I/II clinical trial[37].

NK cells derived from all of these sources have been shown to have advantages and disadvantages for adoptive cell therapy applications. Having various options for source material confers a degree of versatility to the design of therapeutic strategies, allowing platforms to be specifically tailored to address the needs of each patient population and disease indication.

It is important to note that inter-donor variability may influence NK cell profiles and, thus, impact clinical outcomes. Therefore, a thorough understanding of product characteristics is critical to define biomarkers indicative of greater potency and persistence. From an immunological perspective, donor-derived NK cells might prove advantageous when administered across HLA–KIR genotypic boundaries as HLA–KIR ligand disparity might help overcome tumour immune evasion. Early into the journey of NK cell-based immunotherapy, the field recognized a dramatically reduced probability of post-transplant AML relapse in recipients of HLA haplotype-mismatched grafts with KIR ligand incompatibility[53]. Although KIR–HLA genotype-informed donor selection remains an area of active investigation[54–57], evidence suggests a potential benefit of this approach in the setting of T cell-depleted allogeneic haematopoietic stem cell transplantation (alloHSCT)[58–66]. Further work is needed to determine whether this strategy can be successfully adopted into NK cell therapy pipelines.

Enhancing NK cell fitness and antitumour function

Chimeric antigen receptors. Chimeric antigen receptors (CARs) are synthetic fusion proteins comprising an extracellular antigen-recognition domain and intracellular signalling moieties that trigger cell activation. Most commonly, the single-chain variable fragment (scFv) from a desired antibody is used for the antigen-binding domain, although many CAR modalities comprising the extracellular portion of native cellular receptors have also been constructed, leveraging the natural specificity of receptor–ligand interactions[67–69]. CARs can be expressed on immune effector cells for the purpose of reprogramming their specificity to a particular target (FIG. 2a). CAR-engineered T cell therapeutics were the first to emerge, with various products developed for immuno-oncology applications[1–8]. The field has since expanded, and NK cells have also been integrated into gene engineering pipelines[70,71]. CARs conventionally designed for T cells (comprising CD3ζ and T cell

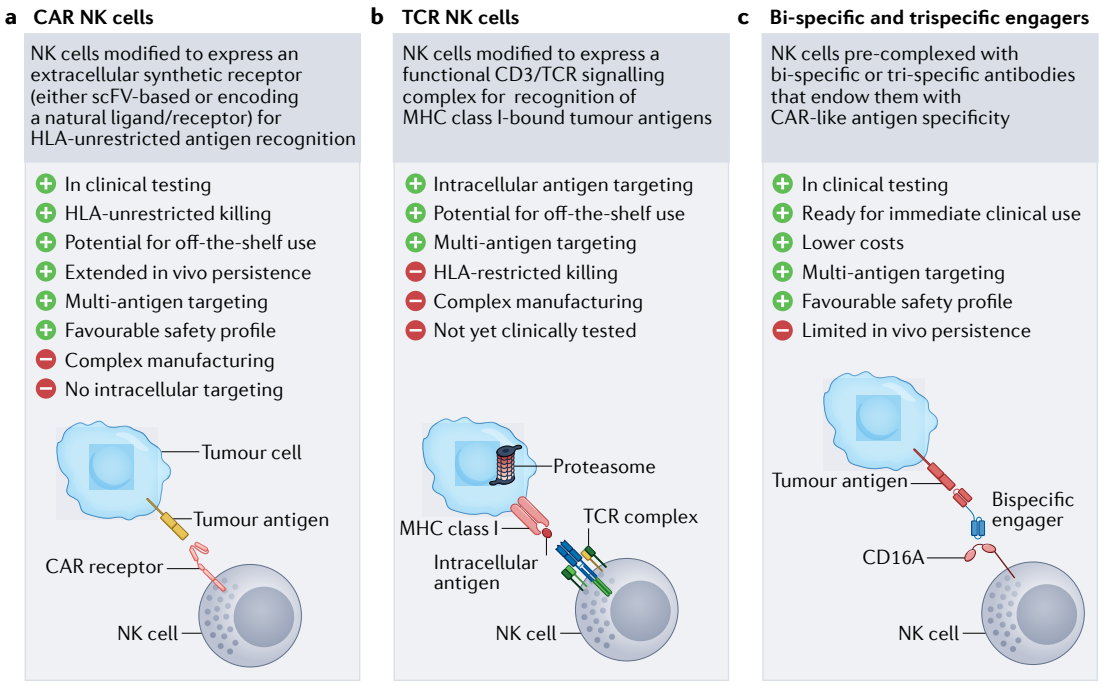


Fig. 2 | **Strategies to redirect NK cell specificity.** Natural killer (NK) cell specificity towards tumour cells can be redirected using different strategies. **a**,**b** | Chimeric antigen receptor (CAR) NK cell[35,37,43,112,264] (panel **a**) and T cell receptor (TCR) NK cell[94] (panel **b**) approaches both build upon stable genetic engineering to endow NK cells with synthetic receptors that recognize extracellular and intracellular tumour antigens, respectively. **c** | Bi-specific and tri-specific engagers[96,98–100,102,103,105,106,275] deploy two-directional or three-directional antibodies which crosslink NK cells with their respective tumour cell targets and circumvent the need for complex genetic editing. HLA, human leukocyte antigen; MHC, major histocompatibility complex; scFV, single-chain variable fragment.

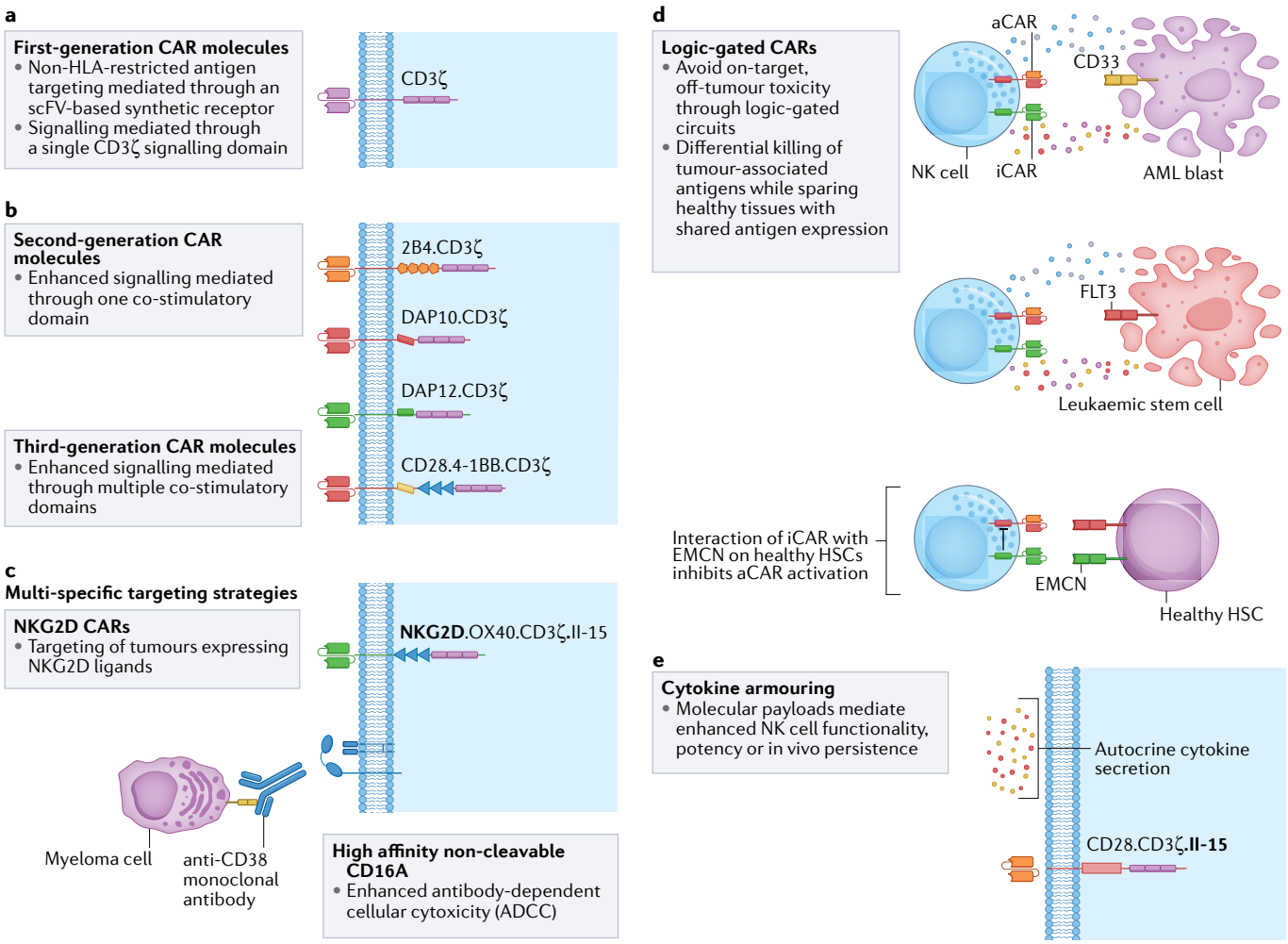


Fig. 3 | **Principles and strategies for CAR design. a**,**b** | Chimeric antigen receptor (CAR) molecules have evolved dramatically over the past two decades, from simple first-generation designs[276] (panel **a**), to second-generation[52,277,278] and third-generation[39,279] CARs with added co-stimulatory molecules (panel **b**) and, finally, to current-generation CAR designs resembling a modular system that encompasses optimized extracellular domains for target recognition, intracellular co-stimulatory molecules for effective natural killer (NK) cell activation and added payloads which can enhance NK cell functionality. **c**–**e** | Current strategies leverage the core principles of CAR signalling and functionality, and provide innovative methods to improve tumour recognition and enhance cell activation, using sophisticated construct designs to allow targeting of multiple tumour antigens[69,280] (panel **c**), provide cytokine support[35,37] (panel **e**) and activate auxiliary cytotoxicity pathways. Integration of logic-gated circuits to guide selective killing of targeted malignant cells while sparing healthy tissues may lead to improved safety profiles[90,92,93] (panel **d**). aCAR, activating chimeric antigen receptor; AML, acute myeloid leukaemia; DAP12, DNAX-activation protein 12; EMCN, Endomucin; HLA, human leukocyte antigen; HSC, haematopoietic stem cell; iCAR, inhibitory chimeric antigen receptor; scFV, single-chain variable fragment.

co-stimulatory molecules) have been used for generation of CAR NK cells, and studies have demonstrated that these cells can target tumours with efficacy and specificity, while maintaining a desirable safety profile[37] (FIG. 3a).

Motivated by these encouraging results, there is growing interest in designing CARs based on activating signals associated with NK cell biology (FIG. 3b). Examples of such an approach include DNAX-activation protein 12 (DAP12) and DAP10, which have been used in place of CD3ζ in some studies[72–76]. These adapter proteins are intracellular signalling domains that function via recruitment of PI3K and are associated with activating molecules such as NKp44, activating KIRs (KIR2DS and KIR3DS) and NKG2C (REFS.[72,76]). Similar to CD3ζ, DAP12 also contains immuno-tyrosine activation motifs (ITAMs) that, once phosphorylated, initiate a cascade of signals that culminate in the release of cytotoxic granules and pro-inflammatory cytokines such as TNFα and IFNγ[72]. DAP10, although not possessing an ITAM domain, has been shown to induce potent killing activity in NK cells via signalling through the NKG2D–DAP10 axis[76].

In the past few years, the behaviour of CARs, once anchored on the cell surface, has garnered considerable interest, especially following observations that, in some cases, CAR T cells demonstrated an elevated basal level of activation independent of antigen engagement, referred to as tonic signalling[77–81]. Although studies continue to explore potential causes of this phenomenon, it has been reported that CARs may incorporate into the TCR–CD3 complex via the CD3ζ domain and may augment T cell activation[82,83]. More recently, studies have shown that the CD28 transmembrane domain, present in some CAR frameworks, mediates CAR heterodimerization

Tonic signalling
The constitutive signalling mediated by a chimeric antigen receptor in a ligand-independent manner.

with endogenous CD28 and, thereby, triggers T cell activation[84,85]. These observations suggest that CARs are not static on the cell membrane but, rather, are prone to interacting with endogenous receptors. Although not much is yet known regarding these types of receptor associations in NK cells, it is conceivable that CARs may form synergistic partnerships with any of the many activating receptors on the membrane of NK cells, resulting in cooperative activation.

Efforts have also focused on refining CAR-based strategies, motivated by learnings from clinical translation of this therapy, which have prompted investigations into approaches to overcome the challenges experienced in the patient. One critical obstacle to CAR-based cell therapy is antigen escape, a process through which tumours, via antigen loss or downregulation, evade the immune response. Various strategies have been employed to address this issue. Targeting multiple antigens is a viable approach to both increase the stringency of tumour detection and extend therapeutic benefit (FIG. 3c). This is often accomplished either by expressing a bi-cistronic construct that encodes for two separate CARs (each specific for a different antigen on the tumour and linked to two separate signalling endodomains) or by expressing a single CAR containing bi-specific recognition domains, each targeting a different antigen in tandem and linked to a single signalling endodomain[86]. Dual-CAR approaches afford greater design flexibility, accommodating combinations of signalling domains and receptor formats. Each CAR may be formatted to provide both activating and co-stimulatory signals upon target engagement. Alternatively, *trans*-signalling formats separate these two signals and require engagement to both antigens for full activation. With bi-specific CARs, the antigen-binding moieties are linked to a single receptor, often a second or third-generation CAR[87,88]. In either approach, considerations regarding antigen density, membrane localization and protein structure are important to ensure both CARs bind their targets efficiently at the immune synapse. Although most of these innovative CAR-based approaches have focused on T cells, it is plausible to anticipate that these systems can also be applied to NK cells.

Strategies for improving the control of CAR-mediated activation have also been explored, especially with a focus on minimizing toxicities resulting from on-target, off-tumour effects. Promoting selective targeting, via the use of logic-gated CARs, has been shown to mitigate some of these problems in the context of CAR T cells[89–93] (FIG. 3d). A recent study presented preliminary findings showing the feasibility of controlling CAR NK activity when applying an 'OR' and 'NOT' logic gated CAR gene circuit approach[92,93]. In this work, the team targeted FLT3 and/or CD33 on AML blasts via bivalent CARs and employed an inhibitory CAR to bind an antigen on healthy haematopoietic stem cells (HSCs) and elicit a 'NOT signal' to prevent cell killing[92,93].

One key limitation of CAR-based approaches is that, for the most part, detection capability is limited to surface proteins. Intracellular antigens, which are presented in the form of peptide–HLA complexes, are naturally detected via the TCR. Engineering NK cells to express a TCR could allow detection of such peptides (FIG. 2b). TCR-guided NK-92 cells have recently been shown to mediate successful antitumour responses[94]. Although additional studies are warranted to validate the clinical applicability of this approach, one potential advantage is that, because NK cells do not possess endogenous TCR, mispairing issues that have been reported in TCR-engineered T cells[95] will likely not be a concern. A downside, however, is that NK cells are not equipped with the full signalling machinery found in T cells, thereby potentially compromising the ability to be potently activated via a synthetic TCR.

NK cell engagers. NK cells can also be directed to tumour sites via engagers that elicit a strong NK cell-mediated antitumour response by triggering an activating receptor on the NK cell, while simultaneously binding a target antigen on the tumour cell[96–100] (FIG. 2c). Additional NK engager strategies include tri-specific and tetra-specific designs that aim to strengthen the antitumour effect by targeting multiple antigens on the tumour or by cross-linking cytokine moieties to support NK expansion and survival[101–105]. The use of cell engagers bypasses the need for engineering and does not require vector-mediated gene transfer, therefore representing a simpler and less costly manufacturing process that can deliver a product capable of inducing CAR-like activity.

Various preclinical studies have shown promising results when employing NK cell engagers to target haematological and solid malignancies[96–106]. Recent work demonstrated a robust NK cell-mediated response against primary patient-derived AML blasts by employing a tri-specific molecule targeting CLEC12A on AML cells and activating NK cells though a humanized anti-CD16 single-domain antibody and IL-15 (REF.[105]). Moreover, a trifunctional engager targeting two NK activating receptors, CD16 and NKp46, was shown to drive potent antitumour response, culminating in efficient control of tumour growth in vivo in the setting of solid and metastatic malignancies in preclinical models[106].

Recently, our group demonstrated that CB-NK cells, when complexed with AFM13 — a bi-specific engager binding CD16 on NK cells and CD30 on leukaemia or lymphoma targets — exhibited enhanced killing of $CD30^+$ tumour cells, leading to CAR-like responses[100]. We have since translated this approach to the clinic for treatment of R/R $CD30^+$ Hodgkin lymphoma and non-Hodgkin lymphoma (NHL) (NCT04074746 (REF.[107])). As more strategies transition into clinical trials, it will be important to also evaluate the durability of the antitumour effect of engager-loaded NK cells and determine whether multiple treatments are required for sustained therapeutic benefit.

Cytokine armouring. Whereas CAR and TCR engineering technologies seek to enhance NK cell function by genetically redirecting their specificity, there are also initiatives that aim to effectively prime NK cells ex vivo and/or in vivo to sustain optimal antitumour function and persistence. It is well described that freshly isolated NK cells have lower cytolytic capacity compared with NK cells that have been primed[108]. One approach to address

Logic gated
A term derived from electronics that refers to implementation of a Boolean strategy to execute a logical function on one or more input signals to generate a single output signal.

this limitation is cytokine-mediated activation, and various methods are currently under investigation[29,100,109–114]. Ex vivo expansion of NK cells with combinations of IL-2, IL-15 and IL-21 supplementation shows that these cytokines enhance cytotoxic function and support high proliferation rates while maintaining cells in a healthy, non-exhausted state[115].

It has now become clear that, when cultured in the presence of IL-12/15/18, PB-NK cells shift to a phenotype referred to as cytokine-induced memory-like NK cells[29]. Cytokine-induced memory-like NK cells have shown clinical efficacy in patients with R/R relapsed myeloid neoplasias both in the pre-transplant and post-transplant setting[29,30]. Engineering memory-like NK cells to express CAR augments antitumour responses leading to increased potency against NK-resistant malignancies[111,112]. Furthermore, in our own clinical trial employing the NK cell engager AFM13, preactivation of NK cells with IL-12/15/18 to induce memory programmes led to enhanced responses against CD30$^+$ lymphomas[100] (FIG. 2c) (NCT04074746 (REF.[107])).

Although these data strongly point to the advantage of cytokine priming, continuous ex vivo stimulation renders NK cells 'cytokine-addicted' and leads to decreased persistence when these cells are infused in the absence of in vivo cytokine support[115]. To avoid this problem and still leverage the benefits of cytokine activation, the field has turned to genetic engineering, through which NK cells are modified to produce cytokines that will support cell potency, proliferation and persistence (FIG. 3e). This autocrine support has garnered notable interest, and CAR-engineered NK cells are emerging armed with supplemental cytokine signalling[35,37,48,49]. Cytokine armouring can be programmed such that soluble cytokines are released into the environment or are engineered in membrane-bound form to induce response upon cell to cell interaction[116]. In soluble form, cytokines that are produced by engineered NK cells can mediate a bystander effect and, thereby, activate other immune effector cells — such as T cells or myeloid cells — that are present in the tumour microenvironment (TME), thus potentially further augmenting the antitumour response[117]. Our group, as well as others, has demonstrated that IL-15-armoured CAR NK cells exhibit superior persistence in vivo in preclinical models when compared with CAR alone[35,48,49]. These findings were confirmed in a clinical study of patients with CD19$^+$ lymphoid malignancies, in many of whom we detected CAR NK cells in circulation a year after treatment[37]. With each cytokine armouring approach, it is important to determine optimal cytokine-dosing strategies that will support increased function and persistence, but will not compromise NK cell functionality by inducing metabolic exhaustion, as has been previously reported[118].

Overcoming immunosuppression. The TME consists of a harsh metabolic landscape characterized by a heterogeneous mix of immunosuppressive metabolites, glucose and amino acid deprivation, hypoxia and acidity, which, in concert, prevent effective antitumour immunity. In solid tumours specifically, hypoxia is a common driver of immune cell dysfunction. It has been shown that NK cell function is impaired in the hypoxic TME, in part due to the increased influx of suppressor cells such as myeloid-derived suppressor cells (MDSCs), regulatory T cells (T_{reg} cells) and M2 macrophages in the TME as well as a direct impact of hypoxia on NK cell function[119–122]. Inhibition of hypoxia-responsive HIF1a signalling in NK cells has been reported to enhance NK cell potency and unleash NK cell-mediated antitumour function[123].

Tumours often display an aberrant metabolic behaviour that results in high levels of lactic acid, depletion of vital nutrients and an increase in concentrations of toxic catabolites, adenosine and reactive oxygen species[109]. In addition, the uncontrolled proliferation, dysfunctional vasculature and presence of immunosuppressive cell subsets contribute to the dismal fate of immune effector cells that venture into the TME. To overcome the detrimental effects of metabolic immunosuppression, current strategies focus primarily on two areas: altering the metabolic constitution of the tumour or modifying gene expression programmes in immune cells to shield them from the suppressive metabolites in the TME (FIG. 4a). Studies have shown that high levels of lactate dehydrogenase (LDH) in the blood and the TME correlate with poor outcomes for patients with melanoma and low response to checkpoint therapy[124]. Although still in the preclinical phase, glycolytic inhibitors and LDH blockers may provide an opportunity to favourably modulate the TME. A recent study showed that treatment of patient-derived melanoma cells with a lactate dehydrogenase A (LDHA) inhibitor, GSK2837808A, led to improved T cell antitumour cytotoxicity both in vitro and in vivo[125]. Lactate levels in the TME can also be decreased by targeting its transporters, MCT1 and MCT4, as has been shown for AstraZeneca's AZD3965 compound, currently under investigation in the clinic (NCT01791595 (REF.[126])).

Adenosine, a by-product of ATP metabolism, is generated via the activity of ectonucleotidases CD39 and CD73. Under the hypoxic conditions of the TME, expression of these enzymes is upregulated as part of the hypoxia-driven purinergic signalling pathway, resulting in the accumulation of extracellular adenosine, which in turn acts as a negative regulator of T cell and NK cell function in the TME, suppressing their metabolism and effector function[127–130]. Interestingly, adenosine contributes to activation of suppressor cells such as T_{reg} cells, M2 macrophages and MDSCs, all resident populations within the TME. Therapeutic strategies for overcoming adenosine-mediated immunosuppression include blocking CD73 on tumour cells via small-molecule inhibitors or with antagonistic antibodies, both of which are currently under clinical investigation (NCT04148937 (REF.[131]), NCT03454451 (REF.[132]) and NCT03616886 (REF.[133])). Furthermore, in preclinical studies, genetic editing to delete the adenosine A2A receptor in both CAR T cells and CAR NK cells has shown promise for improving the potency and antitumour efficacy of these cells[134–137] (FIG. 4a).

TGFβ signalling is another mechanism of immunosuppression within the TME that has a deleterious effect on NK cell function. Knockdown of the TGFβ-induced

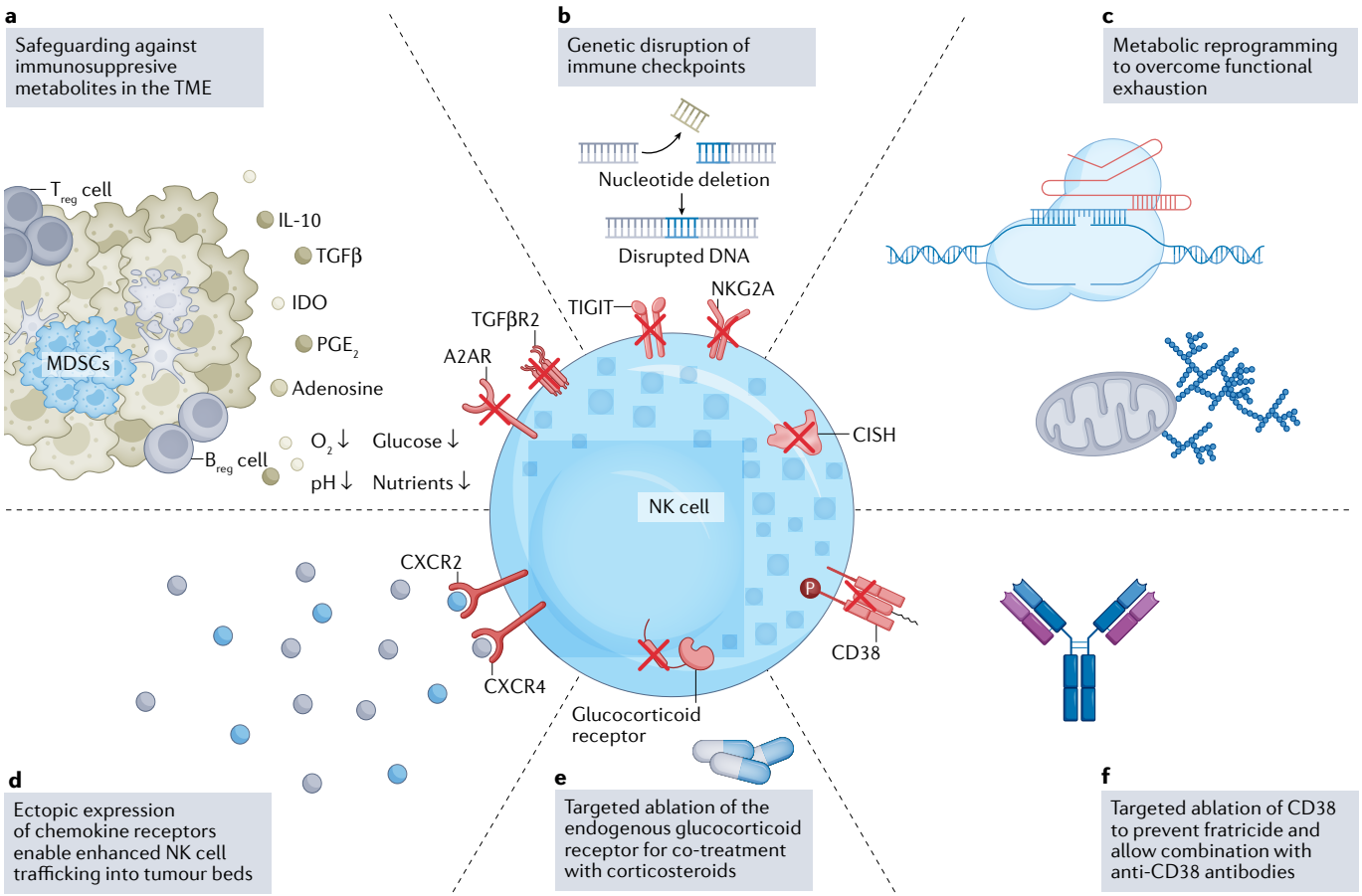


Fig. 4 | **Genetic engineering strategies to overcome suppressors of NK cell function. a–f** | Immune cell function is severely compromised by the hostile tumour microenvironment (TME)[134]. Current strategies leverage engineering tools to disrupt suppressive signals in the TME (panel **a**) and improve immune cell homing into tumour beds by ectopic expression of chemokine receptors (panel **d**). A selection of natural killer (NK) cell-relevant pathways that have been targeted through genetic engineering is shown. Genetic engineering strategies that include targeted ablation of inhibitory checkpoints[156,164,167,168] (panels **b,c**) as well as disruption of extracellular receptors which sense inhibitory stimuli including TGFβ[140,141] and adenosine[135] (panel **a**) have been shown preclinically to effectively target pathways to enhance metabolic fitness and persistence of NK cells, and efforts are ongoing to advance these findings into the clinic. Ablation of endogenous receptors allows for combinatorial therapeutic approaches, such as by rendering immune cells resistant to corticosteroid-induced immunosuppression (panel **e**), a principle previously established in severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-directed cytotoxic T lymphocytes (CTLs)[281]. Knockout of CD38 (panel **f**) renders NK cells resistant to CD38-mediated fratricide, which enables combination strategies of NK cells and anti-CD38-targeting monoclonal antibodies in the context of treating multiple myeloma[50]. B$_{reg}$ cell, regulatory B cell; MDSC, myeloid-derived suppressor cell; NKG2A, CD94/NK group 2 member A receptor; TIGIT, T cell immunoreceptor with immunoglobulin and ITIM domains; T$_{reg}$ cell, regulatory T cell.

miR-27a-5p led to improved NK cytotoxicity in vitro and in vivo[138]. Additionally, NK cells equipped with a high-affinity dominant-negative TGFβ receptor can resist the effects of TGFβ and retain their potency[139]. Similarly, our group successfully rendered CB-NK cells immune to TGFβ signalling by targeted deletion of the TGFβ receptor (*TGFBR2*)[140]. We subsequently provided in vivo evidence that disruption of TGFβ signalling safeguards NK cell cytotoxicity in a mouse model of glioblastoma[141].

As we seek to modulate immunometabolism in the TME, it is important to achieve a physiologic balance, as some metabolites are an essential component of normal metabolism. Although many strategies are still under development, it is plausible that a combined approach involving TME modulation and NK cell engineering will lead to reduced immunosuppression and will support robust immune cell activity.

Checkpoint disruption. Tumours have evolved sophisticated mechanisms to evade immune surveillance, including the engagement of immune checkpoints, which may restrain NK cells in a similar manner as has been described for T cells. Striving to translate the clinical successes seen with the blockade of T cell-associated immune checkpoints PD1 and CTLA4, several groups have explored the potential of modulating these regulatory circuits in the context of NK cells[142–149] (FIG. 4b). Although some studies point to their role as functional suppressors, their overall relevance in NK cell biology is still under debate[146,150]. With some likelihood, the signalling networks that underpin NK cell activation constitute a more nuanced picture in which these predominantly T cell markers act in concert with various other NK cell regulators[151]. Other regulators which have been investigated include TIM3 (REFS.[152,153]), T cell immunoreceptor with immunoglobulin and ITIM domains

Licensing
A process of education in maturing natural killer (NK) cells that is driven by the interaction of inhibitory receptors and self-major histocompatibility complex class I molecules, which potentiates NK cell responses to activating signals.

(TIGIT)[136,153–156] and LAG3 (REF.[157]), and their targeting using monoclonal antibodies has been shown to reverse tumour-induced NK dysfunction in vitro.

Inhibitory KIRs are potent negative regulators of NK cell function and can override any concomitant activating signal when engaged with HLA class I ligands. Given their prominent role in suppressing NK cell function, inhibitory KIRs have attracted considerable interest. Lirilumab, an anti-panKIR2D antibody, functions to skew the diverse and often opposing signalling cues perceived by NK cells towards net activation by blocking these inhibitory KIRs[158]. In two early-phase clinical trials, however, Lirilumab failed to elicit clinically meaningful responses[159,160]. One potential reason for these results might stem from the important role of inhibitory KIRs in NK cell education and licensing. Under this assumption, prolonged KIR inhibition due to continuous KIR blockade by Lirilumab might negatively affect NK cell function[161,162].

Similar to KIR molecules, CD94/NK group 2 member A receptor (NKG2A) is another prominent negative NK cell regulator, which, when bound to its cognate ligand HLA-E, restrains NK cell cytotoxicity. Monalizumab specifically disrupts this interaction and led to a promising 31% objective response rate in patients with previously treated recurrent or metastatic squamous cell carcinoma of the head and neck when administered in combination with cetuximab[163]. Monalizumab is currently being tested against other solid tumours including colorectal cancer and non-small-cell lung cancer as well as post alloHSCT.

Common to these approaches is the reliance on monoclonal antibodies to modulate the patients' immune cells, a strategy that requires multiple infusions owing to their limited in vivo half-life. With advances in genetic editing capabilities, NK cells can be stably modified to regulate biological mechanisms that augment NK cell effector function. One example is the genetic disruption of the inhibitory receptor NKG2A, which led to superior tumour control in xenograft mouse models inoculated with HLA-E$^+$ tumours[164] (FIG. 4b). Moreover, building on seminal work that established CIS (cytokine-inducible SH2-containing protein) as a critical negative regulator of NK cell function[165,166], we and others successfully engineered NK cells[167,168] lacking this intracellular cytokine checkpoint. The resulting CAR NK cells exhibited enhanced metabolic fitness and increased antitumour activity (FIG. 4c).

These studies foreshadow the potential of targeted genetic perturbations to modulate NK cell biology. Going forward, we anticipate that unbiased high-throughput discovery approaches will elucidate the functional consequences of specific genetic interventions in a more systematic manner to, ultimately, inform the design of the next generation of NK cell immunotherapies.

Enhancing NK cell trafficking to tumours. The ability of NK cells to traffic to and penetrate tumour beds is a critical prerequisite for effective antitumour immunity and has been linked to improved clinical outcomes[169–173]. NK cells, similar to other immune cells, are guided towards tumour sites by the dynamic interplay of chemokine receptors and their cognate ligands secreted in the TME[174–177]. A growing body of work has, in recent years, investigated how modulation of these interactions may be harnessed to augment effective homing to the tumour. Whereas earlier studies focused on the expansion-induced upregulation of chemokine receptors[178,179] as well as transient transfection methods[180,181], the swift loss of chemokine expression owing to internalization and degradation[182] has led to the increasing adoption of genetic engineering strategies to stably equip NK cells with ectopic chemokine receptors[182,183] (FIG. 4d). Today, the available studies provide encouraging preclinical evidence to support chemokine receptor modulation to enhance NK cell trafficking into tumour beds across a broad range of hard to treat tumours including multiple myeloma[184,185], glioblastoma[182], renal cell carcinoma[183], pancreatic ductal adenocarcinoma[186] and ovarian cancer[187].

Despite these advances, three important obstacles remain. First, chemotactic gradients rely on adequate tumour perfusion, and microthrombi-induced circulation deficits may require strategies to increase tumour micro-perfusion[182]. Second, the release of chemokines from the TME follows distinct tumour-specific kinetics, and intratumoural chemokine levels may need to be artificially boosted to robustly attract engineered NK cells[179]. Recent work has addressed this concern by locally augmenting chemokine concentrations using a mesothelin-targeting antibody fusion protein loaded with CXCL16, which is cleaved in the TME upon tumour cell engagement[186]. Furthermore, recent work has highlighted the application of radiation-induced CXCL8 to enhance NK cell migration to the tumour[188]. Last, chemokine–receptor interactions may be context-specific, either promoting or abrogating NK cell homing depending on the specific tumour type in question, as has been previously observed[184,185]. Future research will need to address these important questions and, ultimately, validate whether the overall promising findings can prevail in the context of the complex and dynamic interplay of chemokines within the human body.

Clinical lessons learned

CAR T cell therapy has led to remarkable clinical outcomes, with some patients achieving decade-long remissions in the presence of sustained CAR T cell persistence[1–9]. This new form of cellular immunotherapy has also shown potential for treatment of various cancers, driving the development of an ever-expanding number of new strategies to build on these successes[189–191]. The CAR T cell experience has elucidated many requirements for successful development of safe and efficacious cell therapies and has also revealed critical challenges related to mechanisms of resistance and barriers to immune effector cell persistence[192,193]. Knowledge gained from the clinical journey with CAR T cells has contributed to the emergence of novel cell therapy modalities. Over the past decade, the field has seen NK cells emerge as a strong new therapeutic candidate, possessing biological properties that may help overcome some of the limitations seen with T cell-based

approaches. One noteworthy finding is that early clinical data suggest that NK cells are well suited for use in the allogeneic therapy setting as no major adverse events have been reported thus far in ongoing clinical studies[194]. Although safety results are encouraging, further investigations are required to elucidate whether allogeneic NK cells are capable of evading recipient T cell rejection for long-term persistence. A growing number of clinical studies have shown safety and efficacy of NK cells derived from peripheral blood mononuclear cells, cord blood, iPSCs, HSPCs and cell lines in the treatment of haematological malignancies[28–30,33,34,37,39,48,49]. Moreover, we have learned that, similar to their T cell counterparts, NK cells are quite amenable to genetic modification and numerous clinical candidates have been engineered to target the tumour with greater precision (by expressing CAR) and to increase persistence (through cytokine armouring). In addition, continuous efforts to enhance trafficking to tumour sites (through chemokine receptor editing) (FIG. 4d) and to shield against suppressive factors in the TME (through checkpoint inhibition, metabolic reprogramming (FIG. 4a) and hypoxia tolerance) may lead to enhanced benefits in the clinic. NK cells may also differentiate to a memory-like phenotype that naturally extends the persistence of NK cell function and confers the ability to recall an antitumour response upon antigen re-encounter. Memory NK cells are currently under clinical evaluation in the treatment of leukaemia. Moreover, memory-like NK cells pre-complexed with engager molecules provide an alternative to achieving CAR-like specificity without the need for lengthy manufacture[100], and in the clinic, this strategy is driving responses comparable with those seen for CAR T cells and CAR NK cells. Importantly, irrespective of the approach, NK cell therapies have consistently demonstrated a favourable safety profile, and, to date, with no observed CRS or GvHD.

Although tremendous progress has been made, and in a short time NK cells have become an important tool for immuno-oncology, most of the successes reported are limited to haematological malignancies, much like the initial function of CAR T cells. However, there have been challenges. Not all patients respond to NK cell therapy, and some who do, eventually relapse. What are the mechanisms underlying tumour resistance and relapse? Are tumour cells, and, likewise, therapeutic NK cells, evolving in the course of their interactions? Understanding the biological underpinnings that influence patient response is critical to answering these questions. In the current era of single-cell multi-omic capabilities, we are poised to see these challenges as, in fact, opportunities (BOX 1). Interrogating therapy evolution through a combination of genomic, proteomic and epigenomic profiling might offer valuable insights into changes that permanently 'scar' NK cells into a state of exhaustion and hypo-responsiveness to the tumour, as previously observed in T cells[195,196]. Indeed, we might also identify epigenetic roots to metabolic reprogramming that results in low bioenergetic reserves and decreased persistence. Once these mechanisms are elucidated, strategizing ways to reverse their effects and rescue cell function, or identifying favourable gene edits that intercept harmful interactions, will play a pivotal role in maximizing the benefit of cellular immunotherapies. Furthermore, implementing combination therapy approaches may provide a strategy for achieving a synergistic effect and increasing the efficacy of engineered adoptive NK cell therapies (TABLE 2). Integration of antibodies or small-molecule inhibitors to prevent metabolite-driven immunosuppression, or blockade of checkpoint and immunosuppressive mechanisms (FIG. 4b,e), for instance, might lead to a multifaceted outcome: boosting CAR NK cell function, hindering signals from suppressive cells in the TME and supporting the function of other immune effector cells[197–199]. Lastly, engineering NK cells to become resistant to CD38-mediated fratricide will enable NK cell-based treatment approaches to be combined with anti-CD38 monoclonal antibodies and allow NK cell immunotherapy to move into earlier lines of treatment in the setting of multiple myeloma[50] (FIG. 4f).

Although many clinical trials are still in the early stages, through an iterative process we will be able to learn directly from patients what the barriers posed by each cancer are and take this information back to the laboratory where therapies can be further refined into applicable solutions to real-life challenges. Knowledge gained from these investigations will serve as the foundation upon which future clinical studies are built and will be critical to achieving success as we move forward and take aim at solid tumours. Through cellular engineering strategies and combination therapy approaches, NK cell adoptive therapies for solid tumours will also need to address and overcome challenges related to poor

Box 1 | **Forward genetic screens to inform the design of next-generation NK cell therapies**

CRISPR-enabled functional genetic screens allow the systematic interrogation of cancer-intrinsic vulnerabilities towards anti-neoplastic treatments in an unbiased large-scale manner, thereby extending the scope of traditional hypothesis-driven strategies. Over the past decade, many groups have applied these tools to better understand the molecular mechanisms of therapeutic resistance including against T cell immunotherapy[282–289]. Recently, this approach has been adapted to decipher cancer-specific vulnerabilities towards natural killer (NK) cell immunotherapy leveraging an orthogonal multiplexed approach, in which a diverse pool of DNA-barcoded cancer cell lines (PRISM) was examined simultaneously to identify shared transcriptional programmes that correlate with response. These studies hold an intriguing implication as they lay the groundwork for future biomarker-guided approaches to help identify and stratify patients whose tumours might be particularly susceptible to NK cell immunotherapy[290].

Translating CRISPR screening to primary immune cells, however, has proved much more challenging. First, primary immune cells are inherently more resistant to stable genetic editing, a prerequisite to reliably uncover genotype–phenotype relationships. Furthermore, limited ex vivo culture time periods prohibit long-term assays to achieve the necessary phenotypic resolution. In 2018, the work of two groups overcame this long-standing limitation. Guide Swap technology, which delivers Cas9 ribonucleoproteins pre-complexed to non-targeting single guide RNA, was successfully used to screen human primary $CD4^+$ T cells[291]. Another group applied a hybrid approach to screen primary human T cells by combining lentiviral library delivery with Cas9 electroporation[292]. Recently, this approach was translated to chimeric antigen receptor (CAR)-engineered T cells to investigate drivers of functional exhaustion in a reciprocal glioblastoma stem cell screen[293]. In its latest iteration, pooled knock-in screens now allow the concurrent assessment of functional consequences of integrating a multitude of transgenes[294,295]. Going forward, these tools will serve as invaluable platforms to decipher fundamental immune cell biology and, in turn, can inform the design of next-generation cellular therapies.

Table 2 | **Combinatorial strategies to enhance CAR NK cell therapeutic potency**

Type	Combinatorial agents	Molecular rationale	Refs.
IMIDs	Lenalidomide	Enhance ADCC	254,255
Epigenetic modulators	Azacytidine	Upregulate tumour-associated antigens	256,257
	Decitabine		
	Vorinostat (HDACi)	Restore and enhance tumour immunogenicity	
Oncolytic viruses	Oncolytic adenovirus	Augment tumour immunogenicity and enhance recruitment into tumour beds by promoting inflammation in the TME	258,259
		Dendritic cell-mediated NK cell activation/reversal of NK cell anergy	
Small-molecule inhibitors	GSK3i	Upregulate CD57, drive NK cell maturation	260,261
		Enhance ADCC	
Immune checkpoint inhibitors	PDL1 antibody	Direct pro-cytotoxic effect on NK cells	149
	PD1/PDL1 blockade	Unleash NK cell antitumour immunity	148
	Anti-NKG2A antibody		262
	Monalizumab		
	Anti-KIR antibody		159,160,263
	Lirilumab		
	TIGIT		154

An overview of different combinatorial agents that might be used to further enhance CAR NK cell immunotherapy. Although no concrete dosing schedules have yet been established clinically, combination of one or multiple of these agents with CAR NK cell infusions is conceivable to augment NK cell cytotoxicity and increase tumour immunogenicity via pro-inflammatory mechanisms to, ultimately, improve antitumour potency. ADCC, antibody-dependent cellular cytotoxicity; CAR, chimeric antigen receptor; GSK3i, glycogen synthase kinase-3 inhibitor; HDACi, histone deacetylase inhibitor; IMID, immunomodulatory drug; KIR, killer cell immunoglobulin-like receptor; NK, natural killer; NKG2A, CD94/NK group 2 member A receptor; TIGIT, T cell immunoreceptor with immunoglobulin and ITIM domains; TME, tumour microenvironment.

tumour trafficking and limited persistence and effector function in the immunosuppressive TME.

Ensuring genomic fidelity in NK cell therapies

Given the vast scope and the accelerated pace at which novel insights into fundamental NK cell immunobiology are generated, we anticipate an increase in novel engineered cell products. It is therefore increasingly important to establish platforms for screening and characterization of products to identify unintended genetic alterations resulting from off-target nuclease activity. Concerns that some genomic changes may inadvertently lead to oncogenic mutations continue to reverberate in the background as the field advances. To address this need, the US National Institutes of Health (NIH) have launched the Somatic Cell Genome Engineering (SCGE) programme with two main missions: first to provide financial aid to researchers to facilitate the transfer of genome editing technologies to the clinic, and second, to support the development of more comprehensive assays to investigate potential adverse biological effects that could result from the use of these tools[200]. In addition, various platforms to investigate genome editing fidelity have been developed, such as GUIDE-Seq[201], CIRCLE-Seq[202] and rhampSeq[203] that rely on sequencing technologies to unbiasedly identify sites in the genome where double-stranded breaks may have occurred. Moreover, unintended genetic rearrangements may be elucidated by assays such as linear amplification-mediated high-throughput genome-wide translocation sequencing (LAM-HTGTS)[204]. As these tools continue to evolve and newer ones are developed, the implementation of such assays in cell therapy development will be of paramount importance to ensure greater understanding of safety and efficacy of cellular products such as engineered NK cell therapies.

Concluding remarks

Engineered cellular immunotherapies continue to experience tremendous growth, with diverse modalities quickly advancing from preclinical studies into clinical testing. The recent report of in vivo editing of hepatocytes in patients with transthyretin amyloidosis epitomizes the latest frontier in the cell and gene therapy space[205]. In a related effort, an approach to enable T cell-targeted in vivo CAR transfection using lipid nanoparticles has been developed for the generation of fibroblast activating protein (FAP)-redirected CAR T cells to target cardiac remodelling in the context of myocardial injury[206,207]. Moreover, recent work has demonstrated that Nipah lentivirus vectors redirected to CD3, CD8 and CD4 could specifically deliver therapeutic genes (such as CAR and TCR) to T cell populations in vivo[208]. These technical innovations provide a new approach to off-the-shelf personalized therapies, in which targeted viral vectors are readily available to be administered to patients upon need, thereby bypassing the requirement for extensive ex vivo manufacturing, reducing costs and expediting therapy delivery. It is conceivable that targeted in vivo editing may also be applied to tumour cells, driving modifications that will restore their sensitivity to anti-neoplastic agents or eliminate resistance mechanisms. Although current initiatives have mostly focused on CAR T cell therapy, in vivo engineering might also be applicable to NK cell-based therapies, such as to boost endogenous NK cell function and persistence, or to increase tumour sensitivity to NK cell-mediated cytotoxicity.

As the field continues to innovate at a rapid rate, it is important to keep abreast of potential safety risks associated with these various gene editing strategies, as concerns associated with possible off-target effects are very relevant. The recent halt of multiple ongoing cell therapy trials owing to cases of chromosomal abnormalities, treatment-associated AML/myelodysplastic syndrome (MDS) or malignant transformation of the infused CAR T cell product[209] justify these concerns and serve as reminders of the importance of closely studying the safety of engineered products. Moreover, it is critical that cell therapy programmes implement pipelines for systematic screening of products to assess potential unwanted genetic modifications that may result in deleterious effects. Leveraging resources such as the NIH's SCGE programme may provide the initial support needed to launch these initiatives.

Although NK cell-based immunotherapy is well positioned as a safe off-the-shelf antitumour therapy,

important questions remain open. Elucidating the key parameters that determine NK cell potency and persistence will be important as the field progresses into developing approaches to address challenges specific to each disease indication. Moreover, decisions related to use of co-stimulatory signal, cytokine armouring and combination with other therapeutic modalities will play a role in maximizing the longevity and benefit of adoptively transferred NK cells. Finally, it will be critical to develop and implement optimal methods for expansion and cryopreservation of NK cells to ensure that high product quality is sustained. Logistically, for emerging NK cell therapy programmes to succeed, it will be essential to establish multidisciplinary team structures comprising researchers, clinicians and regulatory officials to jointly sketch a wholistic path to clinical translation.

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K.R. and The University of Texas MD Anderson Cancer Center have an institutional financial conflict of interest with Takeda Pharmaceutical and Affimed GmbH. K.R. participates on the Scientific Advisory Board for GemoAb, AvengeBio, Virogin Biotech, GSK, Bayer, Navan Technologies and Caribou Biosciences. The remaining authors declare no competing interests.

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