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REVIEW ARTICLE

Nanoparticle-Based Chimeric Antigen Receptor Therapy for Cancer Immunotherapy

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Abstract Adoptive cell therapy with chimeric antigen receptor (CAR)-engineered T cells (CAR-Ts) has emerged as an innovative immunotherapy for hematological cancer treatment. However, the limited effect on solid tumors, complex processes, and excessive manufacturing costs remain as limitations of CAR-T therapy. Nanotechnology provides an alternative to the conventional CAR-T therapy. Owing to their unique physicochemical properties, nanoparticles can not only serve as a delivery platform for drugs but also target specific cells. Nanoparticle-based CAR therapy can be applied not only to T cells but also to CAR-natural killer and CAR-macrophage, compensating for some of their limitations. This review focuses on the introduction of nanoparticle-based advanced CAR immune cell therapy and future perspectives on immune cell reprogramming.

Keywords Chimeric antigen receptor (CAR) · Nanoparticle · Genetic engineering · Immune cell reprograming · Cancer immunotherapy

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1 Introduction

The U.S. Food and Drug Administration (FDA) approved the first chimeric antigen receptor T cell (CAR-T) product, tisagenlecleucel of Novartis in 2017 As a cell therapy for acute lymphoblastic leukemia, a type of hematologic cancer, tisagenlecleucel is based on the enhanced adaptive immune response of engineered T cell [1]. Since then, five additional CAR-T products have been approved by the FDA for hematologic cancers, including diffuse large B cell lymphoma, mantle cell lymphoma, and multiple myeloma [2–5]. In addition, many new CAR-T products are undergoing clinical trials, and research is under way in laboratories.

CAR, which was first introduced in the mid-1980s, can dramatically enhance T-cell-mediated adaptive immune responses [6]. CAR-Ts can recognize certain tumor ligands and kill the targeted tumor cells specifically. Tumor cells often evade immune responses through a lack of expression of MHC class I molecules. MHC-independent CAR-Ts have significant advantages [6, 7]. CARs also have a higher antigen affinity than typical T cell receptors (TCRs) and form stable and functional immune synapses more quickly [8]. Costimulating molecules incorporated into CARs activate CAR-Ts more potently through the signaling pathway [9]. With these advantages, CAR-Ts have opened a new horizon in cancer immunotherapy, removing tumors in some patients who were unresponsive to chemo-, radiation, and other immunotherapies [10–12].

Nevertheless, CAR-T therapy has several limitations. Toxicity, such as systemic cytokine syndrome and neurotoxicity caused by excessive immune activity, is often present at a level that cannot be ignored [13]. Unlike hematologic cancers, there have been very few successful cases of treating solid tumors [11]. In addition, the high cost of production places an excessive burden on patients and pharmaceutical companies. All commercially available CAR-Ts use virus vectors to deliver CAR genes to T cells, which have high transfer efficiency but incur high costs and high regulatory demands in clinical settings, making it difficult for new CAR-Ts to be approved by the FDA [14].

Nanotechnology provides an alternative to conventional CAR-T therapy [15]. Nanoparticles (NPs) have long been studied for their benefits in cancer drug delivery owing to their size, high surface area, targeting ability, and ability to encapsulate a variety of drugs for controlled release [16, 17]. NPs have fully demonstrated their capabilities in many aspects of cancer immunotherapy, such as diagnosis through *in vivo* tumor imaging [18], reprogramming of the immune-suppressive tumor microenvironment (TME) [19, 20], and cancer vaccine delivery platforms [21]. The physicochemical properties of NPs, such as their size, surface charge, and surface ligands, can be modified by selecting appropriate materials. These modifications make NPs a possible tool for cancer immunotherapy by providing biocompatibility, toxicity control, and efficiency improvement [22–25]. NP-based CAR therapy can be applied not only to T cells but also to CAR natural killer (CAR-NK) and CAR macrophage (CAR-M) therapies, which compensates for some of their limitations (Table 1) [26, 27]. During the past five years, NP-based strategies have shown remarkable potential for CAR-T, CAR-NK, and CAR-M therapies. In this review, NP-based CAR therapies are introduced, and future prospects for CAR technology are discussed (Table 2).

2 Chimeric antigen receptor (CAR)

2.1 CAR structure

CARs are recombinant antigen receptors that redirect target cell surface antigens and the specificity and function of T cells and other immune cells [37]. CARs consist of an extracellular antigen-binding domain, transmembrane domain, and intracellular signaling domain (Fig. 1A). The signal is transmitted through the intracellular signaling domain of the CAR into T cells upon interaction between the single-chain variable fragment (scFv) and antigen. The extracellular antigen-binding domain is the scFv of a monoclonal antibody that recognizes an antigen [38]. The transmembrane domain consists of a hydrophobic α-helix that anchors CARs to the cellular membrane [39]. The intracellular signaling domain is the functional end of the receptor and is typically composed of a CD3ζ chain containing three immunoreceptor tyrosine-based activation motifs (ITAMs) [40]. Similar to conventional TCRs, CAR-Ts are activated when their ITAMs are phosphorylated [6].

CAR generation is typically divided according to the structure and composition of the intracellular signaling domain. The first-generation CAR has a single CD3ζ as an intracellular signaling domain, which results in low cytotoxicity and proliferation [40]. Second-generation CAR enhances the cytotoxicity, proliferation, and persistence of CAR-Ts by adding a costimulatory domain (*e.g.,* CD27, CD28, CD134, or CD137) to the intracellular signaling domain [41]. Third-generation CARs are composed of two or more costimulatory domains and have stronger cytokine production and killing abilities [42].

2.2 Process of conventional CAR-T therapy

Genetic modification of autologous T cells from patients for conventional CAR engineering can be implemented using viral vectors (Fig. 1B) [41, 43]. As of 2022, all six

Table 1 Comparison of CAR T, NK and macrophages (Reproduced from a previous report [28])

Parameter	CAR T cells	CAR NK cells	CAR macrophages
Cell source	Autologous or MHC-matched allogeneic	Autologous, non-MHC-matched allogeneic or NK cell lines	Autologous. Preclinical studies use iPSCs and cell lines
Intracellular signaling domain	CD3ζ plus a costimulatory domain, CD28, 4-1BB and others	Similar to CAR T structure, but can use NKspecific signaling domains, such as 2B4, DAP10, DAP12	Similar to CAR T structure, but can use other ITAM-containing signaling domains
Cytotoxicity mechanisms	CAR-dependent cell killing	Both CAR-dependent and CAR-independent NK-mediated cell kiling	CAR-dependent macrophage-mediated phagocytosis; macrophage-mediated alteration of tumor microenvironment; macrophages as antigen-presenting cells to stimulate immune response
Infiltration into tumors	Usually poor	Usually poor	Usually abundant
Cytokine release syndrome and neurotoxicity	Common and often serious	Less common and serious	No clinical data. But expected to be common

Table 2 Nanoparticles used in CAR therapy

Types of CAR	Types of nanoparticles	Functions	References
CAR-T	Self-assembled nanoparticles (SNPs)	Lower toxicity, higher CAR expression rate and increased specific reactivity to target tumor cells	[29]
	Lipid nanoparticle (LNP)	Lower toxicity than EP and tumor cell killing effect similar to viral vector	[30]
	Polymer-based nanoparticle (PNP)	*In vivo* CAR expression of $CD3^+$ cells, tumor eradication, and survival rate improvement	[31]
	LNP	Preventing the progression of fibrosis and restored heart function	[32]
CAR-NK	Charge-altering releasable transporter (CART) system	Higher expression of CD107a, TNF-α, IFN-γ and enhanced killing of the target cells	[33]
	PEI-coated magnetic NPs (MF-NPs)	Significant level of *in vitro* transfection efficiency and antitumor effect without any specific toxicity to NK cells	[34]
CAR-M	Mannose-conjugated polyethylenimine (MPEI) nanocomplex	Macrophage targeted delivery, M2-to-M1 phenotype shift, higher CD8/Treg ratio, tumor growth inhibitory effect	[35]
	LNP	Higher transfection efficiency to primary BMDM, cytotoxicity against human B lymphoma	[36]

FDA-approved CAR-T products use viral vectors, such as lentivirus and retrovirus, for CAR gene transduction [44–46, 108, 109]. Mononuclear cells collected from patients through leukapheresis are subjected to T cell isolation using T-cell-specific antibody-coated magnetic beads [47, 48]. For separated T cells to become CAR Ts, they must undergo activation and gene modification. T cells are activated by anti-CD3/CD28 monoclonal antibodies (mAbs) in the form of soluble, magnetic bead-coated, plate-coated, or cell-based antigen-presenting cells (APCs) as endogenous antigen-presenting cells are restricted to ex vivo T cell activation by good manufacturing practice [47, 49, 50]. CAR-expressed T cells must undergo an expansion process using bioreactors, prodigy systems, or artificial APCs to ensure the amount is sufficient before being applied to patients [51]. This complex process increases production cost and duration and limits the widespread use of CAR Ts in cancer treatment.

3 Nanoparticle-based CAR-T therapy

3.1 Ex vivo engineering for CAR-T therapy

To solve the challenges caused by the transduction of CAR genes by viral vectors, researchers have begun to focus on nonviral gene delivery systems, such as electroporation (EP), sonication, and NPs [14, 46]. Viral vectors and non-

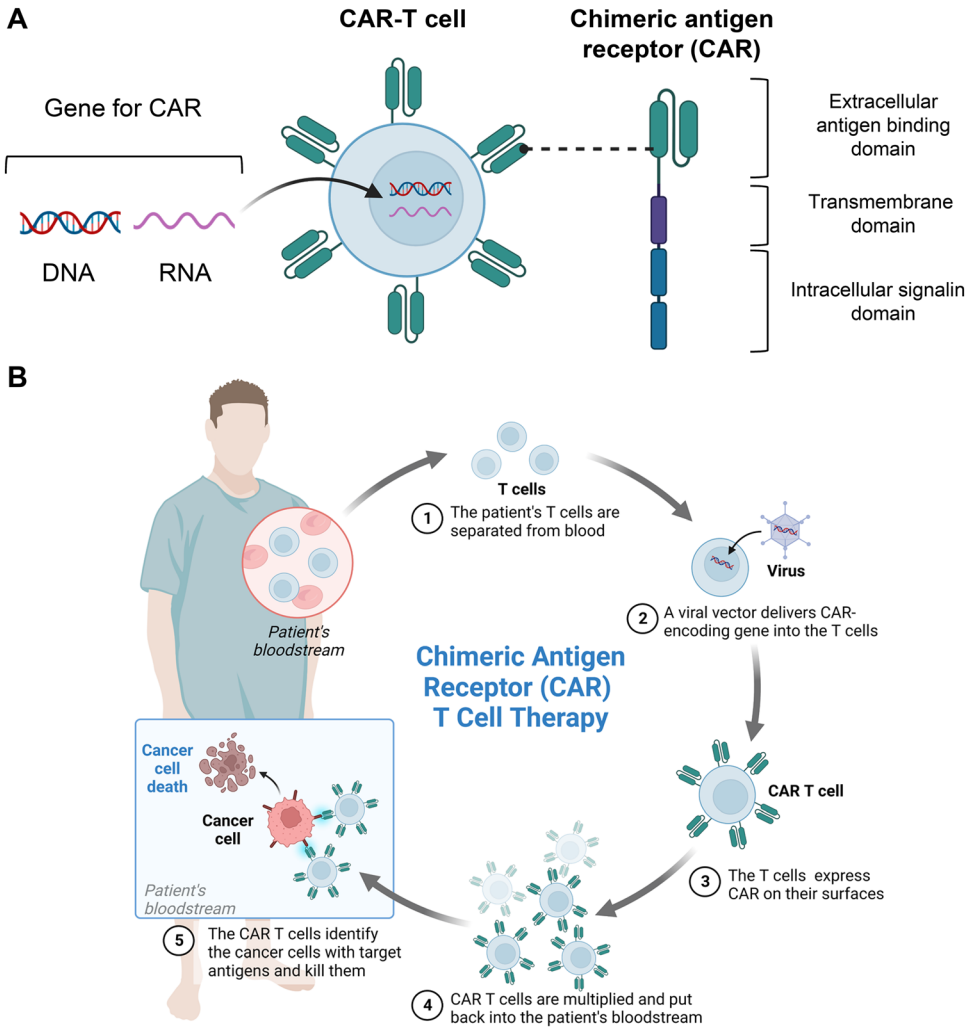

Fig. 1 **A** Schematic illustration of CAR and CAR-T and **B** Schematic diagram of conventional process of CAR-T therapy using viral vectors (created with BioRender.com)

viral vectors are the same in terms of delivering genes into cells, but have various differences (Table 3). Although transfection efficiency is lower than that of viral vectors, nonviral vectors have strengths in terms of manufacturing costs and processes and have low tumorigenicity and immunogenicity. However, physical methods such as EP, microinjection, and sonication have obvious limitations in that they can damage cells. Owing to their advantages, NPs have attracted attention as gene delivery platforms for nonviral vectors [52]. The relatively low transfection efficiency of nonviral vectors can be overcome through various properties of the nanosystem [53]. A high surface-area-to-volume ratio and small size (nanometer scale) facilitate interaction with the cell membrane and penetration into the target [52, 54]. In addition, NPs provide barriers protecting the loaded genes from immediate endosomal degradation that occurs after cell internalization, keeping genes intact [53]. The biggest feature of NPs is their designability. NPs can be designed with a variety of materials, and their surface can be modified in any way desired. NPs that have been proven to be effective and stable through various assays can be mass-produced, which leads to lower costs in the production process [55].

Polymers are materials used in a large proportion of biomedical industry owing to their biodegradability, easy synthesis and modification, diversity of functions, and high possibility of mass production [56]. Cationic polymers at physiological pH, as gene carriers, can form nanoscale polyplexes through electrical interactions with negatively charged genetic materials. Polyethyleneimine (PEI), poly(L-lysine), and poly(2-dimethylamino)ethyl methacrylate are representative polymers widely used as materials for polymer-based nanoparticles (PNPs) [56, 57]. As they are electrically cationic, they are internalized by electrostatic binding to the anionic cell membrane when they meet the target cells. In many cases, they escape the endosomes through the proton sponge effect [58].

Yu et al. [29] reported *in vitro* CAR-T engineering using self-assembled nanoparticles (SNPs) with less toxicity and a higher CAR expression. SNPs were prepared by self-

Table 3 Pros and cons of viral and non-viral vectors

Type of vectors		Pros	Cons
Viral vector	Adenovirus	High transfection	High manufacturing cost
	Retrovirus	efficiency	Highly regulatory demands
	Lentivirus	High stability of expression	Host genome integration
			Limited genetic cargo capability
			Immunogenicity
Non-viral vector	Electroporation	Low manufacturing cost	Low transfection efficiency
	Lipid-based nanoparticles	Simple manufacturing process	
	Polymer-based nanoparticles	High genetic cargo capacity	Toxicity related to materials
	Inorganic nanoparticles	Low immunogenicity	

assembly using plasmid DNA (pDNA), polyethylene glycol (PEG) and the cationic polymers polyamidoamine (PAMAM) and PEI, and pDNA was successfully encapsulated (Fig. 2A). The $SNP^{G1/800}$ (adamantane-grafted polyamidoamine (Ad-PAMAM) dendrimer: G1, CD-PEI: 800 Da) showing the highest T cell transfection efficiency was screened by controlling the generation of PAMAM and the molecular weight of PEI. As a result, the EGFRvIII CAR gene delivered by SNP in Jurkat cells showed much less toxicity than PEI and showed tenfold higher luciferase activity than Lipofectamine 2000 transfection reagent (Fig. 2B). Unlike the control group, Jurkat CAR + cells showed specific recognition of EGFRvIII-positive tumor cells (Fig. 2C). These results demonstrated that jurkat T cells can be effectively engineered using SNP to transiently express CARs that recognize EGFRvIII and exhibit specific reactivity to target tumor cells.

In 2018, the FDA approved a lipid nanoparticle (LNP)-based drug for RNA interference (RNAi) to treat polyneuropathy caused by transthyretin amyloidosis, which opened the era of LNP-RNA [59, 60]. In general, LNPs are composed of ionizable lipids, cholesterol, helper lipids, PEG lipids, and nucleic acids [61]. At acidic pH, positively charged ionizable lipids can condense negatively charged nucleic acids into LNPs and contribute to endosomal escape by fusion with the endosome membrane. Toxicity is minimized because of their electrically neutral properties at physiological pH [62, 63]. Components other than ionizable lipids are involved in the stability of LNP, formation of the bilayer structure, and reduction of aggregation [61].

mRNA has several advantages over DNA in the selection of nucleic acid substances for transfection. Translation occurs directly in the cytoplasm without passage through the nuclear membrane or transcription [64]. In addition, mRNA is unlikely to be integrated into the host genome and enables rapid and transient expression of the target protein [65]. Considering these advantages, the LNP-mRNA-based strategy has potential for effective CAR-T therapy.

Billingsley et al. [30] reported that LNPs can deliver mRNA to primary human T cells with low toxicity compared with EP. Twenty-four different ionizable lipids were combined with cholesterol, phospholipid, and lipid-anchored PEG and mixed using a microfluidic device with CAR mRNA to form LNP (Fig. 3A). The library of 24 ionizable lipids consists of a combination of three alkyl chains and eight polyamine cores; consequently, the combination of C14 (1,2-Epocytetradecane) and the polyamine was determined to be optimal. To determine usability in the CAR-T manufacturing process, the anti-CD19 CAR gene was delivered to primary T cells to evaluate transfection efficiency, viability, and cancer cell killing *in vitro*. Ionizable LNPs expressed CAR with an efficiency similar to that of EP in primary T cells, whereas they had a significant advantage in terms of toxicity to cells when compared with viability (Fig. 3B). The ability to kill tumor cells *in vitro* also did not lag behind other CAR-Ts engineered using conventional methods (Fig. 3C). These results indicate the ability of LNP to deliver mRNA to primary human T cells and the potential of mRNA-based CAR-T therapy.

3.2 *In vivo* engineering of CAR-T therapy

Current methods of ex vivo CAR-T generation are labor-intensive and require considerable cost and time owing to the complexity of the process. As explained above, autologous T cells extracted from patients undergo a series of processes, such as isolation, genetic modification, and expansion. Patients who fail to withstand the excessive cost and long time of these processes cannot even undergo treatment and die [47, 51, 66, 67, 110]. Thus, it was necessary to find an "in vivo engineering" system that would make the T-cell engineering process simpler. With this

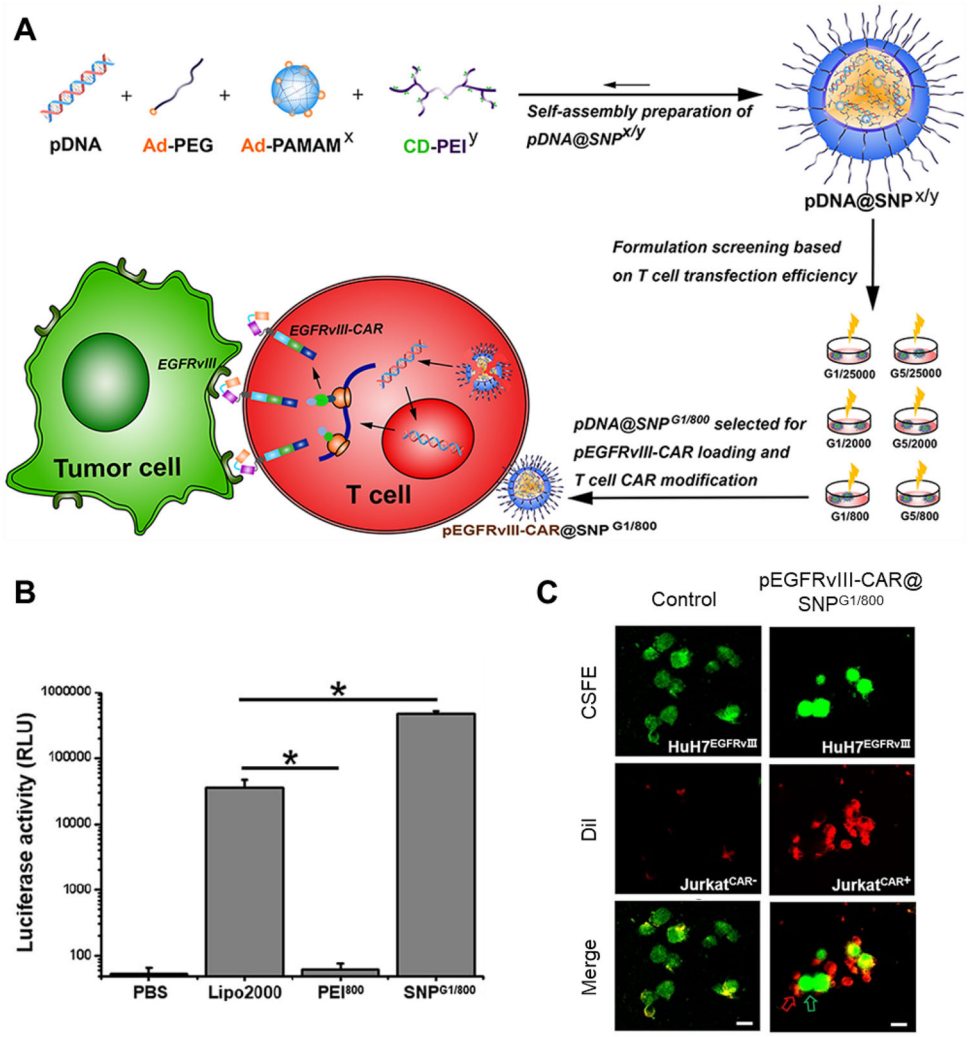

Fig. 2 **A** Schematic diagram of preparation and formulation screening of SNPs by comparing T cell transfection efficiency depending on the type of Ad-PAMAM and CD-PEI, and anticancer effect of anti-EGFRvIII CAR-T. **B** Transfection efficiency of Lipofectamine 2000 (Lipo2000), PEI800, or SNPG1/800 via luciferase activity (*$p < 0.05$). **C** Confocal microscopy images of interaction between EGFRvIII-positive HuH7 cells (green) and Jurkat T cells (red) transfected with or without pEGFRvIII-CAR@SNPs$^{G1/800}$. Scale bars, 20 μm. (Reproduced from a previous report [29] with permission from Dove Medical Press Limited)

concept, it is no longer necessary to extract and isolate T cells from patients, and proliferation of powerfully genetically modified T cells can occur spontaneously in the bodies of patients.

To generate CAR-Ts in vivo, a platform capable of safely transferring genetic material to the target T cells is required. The targeting strategy is considered in addition to the characteristics required for ex vivo engineering. The surfaces of NPs are commonly coated with monoclonal antibodies specific to T cell surface ligands [110]. When NPs loaded with the genetic material are injected, internalization of the NPs occurs through the interaction of the surface antigen of the target T cells and the surface antibody of the NPs while circulating in the living body, so that the T cells can express CARs [68]. The generated CAR-Ts encounter the target cancer cells and are activated, causing rapid cell division and cytotoxicity to the target tumor.

To the best of our knowledge, the first attempt to program CAR-Ts *in vivo* was reported in 2017. Smith et al. [31] devised a polymer-based nanocarrier to deliver CAR-encoded DNA to T cells *in vivo*. Unlike when transferring genes to highly pure isolated T cells outside the living body, gene-loaded nanocarriers must be able to target T cells. Furthermore, if the genetic material is DNA, it should be possible to deliver it to the nucleus. To achieve effective pDNA delivery into T cells, the surface of the NP formed by a cationic polymer, poly-(β-amino ester) (PBAE), was coated with a polyglutamic acid (PGA)-conjugated CD3e f(ab′)2 antibody (Fig. 4A). In the meantime, PBAE was conjugated with peptides composed of microtubule-associated sequences (MTASs) and nuclear localization signals (NLSs), which contribute to the transfer of anti-CD19 CAR pDNA to the nucleus using a microtubule transport mechanism in the cell. In this study, bolus injections of NPs induced rapid and efficient programmed peripheral T cells to recognize leukemia cells (approximately 6% $CD3^+$ on day 6 and approximately 20% $CD3^+$ on day 12; Fig. 4B). The bioimaging results for the distribution of the NPs demonstrate the validity of the targeting capability through CD3 antibody. Most of them without CD3

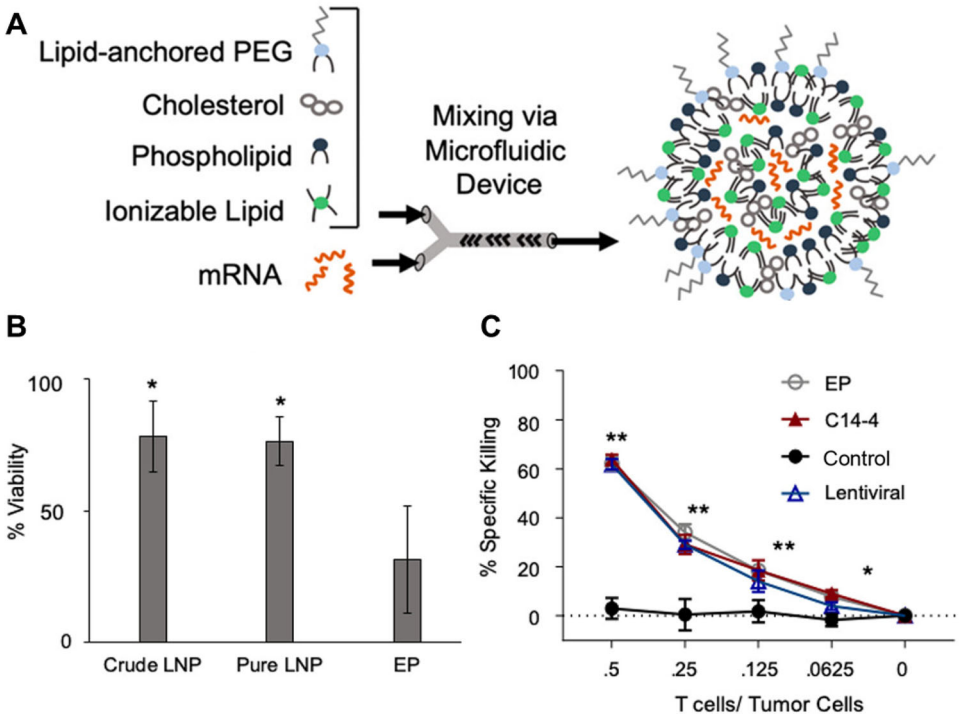

Fig. 3 **A** Schematic diagram of LNP preparation via microfluidic device. **B** Viability of primary T cells treated with crude LNP, pure LNP, and EP group. n = 3 biological replicates, $*p < 0.05$ in paired t-test to EP. **C** Comparison of tumor cell killing ability *in vitro* of CAR-Ts engineered by EP, LNP (C14-4), lentiviral vector, and control. n = 3 wells. $*p < 0.05$, $**p < 0.01$ in the paired t-test to control. (Reproduced from a previous report [30] with permission from the American Chemical Society)

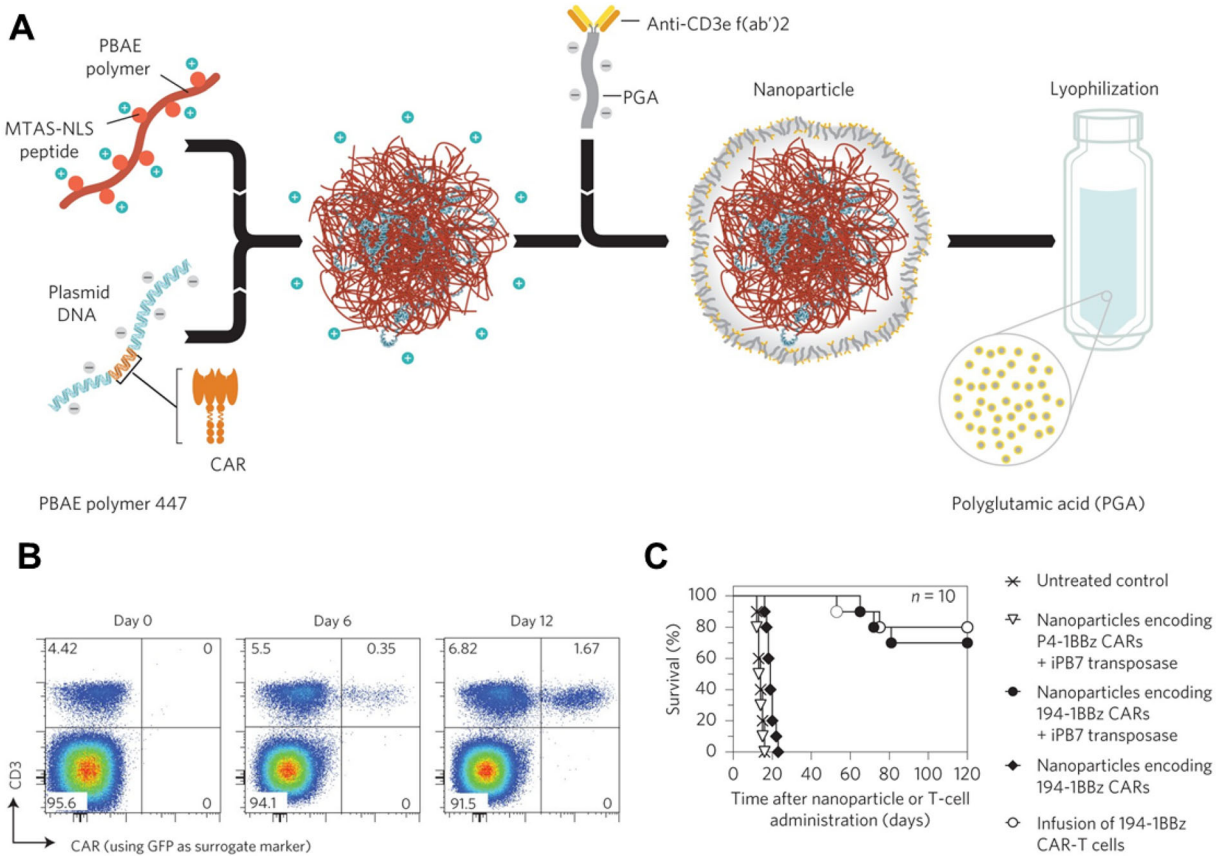

Fig. 4 **A** Schematic diagram of fabrication of anti-CD3e f(ab′)2-coated PBAE NP carrying CAR plasmid DNA for *in vivo* CAR generation. **B** Flow cytometry of peripheral T cells after injection of NP encapsulating 194-1BBz_2A_GFP genes. **C** Survival of animals following the various groups of CAR-T therapy. (Reproduced from a previous report [31] with permission from Nature Publishing Group)

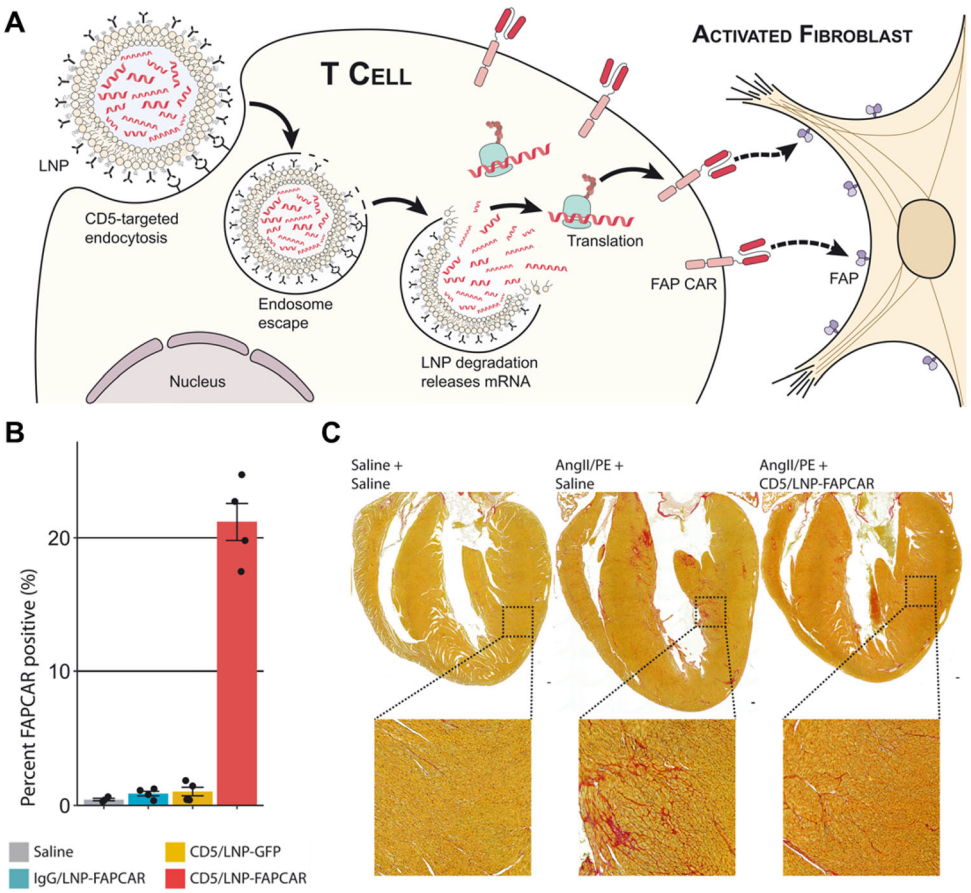

Fig. 5 **A** Schematic illustration of T cell engineering via FAP CAR-mRNA/LNP against activated fibroblast. **B** Percentage of FAPCAR positive T cells isolated from mice 48 h after injection of 10 μg of mRNA-LNPs. **C** Histologic analysis of coronal cardiac sections of animals and quantification of fibrosis. Picrosirius red staining indicates collagen (pink). Inset shows magnification of the LV myocardium. Scale bar, 100 μm. (Reproduced from a previous report [32] with permission from American Association for the Advancement of Science)

antibody moved to the liver, but the others with CD3 antibody were accumulated at significant levels in the spleen, lymph node, and bone marrow. In addition, it was confirmed that CD3-targeting NPs were binded more than 12 times compared to non-targeting NP in the peripheral T cell. As a result of injecting the 194-1BBz(+ iPB7 transposase) nanocarrier into the mouse leukemia model, tumors were eradicated in 7 out of 10 mice, and the average survival rate improved by 58 days (Fig. 4C). Furthermore, PNPs made from PBAE and PGA-conjugated antibodies are available as mRNA carriers for *in vivo* CAR expression [69]. This strategy exhibited disease regression at levels similar to those of ex vivo-engineered lymphocytes.

In vivo CAR-T therapy can be applied not only to cancer, but also to such diseases as cardiac fibrosis [70]. Cardiac fibroblasts activated by myocardial damage secrete excessive extracellular matrix, leading to cardiac fibrosis, and the disease has a significant adverse effect on the state and function of the myocardium, but the level of treatment has been inadequate. Although CAR-Ts targeting activated fibroblasts have been devised and their effectiveness has been demonstrated, continuous *in vivo* expression of fibroblast-specific CARs has been a problem because it can interfere with the normal functions of fibroblasts. In this regard, temporary expression of CAR *in vivo* using the mRNA-LNP system is a promising strategy. Aghajanian et al. reported the treatment of cardiac injury through CD5 targeting LNPs encapsulated with fibroblast activation protein (FAP)-specific CAR-encoding mRNA (Fig. 5A) [32]. The expression of luciferase in the spleen only appeared in the group injected with CD5/LNP-Luc is one of the results supporting the effectiveness of this targeting strategy. In contrast to nontargeted (IgG/LNP-FAPCAR) and targeted LNP with GFP (CD5/LNP-GFP), consistent populations (17.5%–24.7%) of FAPCAR $^{+}$ T cells were identified only in mice that received CD5/LNP-FAPCAR (Fig. 5B). In addition, splenocyte flow cytometry revealed that the expression of FAR in other immune cells was not significant. FAP-CAR T cells prevented the progression of fibrosis and restored heart function by removing FAP from the activated fibroblasts (Fig. 5C). This study is of great significance in that CAR-T engineering *in vivo* is not limited to cancer immunotherapy but has potential as a treatment for various diseases.

4 NP-based CAR-NK therapy

Despite continuous advances, the limitations described above have prompted the application of CAR technology to other immune cells. NK cells play a role similar to that of cytotoxic T cells in the innate immune response. Although there are obvious differences between NK cells and cytotoxic T cells, their functions are similar in that they recognize and destroy target cells. In addition, the differences between NK cells and cytotoxic T cells make the immune responses of the two cells complementary to each other [71]. The unique characteristics of NK cells, which differ from those of T cells, serve as an advantage in the application of CAR technology [28, 72].

First, the target cell recognition method for NK cells is MHC class I independent. Rather, killer Ig-like receptors (KIR) in NK cells can inhibit the activation of NK cells through interaction with MHC class I molecules. This mechanism reduces the aggression of NK cells against host cells that normally express MHC and allows them to maintain their aggression against tumor cells with reduced MHC expression [28, 72, 73]. Furthermore, there is a significant degree of freedom in selecting the cell source to produce CAR-NK compared with CAR-T [28]. In the CAR-T case, if homogeneous T cells are used, there is a risk of developing graft-versus-host disease (GVHD) owing to T cells exhibiting alloreactivity for such reasons as human leukocyte antigen (HLA) mismatch [28, 74–76]. The target recognition of T cells, based on determining whether the antigen originates from itself or not, provides the possibility of attacking cells expressing homogeneous nonself HLA types. To prevent this immunological risk, autologous T cells of patients should be used as a source of CAR-T engineering; however, many patients do not have sufficient T cell populations. There continue to be reports of deaths without attempting treatment owing to the inability to wait for this process, which requires considerable time, or because the conditions are not met [48, 77–79]. However, NK cells, which can only discriminate MHC expression, are free from GVHD pathogenesis after transplantation [80]. This is supported by the fact that GVHD progression was not observed in 11 patients who received HLA-mismatched anti-CD19 CAR-NK treatment for non-Hodgkin's lymphoma and chronic lymphocytic leukemia [81]. Therefore, it is possible to produce "off-the-shelf" CAR-NK cells that can be used immediately when needed by patients with NK cells extracted from a number of possible sources, including cell lines, peripheral blood-derived NK cells, umbilical cord blood-derived NK cells, and stem-cell-derived NK cells, without the need to produce patient-specific products [28, 72, 82, 83].

Second, in terms of toxicity, CAR-NK treatment is safer than CAR-T. One challenge that CAR-T treatment must overcome is the uncontrollable toxicity that sometimes occurs, including cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome. This toxicity results from the excessive release of inflammatory cytokines, such as IL-1, IL-6, IL-10, and TNF from CAR-T immunity [13, 84, 85]. However, cytokines that mediate NK cell immunity differ from these inflammatory cytokines in their profiles, including IFN-γ and GM-CSF [84, 85]. In a previous study that revealed that CAR-NK cells were free from GVHD, there was no CRS or neurotoxicity [81]. Moreover, even if an unexpected "extratarget tumor" phenomenon occurs, the problem can be naturally solved by the rapid depletion of CAR-NK cells owing to the short life cycle of NK cells, such as two weeks. However, the therapeutic effect appears only temporarily, and thus continuous injection of CAR-NK cells is necessary [28, 83, 86].

Third, CAR-NK cells have other routes that can be activated in addition to CAR. KIR, which functions as an important inhibitory regulator of NK cell-mediated cytotoxic immune responses, basically implements "self-tolerance" to stop attacks by MHC class I normal cells. However, when the expression of MHC class I molecules decreases as an immune avoidance method in infected or tumor cells, inhibitory signals through the immunoreceptor tyrosine-based inhibitory motif, an intracellular domain of KIR, are not transmitted, enabling NK cells to maintain their cytotoxicity [87–89]. CD16 expressed on NK cell surfaces mediates antibody-dependent cytotoxicity (ADCC) in NK cells. CD16 (also called FcγRIII) has a low affinity for Ig G1 or G3, which can be combined with the Fc region of the antibody bound to target antigens to activate ADCC of NK [90]. Bi- or tri-specific killer engines, made using a combination of scFVs of antibodies, can improve CD16-mediated ADCC in the NK cells [91]. In conclusion, for CAR-NK cells, anticancer effects can be expected as a route separate from CAR, so it is possible to retain cytotoxicity to target tumors with insufficient expression of CAR-specific antigen.

With the introduction of nanotechnology, CAR-T treatment has made many advances, and it can also be applied to CAR-NK treatment. Transferring CAR genes to immune cells to create CAR immune cells is the first step in CAR therapy. However, the transfection efficacy of viral vectors in NK cells is much lower than that in T cells; hence, alternative transfection methods should be considered [92, 93]. Although the transfection efficacy could be increased by attempting to transfer genes using electroporation, the stress applied to the cell itself cannot be ignored as described above.

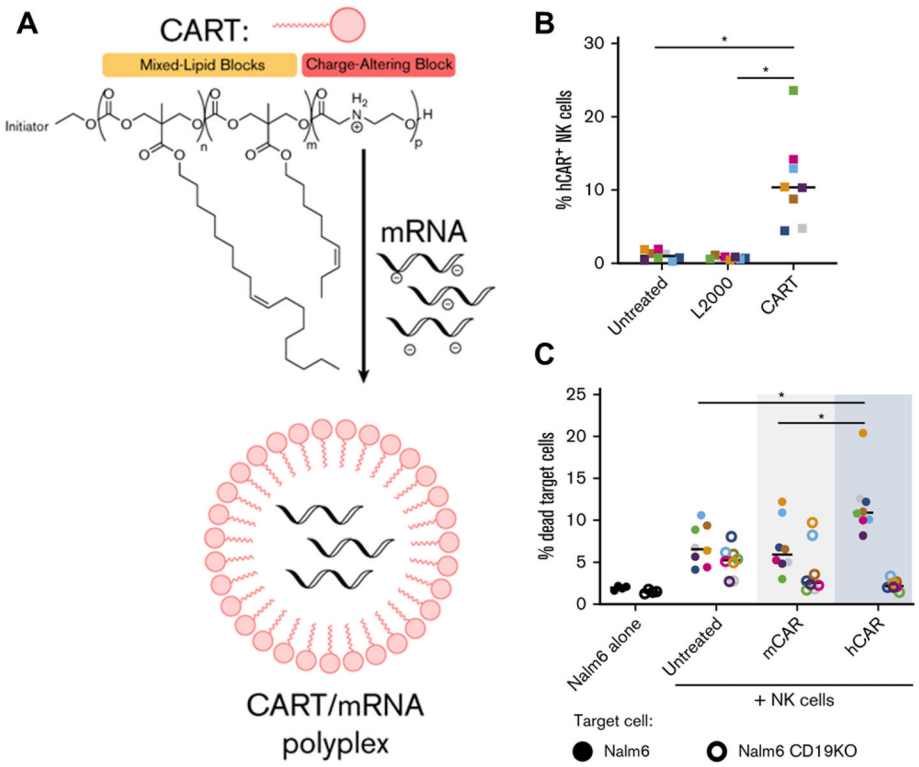

Fig. 6 **A** Schematic diagram of CART/mRNA polyplex preparation. **B** Percentage of antihuman CD19-41BB-CD3ζ CAR expression by isolated primary resting human NK cells. **C** Percentage of dead target cells (CD19 positive Nalm6: filled circles, CD19 knockout Nalm6: open circles) after 20:1 (transfected NK:target) ratio coculture for 6 h before flow cytometric analysis. (Reproduced from a previous report [33] with permission from the American Society of Hematology)

In recent studies, there have been attempts to increase the transfection efficacy through nanoparticles and realize CAR-NK with improved effect [88]. Several characteristics of NPs can be strategically used to increase transfection efficiency. The first is to increase the interaction with the NK cell by controlling the surface and size of the NPs. The addition of targeting moiety, such as anti-CD16 or anti-NK1.1, to the surface of NPs may increase the interaction between NK cells and NPs. Also, the size of NPs affects the endocytosis of NK cells. It is known that the smaller the diameter of NPs, the more advantageous it is to internalize to NK cells through clathrin-mediated endocytosis [94]. The second is that NP can increase endosomal escape. LNPs made of lipids sensitive to the pH environment can mediate endosomal escape of cargo through fusion with the endosome membrane [62, 63]. These strategies have actually been attempted in CAR-T engineering. In addition, since NPs have a significant degree of freedom in selecting cargo, it is also possible to try using surface-active peptides such as melittin [95].

McKinlay et al. developed the charge-altering releasable transporter (CART) as an effective mRNA delivery system [96]. The CART is designed as an oligomer (carbonate-b-α-amino ester) that is cationic at low pH but undergoes rearrangement at pH 7.4. Owing to these properties, oligomers can form complexes with polyanionic molecules, such as mRNA, at low pH levels, protect them, and deliver them intracellularly. Subsequently, proteins are effectively expressed by the translation of the released mRNA, and the oligomers are biologically degraded. Using the CART system, Wilk et al. achieved efficient NK cell transfection and the generation of cytotoxic primary anti-CD19 CAR NK cells (Fig. 6A) [33]. BDK- O_7:N_7:A_{13} was selected from several candidate groups through high-efficiency lipid library screening. Compared with lipofectamine (< 1%), a commercially available transfection reagent, CART BDK-O_7:N_7:A_{13} more successfully delivered antihuman CD19-41BB-CD3ζ CAR mRNAs to isolated resting human NK cells (> 10%) (Fig. 6B). NK cells expressing antihuman CD19 CAR via CART transfection showed superiority in CD107a, TNF-α, IFN-γ, and indicators of activation level. Antihuman hCAR-transfected NK cells showed enhanced killing of the target cells (human CD19 + Nalm6), whereas the negative control antimurine mCAR did not (Fig. 6C). Considering these results, the CART system may be an attractive technology as it can increase the efficiency of transfection through chemical modifications or lipid library alteration [97, 98].

PEI-coated magnetic NPs (MF-NPs) have also been designed for use in CAR-NK therapy in a multifunctional manner [34]. The cationic polymer (PEI) and magnetic core (Zn/Fe) form core–shell structured NPs through adhesive molecules called polydopamine. The PEI shell provides an electrostatic attraction for anti-EGFR CAR pDNA to bind, transfecting NK cells, and the magnetic core is conceived for *in vivo* tracking and imaging through magnetic resonance (MR) (Fig. 7A). Via endocytosis, MF-NP/anti-EGFR CAR pDNA was internalized in NK-92MI

cells, and exhibited a significant level of *in vitro*transfection efficiency (≈60%). This led to a high level of antitumor effect without any specific toxicity to NK cells (Fig. 7B). Stability and efficiency were improved compared with those of the viral vector or EP. The biological behavior of CAR-NK cells *in vivo* can be observed not only through MR but also through a fluorescent imaging device owing to the near-infrared radiation (NIR) fluorescent dye (cyanine 7) conjugated to the PEI shell (Fig. 7C and D). The application of this multifunctional NP can contribute to simplifying and efficiently transforming the process of CAR-NK therapy.

5 Nanoparticle-based CAR-M therapy

Macrophages are the most abundant innate immune cells in solid tumors and constitute up to 50% of the cell mass in the tumor microenvironment (TME) of solid tumors [111]. Since macrophages are phagocytic innate cells and professional APCs, they serve as a bridge between innate and adaptive immunity [99]. Macrophages prime and activate T cells through antigen presentation after phagocytosis by tumor cells [100]. In general, macrophages can be divided into classically activated M1 macrophages and alternatively activated M2 macrophages according to their activation state and function [101]. M1 macrophages are pro-inflammatory, which secrete pro-inflammatory cytokines and chemokines to help eliminate tumor cells, whereas M2 macrophages are anti-inflammatory, which induce immune suppression and promote tumor progression [102].

CAR NK cell therapy has several advantages over CAR-T therapy, but most of the limitations of CAR-T cell therapy can be applied to CAR-NK cell therapy, including target antigen selection, migration to tumor sites, and immunosuppressive TME. In addition, NK cells are not immune cells that are mainly present in the TME and have a short half-life of less than 10 days [103]. This property can be advantageous when severe toxicity occurs; however, repeated administration may be required for a sustained therapeutic response. These limitations made it difficult for NK cells to be selected as an alternative to overcome the limitations of T cells for solid tumors, which eventually led to the emergence of CAR-M [104]. CAR-M therapy refers to the delivery of specific CAR genes to macrophages, with the ability to bind to the tumor cell surface through specific antigen identification and subsequently activate macrophage activity against tumor cells [105]. CAR-M also share

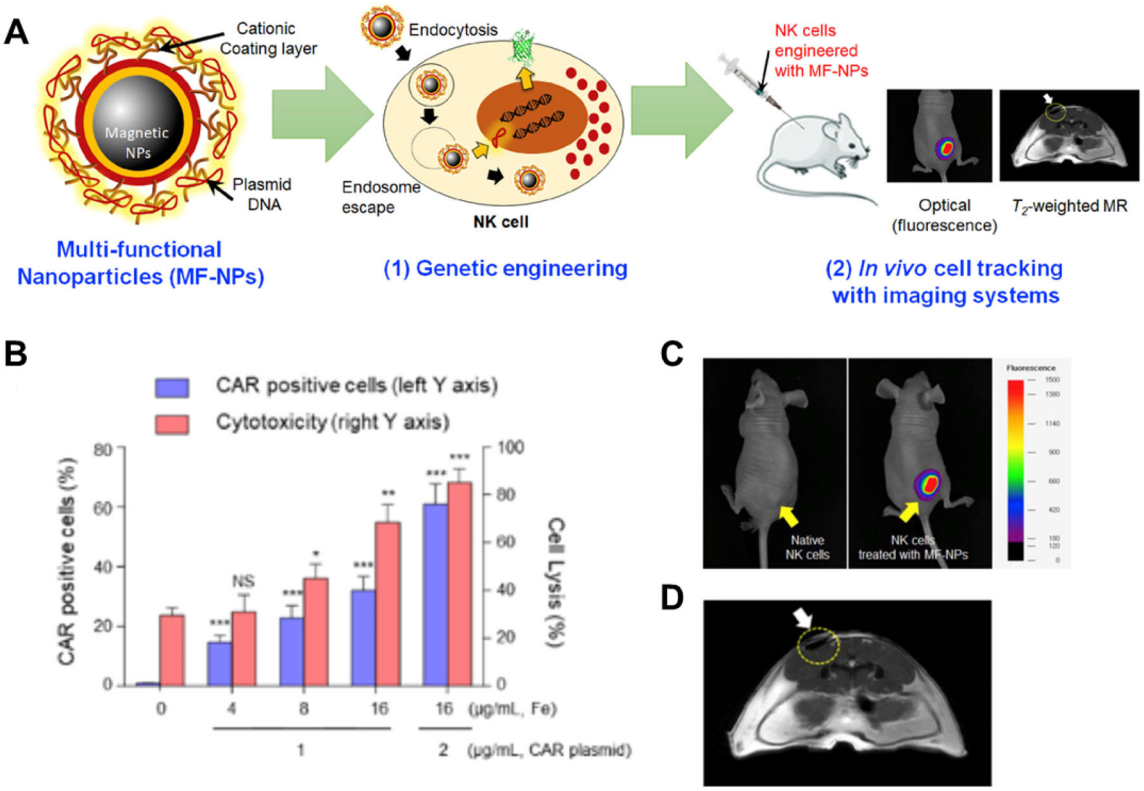

Fig. 7 **A** Schematic illustration of multifunctional nanoparticle (MF-NP) and its bioapplications. **B** Quantitative analysis data of EGFR-CAR expression on NK-92MI surface and cancer cell killing capability of EGFR-CAR expressing NK-92MI cells according to the concentration of MF-NPs. *in vivo* fluorescence, optical imaging **C** and MR imaging **D** of mice transplanted with MF-NP–labeled NK-92MI cells. (Reproduced from a previous report [34] with permission from Elsevier)

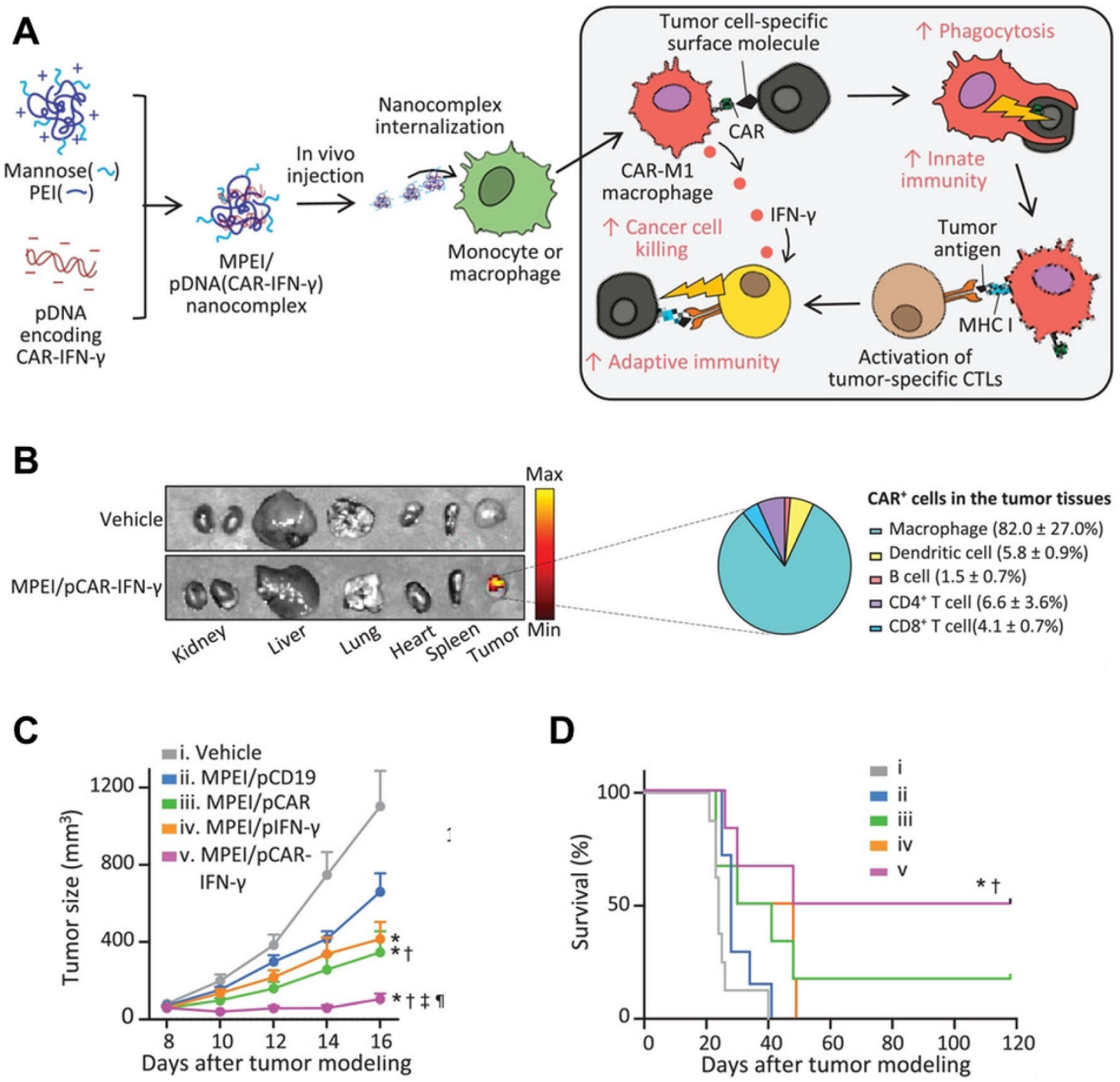

Fig. 8 **A** Schematic diagram of MPEI/pDNA(CAR-IFN-γ) nanocomplex preparation and antitumoral mechanisms of CAR-M1 macrophages. **B** Representative fluorescence images (left) of major organs and tumor 48 h after an intra-tumoral injection of vehicle (negative control) or MPEI/pCAR-IFN-γ. Flow cytometry data (right) of CAR + cell percentages in the tumor tissues (n = 3). **C** Mean tumor growth profiles of Neuro-2a tumor-bearing mice after various treatments (n = 15). **D** Survival rate of Neuro-2a-bearing mice after various treatments (n = 6). (Reproduced from a previous report [35] with permission from Wiley-VCH)

several characteristics and limitations with CAR-T but has two unique advantages over CAR-T: immune cell trafficking and infiltration in (immunosuppressive) TME.

CAR-M is generally produced ex vivo by using viral vectors. This approach is a complex, time-consuming, and expensive process, limiting its clinical application, and there are concerns about the oncogenic potential of viral vectors [106]. In contrast, NP-based strategies have the advantage of using a nonviral vector to reduce the experimental burden and avoid tumorigenicity. Kang et al. reported the *in vivo* programming of CAR-M1 macrophages through nanocomplex-mediated gene delivery (Fig. 8A) [35]. In this study, mannose-conjugated polyethylenimine (MPEI) was used for macrophage-targeted delivery through macrophage-overexpressed mannose receptors [107, 112]. The prepared nanocomplex (MPEI/pCAR-IFN-γ) was formed by electrostatic interactions between the positively charged MPEI and negatively charged CAR-IFN-γ pDNA. The nanocomplex effectively transfected M2 bone-marrow-derived macrophages (BMDMs) to express CAR (with an average transfection efficiency of approximately 14%) and showed a 20-fold increase in IFN-γ expression compared with the control group. Transfected macrophages showed CAR-mediated antigen-specific phagocytosis, and it was confirmed that the M2-to-M1 phenotype shift occurred *in vitro* and *in vitvo* owing to IFN-γ release in an autocrine and paracrine manner. In addition, it was confirmed that 82.0% ± 27.0%

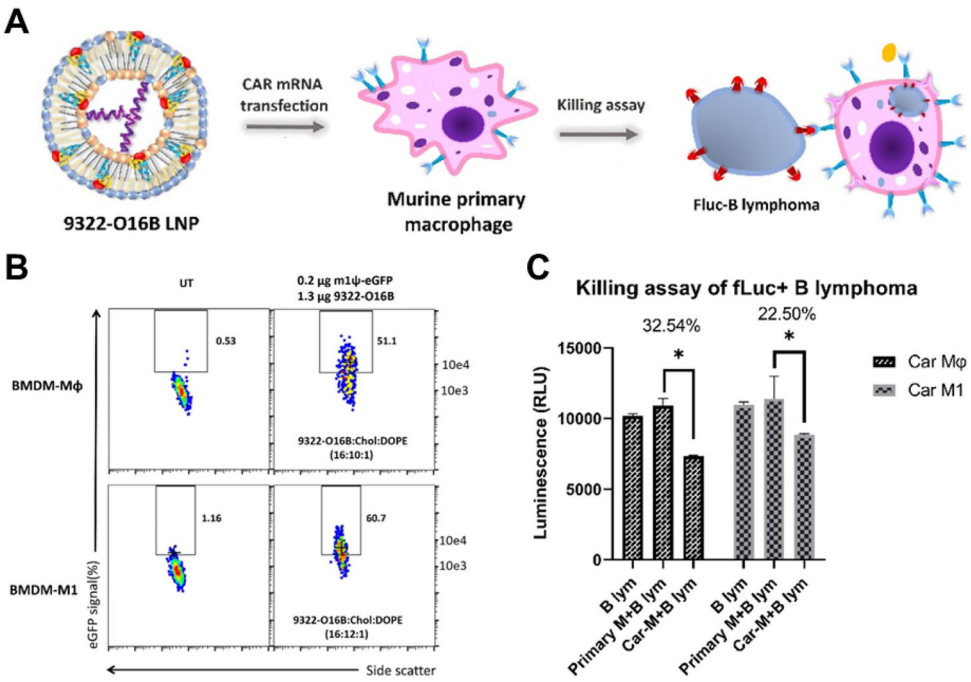

Fig. 9 **A** Schematic illustration of CAR mRNA delivery to murine primary macrophages and killing assay of FLuc$^+$ human B lymphoma. **B** Flow cytometry data of transfection efficiency of m1ψ-eGFP mRNA in Mφ and M1 treated in different formulations of 9322-O16B. **C** Killing assay of FLuc$^+$ B lymphoma following different treatments (Mφ, $p < 0.05$*; M1, $p < 0.05$*). Data are presented as mean ± SD (n = 3). (Reproduced from a previous report [36] with permission from the American Chemical Society)

of CAR expression in the tumor during intratumor injection and 63.4% ± 14.4% after intraperitoneal injection were macrophages, confirming that effective macrophage targeting was achieved (Fig. 8B). MPEI/pCAR-IFN-γ increased the CD8$^+$ T cell immune response, showing the highest CD8/Treg ratio compared with the control group and the best tumor growth inhibitory effect (Fig. 8C and D). This approach has the potential to overcome several challenges of current CAR-T therapy, including the complex procedure of CAR immune cell fabrication and unsatisfactory clinical outcomes of solid tumor therapy.

Ye et al. reported effective CAR expression in primary macrophages through LNP-based mRNA delivery (Fig. 9A) [36]. Through transient CAR expression using LNP-mediated mRNA delivery, an alternative method that can reduce adverse effects (such as host genome integration, on-target, and off-tumor effects) was used [30]. In this study, a top-performing mRNA modification (N1-methylpseudouridine, N1mψ) that showed the highest expression level (53.1%) among mRNA types and an optimized mRNA delivery system (9322-O16B LNP) for macrophages were screened. The LNP (9322-O16B LNP/N1mψ-mRNA) prepared using this method showed a high eGFP-positive cell population of 51.1% for primary BMDM (Mφ) transfection and 60.7% for M1 macrophages (Fig. 9B). Compared with primary BMDM treated with empty LNP, the CAR-Mφ and CAR M1 groups showed significant cytotoxicity against FLuc$^+$ human B lymphoma, for which the luminescence levels were reduced by 32.54% and 22.50%, respectively (Fig. 9C). This study highlights the great potential of adoptive cell therapy for B lymphoma using CAR-macrophages *in vitro*through LNP formulation and mRNA modification.

6 Summary and future perspectives

CAR-T therapy has shown great success in the treatment of hematological cancers. Nevertheless, CAR-T therapy has limitations, such as its limited efficacy for solid tumors, complicated processes, and excessive manufacturing costs. The introduction of nanotechnology can overcome the limitations of conventional CAR-T cell therapy. Owing to their unique properties, NPs can not only serve as a delivery platform for drugs but also target specific cells. NP-based CAR therapy can be applied not only to T cells but also to CAR-NK and CAR-M, compensating for their limitations. In this review, three strategies were introduced in which NPs have been applied to advances in CAR therapy: (i) NP-based CAR-T therapy, (ii) NP-based CAR-NK therapy, and (iii) NP-based CAR-M therapy.

Ex vivo engineering using viral vectors leads to tumorigenicity risks, complicated procedures, and high costs. NP-based CAR-T therapy has been proposed as an alternative to viral vectors in ex vivo engineering. Ad-PAMAM, PEI, PBAE and ionizable LNP successfully produced CAR-T cells *in vivo* or ex vivo by transferring pDNA or mRNA using an NP system and showed lower toxicity, similar treatment effects, and lower cost than conventional CAR-Ts [29, 30]. These results are expected to increase access to CAR-T generation, which is currently only possible in highly specialized centers.

The *in vivo* engineering strategy of CAR-T therapy is designed to compensate for the limitations of ex vivo engineering systems, such as labor intensity and the significant cost and time required because of the complexity of the process. With this concept, it is no longer necessary to extract and isolate T cells from patients, and the proliferation of strongly genetically modified T cells can occur spontaneously in the body of the patient. To generate CAR-T cells *in vivo*, additional targeting strategies that can deliver genetic material to the target T cells are considered. Introduction of pDNA and mRNA delivery using T cell targeting antibody to PNPs. It was confirmed that the anticancer effect of *in vivo* T-cell manipulation was similar to that of the existing method of infusing ex vivo engineered CAR-Ts [31]. In contrast, CD5 targeting LNP encapsulated with FAP-specific CAR-encoding mRNA prevented the progression of fibrosis and restored cardiac function by removing FAP [32]. This research is significant in that CAR-T engineering *in vivo* is not limited to cancer immunotherapy but has potential as a therapeutic agent for various diseases.

NP-based CAR-NK therapy uses the unique properties of T cells and other NK cells. NK cells have an MHC class I independent recognition method, allowing them to maintain aggression against tumor cells with reduced MHC expression. NK cells have a significantly lower transfection efficiency using viral vectors than T cells, and NPs can be an alternative. The CART system delivers CAR mRNA more successfully to isolated resting human NK cells than the commercially available transfection reagent Lipofectamine [33]. PEI-coated magnetic NPs have also been utilized in CAR-NK therapy in a multifunctional manner [34]. These MF-NPs have improved stability and efficiency compared with viral vectors or EPs, and the biological behavior of CAR-NK cells *in vivo* can be observed by MR. Furthermore, CAR-modified NK cells can act as carriers of drugs by themselves, showing synergistic anticancer effects with chemotherapy.

CAR-M therapy involves the delivery of specific CAR genes to macrophages with the ability to bind to the tumor cell surface through specific antigen identification and subsequently activate macrophage activity against tumor cells. CAR-M shares several characteristics and limitations with CAR-T but has two unique advantages over CAR-T treatment: immune cell trafficking and infiltration into the immunosuppressive TME. The MPEI/pDNA nanocomplex was engineered for *in vivo* CAR-M generation, and its effective anti-solid tumor capability showed that CAR expression *in vivo* can be applied to other immune cells as well as T cells [35]. In addition, effective CAR expression in primary macrophages through LNP-based mRNA delivery has been reported previously [36]. This study highlights the potential of adoptive cell therapy for CAR-M B lymphoma *in vitro* through LNP formulation and mRNA modification.

During the past five years, NP-based CAR therapy strategies have shown remarkable growth and potential. Despite high interest, NP-based CAR strategies still fall short compared with viral vectors in terms of experience and sufficient skills to understand and interact with the cellular machinery. Continued research on CAR applications and a better understanding of nanotechnology's interaction with cells will contribute to the development of effective CAR-based anticancer drugs. New nanoparticles designed based on basic research results are expected to maximize the therapeutic effect of not only cancer but also various incurable diseases through effective CAR expression. Recently, gene delivery and vaccine technologies using LNPs encapsulated in mRNA or siRNA have been clinically approved, and LNP-based SARS-CoV-2 vaccines have been a huge success. With this, NP-based genetic engineering technology is expected to develop more rapidly and grow as a promising cancer treatment. Taken together, we believe that the NP-based CAR Therapy introduced in this review will be an effective cancer treatment option in the future.

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Declarations

Conflict of interest The authors declare no conflict of interest.

Ethical statement No animal experiments were carried out for this article.

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