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Nanoparticle-based non-viral CRISPR delivery for enhanced immunotherapy

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The CRISPR Cas9 system has received considerable attention due to its simplicity, efficiency, and high precision for gene editing. The development of various therapeutic applications of the CRISPR system is under active research. In particular, its proven effects and promise in immunotherapy are of note. CRISPR/Cas9 components can be transported in various forms, such as plasmid DNA, mRNA of the Cas9 protein with gRNA, or a ribonucleoprotein complex. Even with its proven gene editing superiority, there are limitations in delivering the CRISPR system to target cells. CRISPR systems can be delivered *via* physical methods, viral vectors, or non-viral carriers. The development of diverse types of nanoparticles that could be used as non-viral carriers could overcome the disadvantages of physical techniques and viral vectors such as low cell viability, induction of immune response, limited loading capacity, and lack of targeting ability. Herein, we review the recent developments in applications of CRISPR system-mediated non-viral carriers in immunotherapy, depending on the targeting cell types, and discuss future research directions.

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Introduction

The discovery and development of clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) systems have provided simple and effective genome editing machinery.[1,2] The CRISPR system is an RNA-mediated adaptive defence system that has evolved in bacteria by capturing snippets of DNA from invading viruses.[3,4] Because of its simplicity, efficiency, and precision characteristics, which are better than the conventionally used gene-editing tools such

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as homing endonucleases, zinc finger nucleases, and transcription activator-like effector nucleases, the CRISPR system has been developed and used widely.[5–7]

The CRISPR system consists of two components; the Cas9 protein, which is an endonuclease, and a single guide RNA (sgRNA) which is a version of the naturally occurring dual guide RNA complex engineered into a single, continuous sequence consisting of CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA).[1] The Cas9 protein contains two nuclease domains, RuvC and HNH, the active sites of which are responsible for the cleavage of opposite DNA strands to induce double-stranded breaks (DSBs).[8] The crRNA in sgRNA consists of a 20-nucleotide guide sequence complementary to the target gene of Cas9, which is located next to the protospacer adjacent motif, while tracrRNA acts as a scaffold that works as an anchor to the Cas9 protein.[9] In this way, tracrRNA assists in the assembly of sgRNA with the Cas9 protein; the complex thus formed is referred to as the ribonucleoprotein (RNP) complex. The resulting RNP induces a DSB at the target gene, followed by endogenous DNA repair through non-homologous end joining (NHEJ) or homology-directed repair (HDR) (Fig. 1). Of these, NHEJ, which induces random insertion or deletion of base pairs at the repair site, occurs predominantly. HDR induces gene repair or insertion with the DNA strand, which has a homologous sequence at the repair site.[10,11]

Due to its simple and precise genome engineering potential, the CRISPR system has been used and applied in various fields. CRISPR systems allow genetic manipulations across the central dogma, genome editing and RNA imaging, RNA editing, and transcription activation or repression with programmable RNA-guided nucleases.[12] For example, researchers have achieved successful gene editing of a plant to increase the crop yield and diversification of varieties by utilizing the CRISPR system.[13] As the CRISPR system can edit the human genome efficiently,[14] its usage is also effective in therapeutic applications.[11] In the clinical field, the CRISPR system can edit

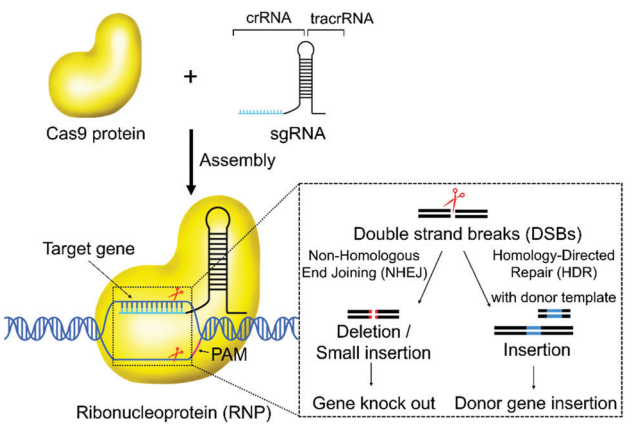

Fig. 1 Mechanism of CRISPR/Cas9. The Cas9 endonuclease protein forms a complex with sgRNA to form a double stranded break at the target DNA sequence, and endogenous DNA repair follows; non-homologous end joining resulting in a gene knockout, or homology directed repair resulting in donor gene insertion.

gene deficiencies or gene mutations that induce diseases. For example, in animal models, Duchenne muscular dystrophy mutations have been treated using the CRISPR system.[15–18] Additionally, the CRISPR system has shown its potential as an effective treatment even in viral infections such as hepatitis B virus, human immunodeficiency virus, and endogenous retrovirus.[19,20]

Although the CRISPR system is universally utilized and effective results are being produced, there are several drawbacks in using viral delivery to cells.[21] There are several viral vectors for CRISPR delivery, such as lentivirus, adeno-associated virus (AAV), and retrovirus. The major limitations of viral CRISPR delivery include the limited CRISPR gene size, its immunogenicity, the undesired integration risk, the sustained expression that can lead to off-target effects, and the high costs associated with challenging manufacturing processes.[22] Although lentivirus or AAV vectors have been widely used for CRISPR delivery owing to their broad tropism and low inflammatory potential, one major drawback is the limited maximum packing capacity of CRISPR genes: 8 kb for the lentivirus vector and 5 kb for the AAV vector.[23] Since the vectors encoding the components necessary for CRISPR/Cas genome engineering are usually large (9–19 kb), the limited CRISPR gene size is a major drawback of using a viral vector.[24] In addition, the immunogenicity of the viral vector as well as any pre-existing T cell and antibody-mediated immunity to it can negatively influence the ability of subsequent administrations of recombinant viruses to boost immune responses further.[25] Viral replication of the viral vector may also increase the risk of genomic integration of DNA derived from certain viruses into the host genome, with its associated risk of insertional mutagenesis or the transactivation of neighbouring genome sequences.[26] The risk of sustained expression of Cas9, elevating its concentration and escalating the risk of off-target editing, is high with viral vectors because they insert the gene encoding the Cas9 protein in the host genome sequence.[27]

To circumvent these limitations of viral vectors in the CRISPR system, non-viral carriers for gene delivery have recently been investigated. Various nanoplatforms such as lipoplexes, polyplexes, and inorganic nanoparticles have been studied in the gene delivery field.[28–31] Non-viral carriers have high loading capacity, chemical design flexibility, high safety and stability, biocompatibility, and low immunogenic response.[32–34] Another beneficial feature of non-viral carriers is the ability to target specific cells by modifying their chemical or physical characteristics.[35–37] These advantages of non-viral vectors extend to the CRISPR delivery system, which could overcome the drawbacks of the current viral CRISPR system.[38–42] For example, lipid nanoparticles loaded with CRISPR mRNA[41] or polyplex nanoparticles loaded with CRISPR plasmid[42] were successfully applied for the treatment of cardiovascular disease or inflammatory disease, respectively, by intravenous injection of the corresponding nanoparticles.

Recently, immunotherapy has attracted significant interest because of its huge potential for the treatment of diverse diseases. Immunotherapy aims to boost adaptive immune responses and retrain the disease-affected immune system to recover and reinforce disease defences.[43–45] The immune system consists of the innate and adaptive immune systems.

The innate immune system functions as an early response to immune reactions, and its major arsenals are composed of macrophages, neutrophils, and monocytes capable of phagocytosis and local killing. The adaptive immune system effects antigen-specific adaptation and memory and utilizes lymphocytes such as B cells and T cells.[46,47] Antigen-presenting cells, particularly dendritic cells (DCs), mediate the connection between innate and adaptive immunity *via* taking up, processing, and presenting antigenic information to B and T cells. Immunotherapy can be applied to treat cancer, autoimmune diseases, and allergic diseases by activating or regulating these immune cells depending on the condition of the disease.[48,49] Recently, immune checkpoint blockade and CAR T-cell therapy have been approved by the FDA as promising cancer therapy methods. Since CRISPR can precisely edit the target genes of immune cells, its application could further extend the potential of immunotherapy.[43]

In this review, we focus on the recently reported non-viral CRISPR delivery systems developed for enhanced immunotherapy. First, we will briefly introduce the non-viral CRISPR delivery mechanism depending on the type of CRISPR-associated cargo (DNA, mRNA, or protein). Next, we discuss the application of non-viral CRISPR delivery to immunotherapy depending on the target cells (cancer cells, innate immune cells, or adaptive immune cells). Finally, future research directions and perspectives on non-viral CRISPR delivery in the field of immunotherapy will be proposed.

The mechanism of non-viral CRISPR delivery depends on the types of CRISPR components

The non-viral CRISPR delivery system consists of non-viral carriers and different forms of CRISPR-associated cargo such as DNA, mRNA, or proteins. Depending on the type of CRISPR-associated cargo, the appropriate design, composition, and physical and chemical properties of non-viral vectors should be considered. Various non-viral carriers, including lipid nanoparticles, polyplex, and inorganic (silica, gold, iron, *etc.*) nanoparticles have been used for different types of CRISPR-associated cargoes.[50–55]

The non-viral carriers, loaded with CRISPR cargos, are internalized in cells mostly through endocytosis, regardless of the cargo form. After endosomal escape followed by the release of the cargo in the cytoplasm, the immediate next events vary depending on the type of cargo (Fig. 2). In any case, the resulting RNP is ultimately translocated to the nucleus to edit the target gene. The typical CRISPR DNA form is a plasmid encoding the Cas9 protein and sgRNA. The Cas9 protein and sgRNA could be encoded in one plasmid DNA (pDNA) molecule or in two different plasmids separately.[56] The strategy for loading plasmids on non-viral carriers mainly uses nanoparticles with positive surface charges that induce electrostatic interactions with negatively charged plasmids.[57] Once the plasmid-loaded non-viral vectors are taken up by eukaryotic cells through endocytosis, endosomal escape to release the plasmid in the cytoplasm follows (Fig. 2A). The released pDNA is transported into the nucleus to access the transcriptional machinery[58] and subsequently transcribed to Cas9 mRNA and sgRNA, which are transported to the cytoplasm, where the mRNA is translated to Cas9 protein. Then, Cas9 assembles with sgRNA to form an RNP complex. The RNP is translocated back to the nucleus and finally starts the designed CRISPR gene editing.[59] The plasmid DNA form is more stable than the RNA form and more cost-effective than the protein form; however, limitations include the risk of genome integration as an off-target effect, and delayed onset.[60]

The other CRISPR cargo that can be combined with non-viral carriers is mRNA encoding the Cas9 protein along with the sgRNA sequence. Cas9 mRNA and sgRNA are mainly assembled with non-viral carriers *via* electrostatic interactions, similar to the plasmid loading strategy. After internalization and endosomal escape, the mRNA is released from the non-viral carrier translated by ribosomes to produce Cas9 protein (Fig. 2B).[61,62] The resulting Cas9 assembles with the delivered sgRNA to form the RNP, followed by translocation in the nucleus and CRISPR gene editing.[63] Delivering Cas9 mRNA and sgRNA is more effective than pDNA in producing RNPs, as it does not require plasmid transcription to RNA in the nucleus and raises less concern for off-target effects because of the transient expression of Cas9. However, RNA is relatively unstable compared to DNA and has lower gene editing efficiency due to its briefer Cas9 protein expression period which leads the short duration of gene modification.[64,65]

The last type of CRISPR cargo is the whole CRISPR RNP, a Cas9 protein/sgRNA complex. RNP delivery enables rapid gene editing by omitting transcription and translation. However, it is difficult to load in non-viral carriers because of its large size and heterogeneous charge.[66] The strategies for RNP loading in non-viral carriers include its encapsulation within the carriers or the utilization of ligands or DNA with high affinity to the protein part of RNP. Once RNP-loaded non-viral carriers are internalized in cells, RNP is expected to be released into the cytoplasm and translocated into the nucleus, and to subsequently initiate CRISPR gene editing (Fig. 2C).[30,67,68]

Application of non-viral CRISPR delivery for enhanced immunotherapy

Targeting cancer cells

Cancers normally develop due to mutations in genes, which are then called oncogenes.[69] Oncogenic mutations lead the cell to survive and proliferate even in abnormal circumstances which would otherwise be deleterious.[70] Cancers also create an environment that is advantageous for survival by selectively controlling the surrounding circumstances. For example, programmed cell death-ligand 1 (PD-L1) on cancer cells plays a critical role in inhibiting the immune response specifically by modulating the activity of T cells *via* binding with PD-1 on T cells.[71] Immune checkpoint inhibitors such as anti-PD-L1 can successfully inhibit PD-1/PD-L1 signalling between cancer cells

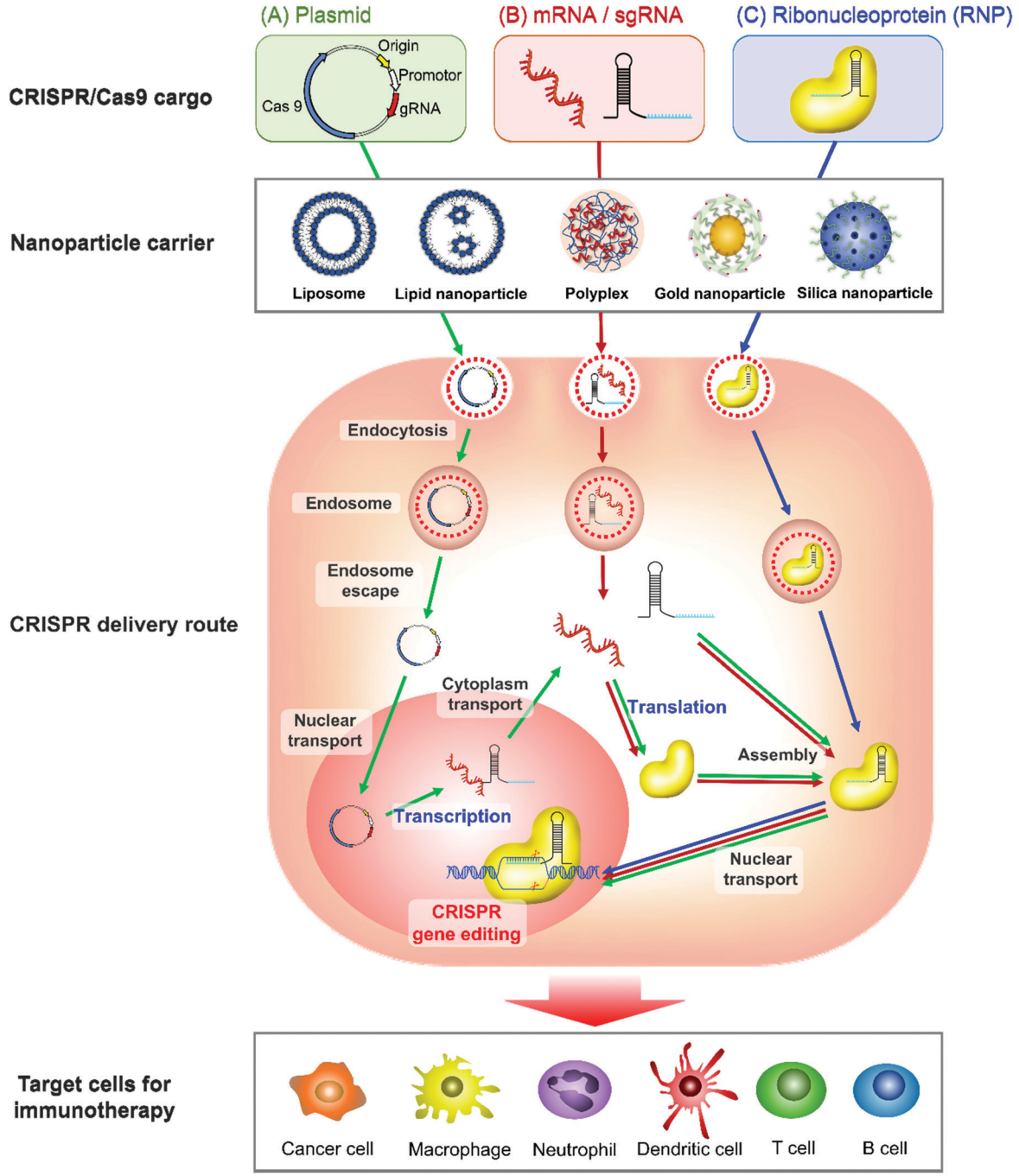

Fig. 2 Intracellular pathways of non-viral carrier mediated CRISPR system delivery depend on the cargo forms, which are (A) plasmid, (B) mRNA, or (C) RNP to target different types of cells for enhanced immunotherapy.

and T cells, making cytotoxic T cells eradicate cancer cells efficiently.[72] CRISPR systems can be used to treat cancer by deleting the gene encoding PD-L1 in cancer cells to enhance cancer immunotherapy.[73–77]

In one study, polyplex nanoparticles delivering pDNA encoding Cas13a and sgRNA for PD-L1 (Cas13a/sgPD-L1 pDNA) have been demonstrated to knock out PD-L1 RNA in cancer cells to enhance immunotherapy efficiency.[77] Cas13a is another type of CRISPR protein that effects the cleavage of single-stranded RNA in the cytoplasm.[12,78] For efficient delivery of pDNA polyplex nanoparticles, the polyplex nanoparticles were encapsulated in dual-locking nanoparticles (DLNPs), which can be selectively activated upon entry into tumour microenvironments (TME) with both low pH and high H2O2 (Fig. 3). The polyplex consisting of pDNA and 4-(hydroxyethyl) phenylboronic acid (HPBA)-modified polyethyleneimine (PEI) was coated with poly(ethylene glycol)-*b*-polylysine polymer, modified with either *cis*-aconitic anhydride (CA) or sodium glucoheptonate dehydrate (SGD). The low pH induced

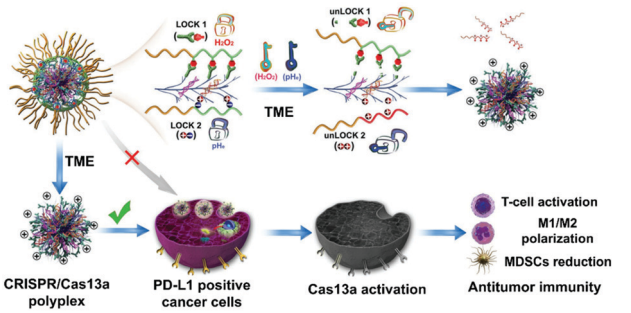

Fig. 3 Dual locking nanoparticle mediated CRISPR pDNA delivery to cleave PD-L1 RNA in tumours for cancer immunotherapy. The intravenous injection of CRISPR pDNA with DLNP induced antitumour immunity, which induced tumour suppression. The dual locking system can only release the CRISPR system in TME with low pH and high H2O2 concentration. Reproduced from ref. 76 with permission from John Wiley and Sons 2019.

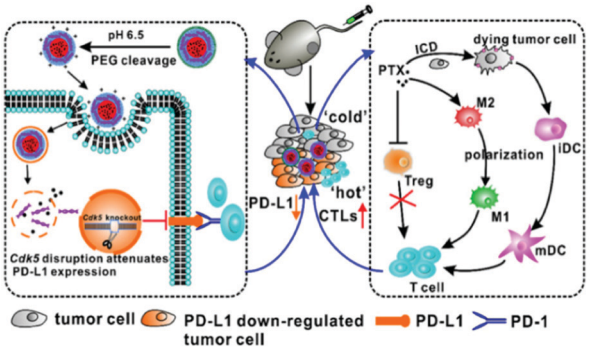

Fig. 4 Co-delivery of the CRISPR system in plasmid form and a small-molecule drug with a polyplex nanoparticle to induce PD-L1 gene knockout and synergistic tumour suppression for cancer immunotherapy *via* intravenous injection. Polyplex nanoparticles release Cas9/sgPD-L1 pDNA and the small-molecule drug only in tumour cells with cleavage of amide bonding of the PEG polymer within the core of the polyplex nanoparticle. Reproduced from ref. 76 with permission from American Chemical Society 2020.

the decomposition of the negatively charged CA groups, leading to the conversion of the anionic polymer to a cationic polymer, which helped the detachment of the coated polymer from the core polyplex. In addition, the high H2O2 concentration induced the cleavage of chemical bonds between the HPBA and SGD groups, resulting in the detachment of the polymer from the core polyplex. The ribosomal RNA cleavage-based assay data showed that RNA cleavage in cancer cells by Cas13a was only possible at pH 6.8 and in high H2O2 conditions. When the DLNP delivering pDNA Cas13a and sgRNA targeting the *PD-L1* gene was intravenously injected into B16F10-bearing mice, the resulting suppression of the tumour cell immune checkpoint protein (PD-L1) led to reduced T cell exhaustion, enhanced T cell-mediated antitumour immunity, and reshaped the immunosuppressive TME (with a reduction of myeloid derived suppressor cells and polarization of M2 macrophages to M1 macrophages). This resulted in enhanced tumour suppression and improved survival rates.

Anti-tumour treatment may be synergistically enhanced by the delivery to tumours of not only the CRISPR system for *PD-L1* knockout but also of anti-cancer drugs. Polyplex nanoparticles delivering the CRISPR/Cas9 pDNA to knock out *PD-L1* and paclitaxel, an anti-cancer drug, have been demonstrated to promote enhanced cancer immunotherapy (Fig. 4).[75] The Cas9/sgPD-L1 pDNA polyplex core nanoparticles loaded with hydrophobic paclitaxel were surface-conjugated with poly(ethylene glycol) *via* amide bonds, forming pH-responsive nanoparticles. The PEG polymer was detached from the core polyplex through the cleavage of amide bonds in the acidic TME. In IFN-γ-stimulated B16-F10 melanoma cells, CRISPR/Cas9 pDNA-delivering nanoparticles were found to induce enhanced *PD-L1* knockout. A melanoma mouse model treated with pH-responsive nanoparticles carrying CRISPR/Cas9 pDNA and paclitaxel *via* intravenous injection showed synergistic tumour suppression. The knockout of *PD-L1* in tumours converts cold tumours into hot tumours, which activates dendritic cells, reduces the population of regulatory T cells, and enhances the polarization of M2 to M1 in the TME, leading to efficient anti-cancer therapy.

Inorganic mesoporous silica nanoparticles (MSNs) have been demonstrated to simultaneously deliver the large Cas9/sgPD-L1 RNP form and axitinib, an anti-cancer small-molecule drug.[76] Mesoporous silica nanoparticles (MSNs) were first loaded with axitinib, conjugated with Cas9/sgPD-L1 RNP by disulphide bonds, and coated with a PEGylated lipid layer (Fig. 5). Upon internalization in B16-F10 melanoma cells, Cas9/sgPD-L1 RNP was dissociated from MSNs by cleavage of disulphide bonds in the reducing microenvironment of the intracellular space, leading to *PD-L1* knockout in melanoma cells. The passive tumour accumulation of the nanoparticles carrying Cas9/sgPD-L1 RNP in B16-F10 tumour-bearing mice after intravenous injection resulted in tumour suppression due to the synergistic effect of *PD-L1* knockout and the anti-cancer drugs. The *PD-L1* knockout converted a cold immunosuppressive tumour into a hot tumour, which is more susceptible to CD8+ cytotoxic T cells. Furthermore, axitinib suppressed the immunosuppressive regulatory T cells, further unleashing T-cell mediated antitumour immunity, and eventually enhanced tumour growth inhibition.

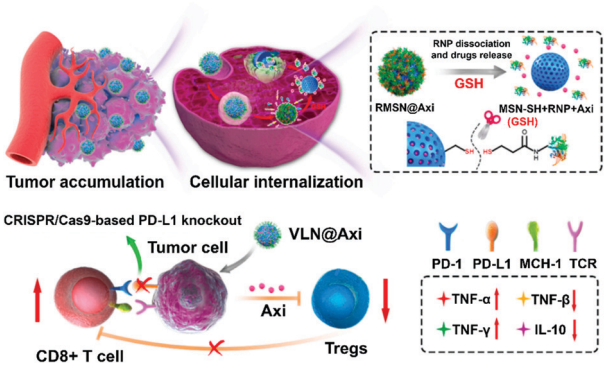

Fig. 5 Co-delivery of the CRISPR system in RNP form and a small-molecule drug with virus-like nanoparticles to induce PD-L1 gene knockout in tumour cells and suppress the proliferation of regulatory T cells for cancer immunotherapy *via* intravenous injection. Virus-like nanoparticles release Cas9/sgPD-L1 RNP and the small-molecule drug only in tumour cells, with cleavage of disulfide bonding of RNP from the nanoparticle. Reproduced from ref. 75 with permission from Elsevier 2020.

Targeting innate immune cells

Innate immune cells comprise NK cells, macrophages, neutrophils, dendritic cells (antigen-presenting cells), and granulocytes.[79] Here, we introduce recent studies on CRISPR delivery based on non-viral carriers to knock out specific genes in innate immune cells for the treatment of cancer and diabetes and reduction of side effects of transplantation.

Targeting macrophages. Macrophages are myeloid immune cells that engulf and degrade dead cells, debris, and foreign materials. They also modulate inflammatory processes.[80] M1-polarized macrophages are usually considered anti-tumour, pro-inflammatory, classically activated macrophages, whereas M2-polarized macrophages are commonly deemed tumour-associated, anti-inflammatory, and alternatively activated macrophages.[81] The tumour-associated macrophages (TAMs), mostly belonging to the M2 phenotype, are one of the key cell types that generate an immunosuppressive TME by producing immunosuppressive cytokines, chemokines, growth factors, and triggering the release of inhibitory immune checkpoint proteins in T cells, which inhibit cancer immunotherapy.[82] Genetic editing by the viral CRISPR system, modulating anti-inflammatory M2 macrophages to anti-tumour M1 macrophages can slow or stop cancer growth with the direct activity of M1 macrophages to stimulate Th1 cytotoxic T cells and other effector cells.[83]

The non-viral CRISPR system targets inhibitory receptors on macrophage surfaces to regulate the activity of immunosuppressive macrophages. Signal regulatory protein alpha (SIRP-α), expressed on TAMs, is a critical regulator of phagocytic macrophage activation and serves a broader role as a myeloid-specific immune checkpoint.[84] SIRP-α on TAMs binds with CD47 over-expressed by most cancer cells, allowing macrophages to bypass cancer cells. Therefore, downregulation of SIRP-α expression in macrophages could enhance macrophage activation, which could be useful in cancer immunotherapy. For this purpose, cationic arginine-coated gold nanoparticles were selected to deliver the Cas9/sgSIRP-α RNP in macrophages for cancer immunotherapy (Fig. 6).[85] The Cas9 protein with glutamic acid peptide tags was mixed with arginine-coated gold nanoparticles, and self-assembled superstructures were generated *via* carboxylate–guanidinium binding. The phagocytic ability of *SIRP-α* knockout RAW 264.7 cells against human osteosarcoma cells was 4-fold higher than that of non-edited RAW264.7 cells. The validation of arginine-coated gold nanoparticle-mediated CRISPR RNP delivery in an *in vivo* model to enhance anti-cancer treatment remains to be validated.

Non-viral CRISPR delivery to macrophages for M1 polarization has been shown in a 3D co-culture system of breast cancer cells and macrophages mimicking the TME. mTORC2 signalling regulates the generation of M2 macrophages; Rapamycin-insensitive companion of mammalian target of rapamycin (RICTOR) is an mTORC2 adaptor protein. Deletion of the *RICTOR* gene in macrophages induces M1 macrophage polarization.[86] The M2 macrophages in an immunosuppressive 3D TME formed by co-culture of breast cancer spheres and M2 macrophages were transformed into M1 macrophages by the infiltration of liposomes loaded with Cas9/sgRICTOR RNP.[87] These nanoparticles

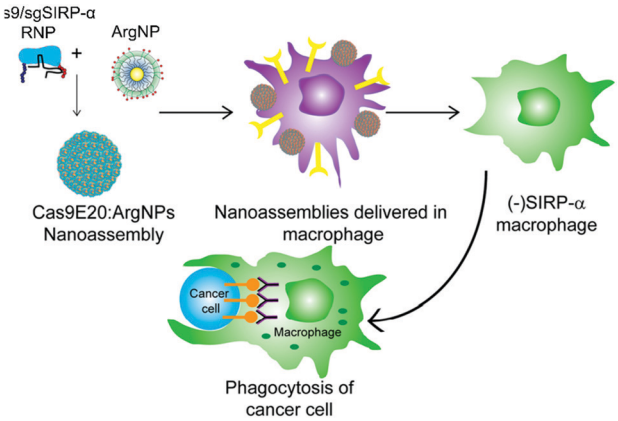

Fig. 6 Arginine-coated gold nanoparticle-mediated delivery of CRISPR RNP to SIRPα gene knockout macrophages for enhancing macrophage phagocytosis of cancer cells *in vitro*. Reproduced from ref. 84 with permission from American Chemical Society 2018.

were demonstrated in an *in vivo* subcutaneous tumour model to induce successful M1 polarization after intratumoral injection.

Macrophages are important pro-inflammatory cells during the development of type 2 diabetes (T2D) and are believed to contribute to the pathogenesis of various diabetic complications.[88] Macrophage accumulation in adipose tissue induces chronic inflammation and insulin resistance associated with T2D.[89] As the progression of diabetes is correlated with the severity of inflammatory reactions, diabetes treatment may be possible by suppressing adipose tissue inflammatory reactions.[90]

The netrin 1 protein, encoded by the *NTN1* gene, is secreted by adipose tissue macrophages, and promotes the recruitment of macrophages, which leads to chronic inflammation. Therefore, downregulation of netrin 1 expression has been reported to be a potential therapeutic target in macrophages for treating T2D.[91,92] Macrophage-specific knockout of the *NTN1* gene was achieved using lipid-based nanoparticles for immunotherapy of T2D.[91] In one study, macrophage-specific promoter (CD68)-driven Cas9/sgNtn1 plasmids were encapsulated in cationic lipid-assisted PEG-*b*-PLGA nanoparticles.[88] Although the resulting lipid-based nanoparticles could be taken up by various types of cells, Cas9/sgNtn1 plasmids could be expressed only in macrophages, which activate the CD 68 promoter, leading to successful macrophage-targeted CRISPR (Fig. 7). Intravenous injection of Cas9/sgNtn-delivering nanoparticles into type 2 diabetic mice improved glucose tolerance and insulin sensitivity by inhibiting NETRIN-1 expression in macrophages and subsequently reducing macrophage retention in adipose tissue. Although intravenously injected nanoparticles accumulate in various tissues, using the CD68 promoter helped avoid off-target effects.

Surface engineering of CRISPR nanoparticles to target macrophages has also been proposed as another strategy to enhance macrophage-specific gene knockout. The NLRP3 inflammasome cleaves interleukin (IL)-1β and IL-18 precursors into their mature forms and causes the release of several pro-inflammatory cytokines.[93] Thus, the NLRP3 inflammasome plays critical roles in the initiation and progression of diverse

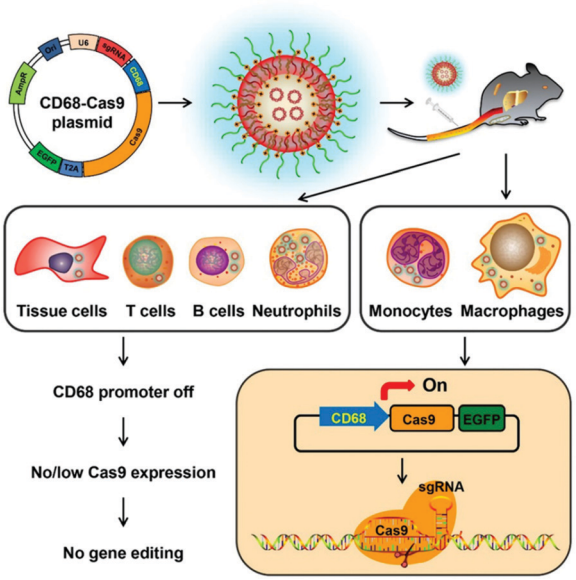

Fig. 7 Macrophage-specific gene editing with the delivery of cationic lipid-assisted PEG-*b*-PLGA nanoparticles loaded with CRISPR pDNA for macrophage-specific promotor-driven NTN1 gene knockout to achieve type 2 diabetes immunotherapy. Reproduced from ref. 90 with permission from American Chemical Society 2018.

inflammatory diseases. Macrophage-targeting lipid-based nanoparticles loaded with CRISPR mRNA to knock out the *NLRP3* gene encoding the NLRP3 inflammasome in macrophages have been demonstrated for the treatment of T2D (Fig. 8).[94] The various combinations of surface charge and PEG density of polymeric nanoparticles were tested and optimized to enhance their internalization in macrophages. The optimized nanoparticles, after intravenous injection, could preferentially deliver Cas9 mRNA with NLRP3 sgRNA to macrophages and inhibit the production of serum IL-1β and Caspase-1 p-10. Glucose tolerance was recovered in high-fat-diet-induced T2D mice.

Targeting neutrophils. Neutrophils are the first line of innate immune defence against infectious diseases. Activated neutrophils provide signals for the activation and maturation of macrophages and dendritic cells. Neutrophils play a crucial role in the regulation of both innate and adaptive immunity during inflammatory conditions.[95] As reducing neutrophil activity could minimize inflammation, genetic editing of neutrophils to reduce their activity can be a promising route for effective diabetes treatment.[96]

Lipid-based nanoparticles with surface engineering to enhance targeting were used to deliver the CRISPR system to neutrophils to mitigate insulin resistance in T2D mice (Fig. 9).[97] Neutrophil elastase is a serine proteinase that proteolyzes collagen-IV and elastin in the extracellular matrix, facilitating the infiltration of neutrophils into the tissue. Therefore, the knockout gene encoding neutrophil elastase in neutrophils could be a target for decreasing infiltrated neutrophil-mediated inflammation.[98] The CRISPR Cas9 plasmid encoding the Cas9 protein and sgRNA targeting the neutrophil elastase gene were loaded into neutrophil-targeting lipid-based nanoparticles. Intravenous injection of the resulting nanoparticles could downregulate neutrophil elastase expression in the white adipose tissue and liver. The modulation of neutrophil-related inflammation increases glucose tolerance in high-fat diet-T2D mice. This shows that weakening the inflammatory microenvironment by directly modulating innate immune cells with nanoparticles carrying the CRISPR system could potentially treat T2D.

Targeting dendritic cells. DCs are antigen-presenting cells that initiate antigen-specific adaptive immune responses.[99] They activate T cells by presenting antigenic peptides in the context of MHC molecules to the T cell receptor and simultaneously provide co-stimulatory signals to induce T cell priming, proliferation, and differentiation. In transplant surgery, graft rejection is mostly caused by T cell-mediated immune responses, and DC-mediated antigen presentation plays a key role in T cell activation.[100] However, when antigenic peptides are present in the absence of co-stimulatory signals, T cell activation can be inhibited, resulting in energy and immunoregulation.[101] Transplantation is usually

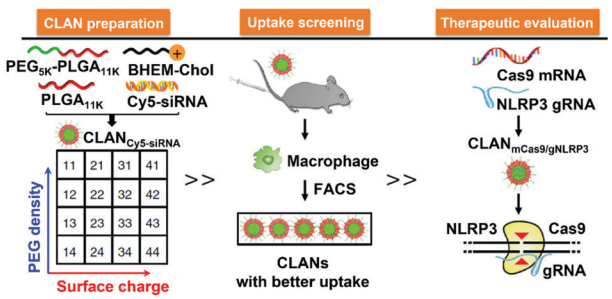

Fig. 8 General scheme for obtaining macrophage-targeting lipid-based nanoparticles delivering CRISPR to knock out NLRP3 in macrophages. Different surface charges or PEG densities were screened to evaluate macrophage uptake of the nanoparticles. The optimized nanoparticles were loaded with mCas9/sgNLRP3 and used for T2D treatment. Reproduced from ref. 92 with permission from Springer Nature 2018.

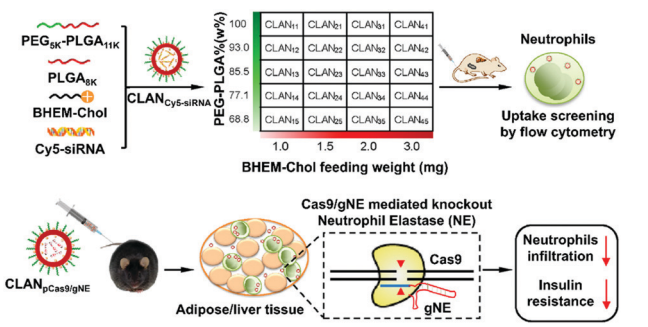

Fig. 9 General scheme for obtaining neutrophil-targeting lipid-based nanoparticles delivering CRISPR to knock out neutrophil elastase in macrophages. Different nanoparticle compositions were screened to evaluate neutrophil nanoparticle uptake. The intravenous injection of optimized nanoparticles loaded with the Cas9/sgNE plasmid results in suppressed neutrophil infiltration of adipose and liver tissue. This leads to the decrease of inflammation and subsequent reduction of insulin resistance for T2D treatment. Reproduced from ref. 96 with permission from Elsevier 2018.

accompanied by immune rejection, in which recipient DCs initiate the activation of T cells with indirect allospecificity.[102,103] Therefore, genetic editing of DCs in organ recipients to suppress the expression of co-stimulatory molecules could be a promising way to circumvent transplant rejection.

In one study, Cas9 mRNA and an sgRNA targeting the co-stimulatory molecule CD40 of DCs were delivered using lipid-based nanoparticles to reduce CD4 T cell activation in the recipient against skin grafts (Fig. 10).[104] An acute graft rejection mouse model was constructed using BALB/C mice as graft donors and C57BL/c mice as recipients of skin transplantation. CD40 protein expression in DCs retrieved from lymph nodes and the spleen was significantly decreased after intravenous injection of mCas9/sgCD40 nanoparticles, indicating that mCas9/sgCD40 was successfully delivered into the DCs by the systemic administration of the nanoparticles. This subsequently inhibited T cell activation, indicated by a decline in the expression of the activation (CD69) and memory (CD44) markers of T cells. The histology of the skin grafts showed more intact tissue alignment and less graft damage in mCas9/sgCD40 nanoparticles. Skin graft rejection was recorded every day using the scoring system, and treatment was found to significantly relieve graft rejection. Graft survival was also dramatically prolonged with intravenous injection of mCas9/sgCD40 nanoparticles.

Targeting adaptive immune cells

Targeting T cells. T cells are a representative population of adaptive immune cells. Naive CD8+ T cells can be activated by binding and recognizing antigenic peptide fragments presented from the major histocompatibility complex (MHC) of DCs, differentiating into cytotoxic T cells that can kill pathogen-infected cells or cancer cells.[45] However, the immune check-points such as the binding of PD-1 on cytotoxic T cells and PD-L1 on cancer cells inhibit the cytotoxic T cell from eradicating cancer cells efficiently. Therefore, genetic editing of T cells to suppress PD-1 expression to evade immune check-points has been proposed for cancer immunotherapy.

Liposomes encapsulated with CRISPR/Cas9 in RNP form, targeting PD-1 in T cells, has been proposed for enhanced cancer immunotherapy (Fig. 11).[105] A plasmid co-expressing a human codon-optimized Cas9 gene together with PD-1 sgRNA was encapsulated in liposome nanoparticles. *PD-1* knockout T cells *via* CRISPR nanoparticles showed significantly greater cytotoxicity against cancer cells than control T cells. Although *PD-1* knockout T cells showed less proliferation and greater apoptosis both *in vitro* and *in vivo*, they exhibited significantly higher tumour suppression ability than the controls. The liposome-mediated CRISPR system delivery to knock out *PD-1* in T cells thus demonstrated the potential of the CRISPR system in cancer immunotherapy.

Targeting B cells. B cells include short- or long-lived plasma cells, germinal centre cells, and memory cells.[106] B cells also induce autoimmune diseases, such as haemolytic anaemia and rheumatoid arthritis. Autoimmunity results from a breakdown of self-tolerance involving humoral or cell-mediated immune mechanisms. The consequences of failure in central or peripheral tolerance include the survival and activation of self-reactive B cells. Such B cells produce pathogenic autoantibodies, which can form complement-fixing immune complexes that contribute to tissue damage.[107] The depletion of B cells using the CRISPR system is a potential treatment for autoimmune disease.[108]

Lipid nanoparticle-mediated delivery of CRISPR pDNA to disrupt B-cell activating factor receptor (BAFFR) expression in B cells has been demonstrated to have a therapeutic effect in rheumatoid arthritis (Fig. 12).[109] The BAFFR protein, encoded by the *B220* gene, is required for B-cell maturation and survival. BAFFR-dependent pro-survival signals are necessary to rescue immature B cells from premature cell death at an early stage of B cell development.[110,111] In this study, C57BL/6 mice were

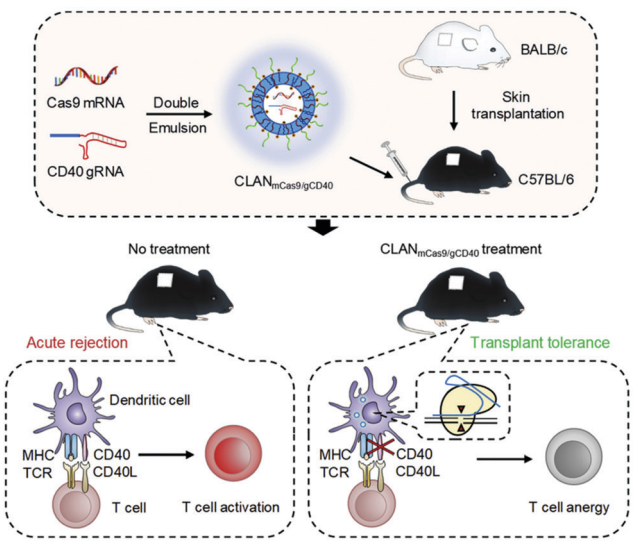

Fig. 10 Specific dendritic cell gene editing using lipid-based nanoparticles targeting dendritic cells loaded with mCas9/sgCD40 for CD40 gene knockout to achieve transplant tolerance. Reducing the expression of CD40 inhibited T-cell activation, reduced graft damage, and subsequently prolonged graft survival. Reproduced from ref. 103 with permission from Elsevier 2019.

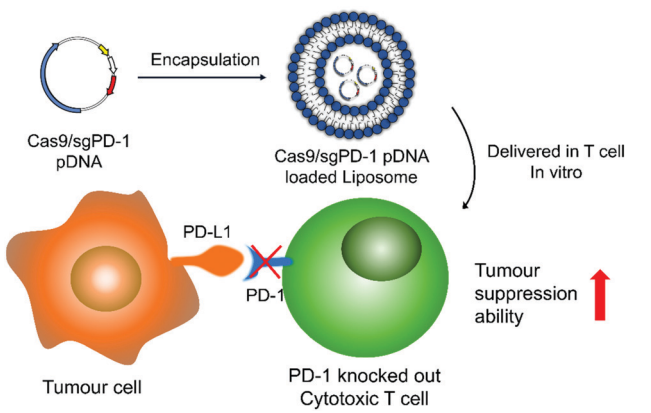

Fig. 11 Liposome mediated delivery of CRISPR pDNA to PD-1 gene knock out in cytotoxic T cell for avoiding the immune check point with cancer *in vitro*. Subsequently, PD-1 knocked out T cells showed high tumour suppression ability.

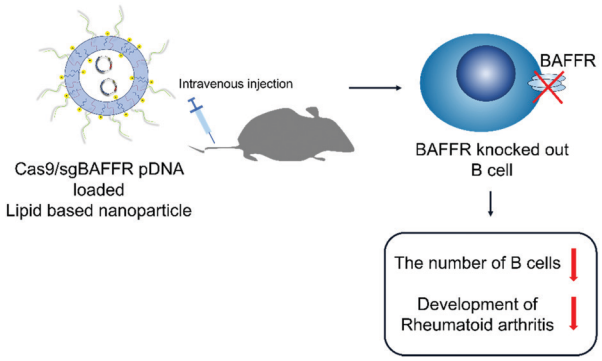

Fig. 12 Lipid based nanoparticle mediated delivery of CRISPR pDNA to BAFFR knock out in B cells *via* intravenous injection for treatment of autoimmune disease, rheumatoid arthritis. Knocking out of BAFFR in B cells induced decrease of the number of B cells and decrease of rheumatoid arthritis development.

intravenously injected with nanoparticles encapsulated with the Cas9/sgB220 plasmid and Cy5 siRNA daily for 5 days, and mononuclear cells were isolated from the spleen and lymph nodes. Cy5+ B cells in the spleen and lymph nodes from NP cas9/sgB220 treated mice had a higher percentage of B 220 negative cells than other groups, demonstrating the decrease of B cells was achieved by delivery of nanoparticles loaded with Cas9/sgB220 pDNA. As a result, the therapeutic effects in a rheumatoid arthritis model by downregulating the number of B cells was demonstrated.

Conclusions

Immunotherapy has attracted significant interests in recent years because of its huge potential to boost adaptive immune responses and to retrain the disease-affected immune system for the treatment of various diseases. Due to its extraordinary capability for specific gene editing, CRISPR system have been applied to increase the efficacy of immunotherapy. We have reviewed the use of various non-viral carriers designed to deliver CRISPR to specific target cells including cancer cells, macrophages, neutrophils, dendritic cells, T cells, and B cells for enhanced immunotherapy. Depending on the types of CRISPR molecules, target cells, and target organs, the physical and chemical characteristics of non-viral carriers such as surface charge, composition ratio, size, and composition, have been appropriately designed to improve the loading efficiency of CRISPR molecules and targeting efficiency to the cells. The recent works have demonstrated its potentials in preclinical studies of cancer, diabetes, and organ transplantations, though more investigations are needed.

Despite these achievements, there are several points to be considered and investigated to determine the feasibility of successful application of non-viral carriers to CRISPR-mediated immunotherapy. Although most non-viral carriers are based on high biocompatibility, the immunogenicity of biomaterials and immune responses associated with the payload must be further investigated. There are diverse compositions such as liposome, lipid nanoparticles, polymers, inorganic materials (gold nanoparticles, silica nanoparticles) that have been studied so far for CRISPR delivery, however, FDA-approved materials composition would be potentially more applicable to *in vivo* gene editing *via* CRISPR delivery in clinical study.

The targeting efficiency to specific immune cells needs to be improved. As there have been accumulated information on the nanoparticle targeting to tumor and cancer cells, this would be beneficial for nanoparticle-mediated CRISPR delivery to cancer cells. However, it is relatively more difficult to target and deliver the CRISPR system to lymphocytes such as T cells and B cells using nanoparticles, as they are not phagocytic cells and are not efficient to uptake carriers. *In vivo* lymphocyte targeting using non-viral nanoparticles has been mostly accomplished *in vitro*, and there are only a few studies to specifically target those cells *in vivo*. As T and B cells play important roles in the adaptive immune system and are promising targets for immunotherapy, more researches should be conducted to validate the targeted delivery of CRISPR-based on non-viral carriers to T and B cells. For disease-specific applications of the CRISPR system in immunotherapy, specific targeting to a particular type of immune cells must be designed in the future. Cell-specific gene editing could be further assisted by combining selective genetic manipulation using plasmids with promoters; the non-viral carriers would be capable of more specific gene editing in the target immune cells.

Given the high potential of the CRISPR system as a promising technique for immunotherapy and the accumulated understanding of nanotechnology applied to living organisms up to now, the synergy of these technologies is highly anticipated to achieve enhanced immunotherapy.

Conflicts of interest

There are no conflicts of interest to declare.

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