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Review

Direct *in vivo* CAR T cell engineering

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T cells modified to express intelligently designed chimeric antigen receptors (CARs) are exceptionally powerful therapeutic agents for relapsed and refractory blood cancers and have the potential to revolutionize therapy for many other diseases. To circumvent the complexity and cost associated with broad-scale implementation of *ex vivo* manufactured adoptive cell therapy products, alternative strategies to generate CAR T cells *in vivo* by direct infusion of nanoparticle-formulated nucleic acids or engineered viral vectors under development have received a great deal of attention in the past few years. Here, we outline the *ex vivo* manufacturing process as a motivating framework for direct *in vivo* strategies and discuss emerging data from preclinical models to highlight the potency of the *in vivo* approach, the applicability for new disease indications, and the remaining challenges associated with clinical readiness, including delivery specificity, long term efficacy, and safety.

Highlights

Adoptive cell therapy using chimeric antigen receptor (CAR) T cells is effective against B cell malignancies; however, the complex manufacturing process and financial realities constrain the scalability of the approach.

The *in vivo* generation of CAR T cells, and possibly other immune cells, using off-the-shelf products therefore has numerous logistical and functional advantages.

In preclinical models, *in vivo* gene delivery using nanoparticles or viral vectors has yielded CAR T cells with therapeutic equivalency to *ex vivo* generated CAR T cells.

Ongoing research efforts are attempting to determine how to best target and leverage effector cells of interest (T cells, macrophages etc.), understand how direct *in vivo* CAR generation interfaces with other immune cells, and optimize design elements of the viral vectors or nanoparticle and nucleic acid formulations.

Therapeutic retargeting of T cells with synthetic receptors

CAR T cell therapy has been a breakthrough approach for the treatment of relapsed/refractory (r/r) B cell malignancies. The synthetic CAR molecule fuses a targeting moiety, typically an antibody-derived **single chain variable fragment (scFv) (**see Glossary), to intracellular CD3ζ and co-stimulatory domains (such as CD28 or CD137; also known as 4-1BB) to redirect T cell function toward tumor surface-expressed antigens. Although the greatest progress has been made in the treatment of leukemia, lymphoma, and myeloma (Box 1), the modularity of the CAR molecule renders it highly versatile, and the preclinical and clinical pipeline is full of novel CAR targets and constructs for hematologic malignancies, solid tumors, and nononcology applications (i.e., autoimmune diseases, infectious diseases, etc.) [1]. Within the past 10 years, innovative new CAR designs and long-term clinical results have emerged that have cemented the therapeutic power of the synthetic immune receptor. However, this success raises the enormous challenge of how to clinically implement a bespoke *ex vivo* manufactured cell product at a broad scale for cancer indications and beyond. Here, we describe the difficulties associated with current *ex vivo* CAR T cell manufacturing practices and discuss two technological advances, nanoparticles encapsulating CAR encoding nucleic acid and viral vectors encoding the CAR, that can be infused directly into a patient to address this unmet need for a universal off-the-shelf product.

Current CAR T cell manufacturing practices

All currently approved CAR T cell products, and a majority of cell therapies undergoing clinical testing, are produced *ex vivo* from patient autologous T cells. Preparation of this bespoke treatment involves a multistep process that requires highly skilled technicians, strict quality management systems, and specialized facilities and equipment (Figure 1A) and is reviewed in [2]. Since viral transduction methods are commonly used to deliver the CAR transgene to T cells, **γ-retroviral** or **lentiviral vectors** must be manufactured by introducing source plasmids into producer cells to generate viral particles. The T cells from a patient are collected by leukapheresis, transported to a manufacturing facility, activated in culture by CD3 and CD28 co-stimulatory molecules and cytokines, transduced with virus, and expanded to yield an individualized cell

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Box 1. Clinical state of CAR T cell therapy for hematological cancers

CAR T cells targeting CD19, which is expressed on B cells, including malignant B cells, have generated complete response (CR) rates ranging from 40 to 54% in r/r aggressive B cell lymphomas and 71–81% in patients with r/r B cell acute lymphoblastic leukemia (B-ALL) [87–91]. CAR T cells targeting B cell maturation antigen (BCMA), which is uniquely expressed on plasma cells, including malignant plasma cells, have also yielded remarkable successes in patients with r/r multiple myeloma [92,93]. Long-term data now show that CAR T cells can persist *in vivo* and this therapy can maintain remissions for close to a decade in patients who were previously thought to be incurable [94,95]. Since the first CAR was approved in 2017, there are now six FDA-approved products: axicabtagene ciloleucel, tisagenlecleucel, lisocabtagene maraleucel and brexucabtagene autoleucel (targeting CD19), and idecabtagene vicleucel and ciltacabtagene autoleucel (targeting BCMA) (a detailed review of clinical outcomes is described in [96]). In addition to commercial products, manufacturing CAR T cells locally, in a point-of-care hospital setting, has been demonstrated to be feasible in a number of jurisdictions [97–100] and reviewed in [101].

product for the patient comprising hundreds of millions of modified T cells. Patient-to-patient variability, which can be exacerbated by previous treatments, as well as semi-random integration of the virus and variegated expression all contribute to heterogeneity in product quality and yield, and overall response [2–4]. While the cells are being modified, the patient undergoes **lymphodepletion** to facilitate engraftment and promote the function of their newly modified cells [5]. The CAR T cells must pass a variety of release tests to ensure their quality and safety [6], then be transported to the treatment site and infused into the patient. Due to the complexity of this process, the time between T cell harvest and CAR T cell injection (vein-to-vein time) is often several

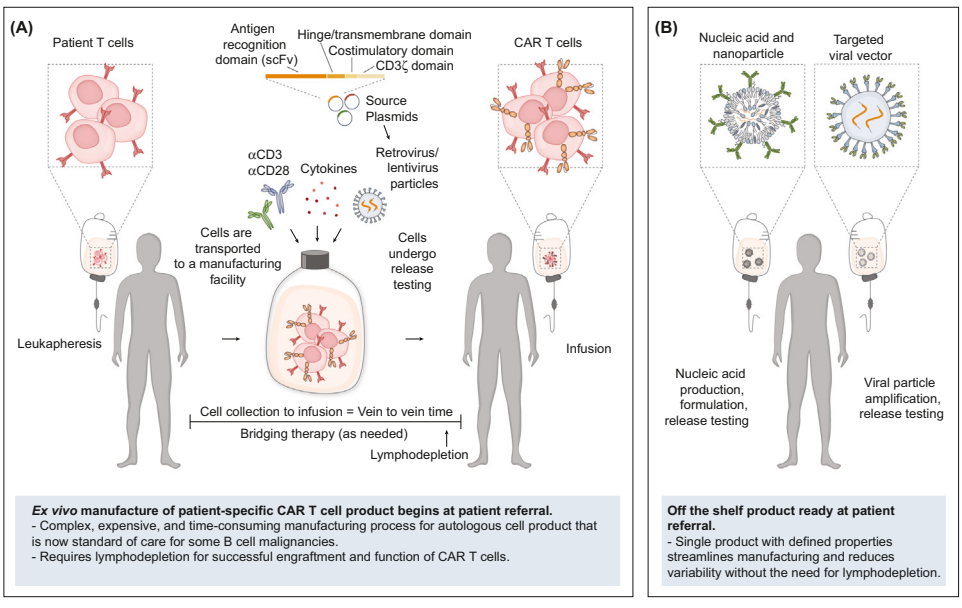

Figure 1. Overview of manufacturing strategies for *ex vivo* and *in vivo* cell therapy. (A) Once a patient is referred to receive autologous *ex vivo* manufactured chimeric antigen receptor (CAR) T cell therapy, their T cells are harvested by leukapheresis. The cells are transported to a centralized manufacturing facility where they are stimulated to proliferate and transduced with a viral vector encoding the CAR genetic material. Following release testing, the cells are transported back to the treatment facility and are ready for infusion. The time between harvest and infusion varies from 2 to 7 weeks and is termed the vein-to-vein time, during which patients may need bridging therapy for rapidly progressing disease. Immediately prior to CAR T cell infusion, the patient receives lymphodepleting chemotherapy (commonly with cyclophosphamide and fludarabine) to enhance the engraftment and function of the engineered T cells. (B) Either CAR encoding nucleic acid formulated in a nanoparticle, or a CAR encoding viral vector would be produced and undergo release testing in a large batch and be ready as an off-the-shelf product for when a patient receives a referral.

Glossary

Adeno-associated virus (AAV): nonenveloped virus, from the parvovirus family, used to deliver a transgene to target cells.

Cytokine release syndrome (CRS): systemic inflammatory response caused by the mass release of cytokines from activated immune cells. Represents a significant CAR T cell therapy-associated toxicity.

Designed ankyrin repeat protein (DARPin): engineered single-domain proteins with a naturally occurring ankyrin-derived scaffold and a binding surface, which can be selected *in vitro* to display high affinity and specificity to a target. Used as a targeting ligand on the surface of the AAV viral capsid to enable cell type specific transduction.

Immune cell-associated neurotoxicity syndrome (ICANS): potentially life-threatening CAR T cell therapy toxicity that is associated with blood-brain barrier disruption and increased cytokines in the cerebral spinal fluid.

Lentiviral vector: construct that is derived from HIV and can be used to introduce a gene of interest (i.e., CAR) into the genome of both replicating and nonreplicating cells. As with γ-retroviral vectors, to increase the safety of the platform, the components necessary to produce viral particles are split between plasmids and provided in *trans*.

Lymphodepletion: chemotherapy regimen generally consisting of fludarabine and cyclophosphamide used to eliminate lymphocytes that act as homeostatic cytokine sinks and immunosuppressive cells, thus maximizing CAR T cell expansion, function and persistence *in vivo*.

Onpattro: transthyretin targeting siRNA encapsulated in an LNP used to treat hereditary transthyretin-mediated amyloidosis.

Pattern recognition receptor: receptors capable of recognizing conserved features of pathogens or damaged cells that elicit a type I interferon response (i.e., unmodified single-stranded mRNA activates endosomal and cytosolic sensors).

PiggyBac transposon: movable genetic element that can be integrated into TTAA sites in the genome with the help of a transposase enzyme. Alternative nonviral method to transfer the CAR gene into the genome.

weeks [7], and rapidly progressing patients require bridging therapy. The list price for the approved commercial CAR T cell products surpasses USD $500 000 per patient and the cost of additional procedures, including pretesting, leukapheresis, treatment with lymphodepleting chemotherapy, possible intensive care unit admission, and response assessments [i.e., positron emission tomography (PET)/computed tomography (CT) scans or bone marrow biopsy and flow cytometry] has been reported to be in excess of USD $1–1.5 million (inclusive of the CAR cell product) [8]. The scalability of a patient-specific product is thus limited by the intricacy of the manufacturing and quality assurance processes and the associated financial burden which poses equity and accessibility barriers.

Allogeneic CAR T cells (produced from healthy donor T cells) represent an attractive universal strategy that reduces product variability, simplifies release testing and shortens the time to treatment [9]. However, additional complex genetic engineering maneuvers are required to mitigate graft-versus-host reactions (caused by the reactivity of donor cells to the recipient alloantigens) and host-versus-graft reactions (caused by the reactivity of recipient endogenous T cells to donor cell alloantigens). To avoid graft-versus-host reactions, virus-specific T cells, gene editing of T cells to remove the T cell receptor (TCR) in αβ T cells, and alternative non-TCR expressing lymphocytes [including natural killer (NK) cells] [10,11] have all been approaches that have been met with success. To avoid host-versus-graft reactions and prolong the persistence of the CAR T cells, broad HLA-matched donor banks [12], or disruption of genes involved in the expression of MHC or CD52 (to enable the administration of alemtuzumab to deplete any host T cells) [13,14] have been explored with compensatory HLA-E overexpression [15]. Despite these efforts, it is not clear that all these issues have been fully resolved and clinical results have sometimes been limited [9]. Moreover, like autologous CAR T cell therapy, the administration of allogeneic CAR T cells also necessitates lymphodepletion which can be associated with prolonged cytopenias and neutropenias and frequent infectious complications [16].

Bypassing the *ex vivo* manufacturing step with *in vivo* CAR T cell engineering

The *in vivo* delivery of synthetic immune receptors has the potential to transform the treatment of cancer and many other diseases. This approach is enabled by advances in viral vector engineering, and nonviral nucleic acid delivery platforms, such as lipid nanoparticles (LNPs) or polymer-based nanoparticles (Figure 1B). An off-the-shelf product that generates CAR-redirected immune cells *in vivo* would have numerous advantages related to simplifying the logistics of the manufacturing process, reducing costs, improving the biology of the therapy, and increasing the safety of the approach, yet also create novel challenges (Table 1).

Logistical advantages

The generation of a single vector or nanoparticle drug product that has defined physicochemical properties and can be used to treat many patients streamlines manufacturing and release testing, truncates the supply chains, reduces cost, and provides rapid access for patients with aggressive disease. This simplified process could facilitate broad-scale implementation that would democratize access.

Biological and safety advantages

In addition to the logistical advantages of *in vivo* CAR T cells, the strategy theoretically may also address some of the current therapeutic challenges of *ex vivo* prepared CAR T cells. *In vivo* CAR T cell therapy necessitates the presence of T cells in the patient and thus obviates the need for lymphodepleting chemotherapy and its associated downstream sequelae (including infectious complications [16]). In turn, the presence of an intact immune system may better support epitope spreading and the generation of a broad antitumor immune response that provides insurance against CAR-target antigen escape, a common mechanism of therapeutic failure [17]. The

Pseudotyped viral vector: viral particles packaged with a foreign glycoprotein envelope to change the binding and entry tropism. Lentiviruses are pseudotyped to transduce T cells.
γ-Retroviral vector: a vector that resembles the parental murine leukemia virus (MLV) genome, where a gene of interest (i.e., CAR) is encoded in place of the gag, pol, and env genes. To generate virions, these genes are supplied in *trans* in producer cells. Proviral sequences integrate into the genome of transduced cells enabling expression of the gene of interest as any other cellular gene.
Single chain variable fragment (scFV): recombinant antibody fragment composed of heavy and light chain variable regions connected with a peptide linker. Used as the antigen binding domain in a CAR and a method to target viral vectors to given cell types.
Untranslated regions (UTRs): 5′ and 3′ mRNA domains responsible for post-transcriptional gene regulation processes including but not limited to mRNA processing, stability, and translation efficiency.

Table 1. Overview of platforms to deliver the CAR genetic information

	Delivery system	Cargo/payload	Example targeting mechanism	Advantages	Disadvantages
Viral vector	Lentivirus	Single-stranded RNA genome	Engineered envelope with scFv (anti-CD3/ CD4/CD8) [64–68,70]	- Quiescent cells, including naïve/stem memory T cells are transducible - The integrated transgene is maintained throughout T cell expansion and can therefore support long-term activity of redirected T cells - Vector manufacturing protocols are well established.	- Transgene integration into bystander cells (i.e., germ cells, malignant cells, inhibitory immune cells) is a safety risk - Insertional mutagenesis is a safety risk - Delivery may be limited by pre-existing neutralizing antibodies to pseudotyping components
	AAV	Single-stranded DNA genome	Capsid bound scFv or DARPin [64,72]	- Episomal persistence without integration provides long term transgene expression while minimizing the risk of insertional mutagenesis - Significant clinical experience exists with AAV gene therapy vectors for other indications. - Vector manufacturing protocols are well established.	- Mutation of the capsid is required to avoid nonspecific cell uptake. - Delivery to and persistence of transduced cells may be limited by pre-existing immunity to certain serotypes, or by immunity induced by repeat dosing [102]
Non-viral nanoparticle	LNP	mRNA	Antibody (anti-CD3, CD8, CD5) [28–31,103]	- Transient expression is a built-in safety feature - Well-defined and highly scalable manufacturing methods are informed by SARS-CoV-2 vaccine pipelines.	- Transient CAR expression may necessitate extensive serial dosing - Antibody modified LNPs face additional manufacturing challenges - Activation of PRRs must be minimized for sustained mRNA expression.
	Polymer	mRNA DNA	Antibody (anti-CD3) linked via polyglutamic acid to polymer [26,27]	- Suitable for the delivery of both DNA and mRNA - DNA payload can be integrating transposon DNA or non-integrating plasmid DNA - Transient expression of mRNA or non-integrating DNA is a built-in safety feature - PBAE polymer nanoparticles are biodegradable with a short half-life (1–7 h) and are thus nontoxic - Polymer is amenable to the addition of targeting ligands.	- Transient CAR expression may necessitate extensive serial dosing - Clinically unproven - Risk of insertional mutagenesis by transposon DNA payloads

off-the-shelf nature of *in vivo* CAR T cell platforms could also simplify simultaneous or serial multi-antigen targeting through the multiplexed use of LNP formulated mRNA or viral vectors versus engineering dual targeting viral vectors for *ex vivo* CAR T cells. The short-term expression of mRNA-based *in vivo* CAR T cells will likely require repeat treatments, thus enabling real-time dose adjustment to increase efficacy or to reduce toxicities such as **cytokine release syndrome (CRS)** and **immune effector cell-associated neurotoxicity syndrome (ICANS).** Simultaneously, transient CAR expression will limit the induction of exhaustion in CAR-bearing T cells, another major mechanism of CAR T cell therapeutic failure resulting from chronic antigen stimulation, tonic signaling, and extrinsic immunosuppression mediated by the microenvironment [18–20]. Serial dosing creates a built-in rest period that has proven efficacious in maximizing T cell function during bispecific molecule (blinatumomab) therapy [21] and CAR T

cell therapy [22]. *Ex vivo* manufactured CAR T cells sample a small fraction of T cells from the circulation, whereas *in vivo* therapy may, in theory, access T cells with various differentiation states and properties that could be therapeutically favorable (i.e., tissue-resident memory T cells). Last, since *ex vivo* expansion and exposure to supraphysiologic levels of cytokines can promote differentiation and exhaustion, rapid protocols generating minimally manipulated CAR T cells have been shown to preserve T cell stemness, promote superior *in vivo* expansion, and enable effective clinical responses at considerably lower doses than CAR T cells produced by conventional methods [23,24]. It stands to reason that unmanipulated T cells redirected by viral-vector-based *in vivo* CAR therapy would possess the same advantages.

Delivery and expression challenges

The genetic cargo must be delivered with sufficient efficiency and specificity to desired cell types. Off-target delivery could lead to dilution that compromises efficacy, and inadvertent transduction or transfection of tumor blasts could lead to epitope masking where the CAR binds to the target in *cis* and blocks engagement with T cells [25]. Transduction or transfection of bystander immune cell types with inhibitory properties (i.e., regulatory T cells) also creates the possibility of exacerbating disease.

T cell targeted nanoparticles and nucleic acids can deliver CAR genetic cargo *in vivo*

Particle, cargo, and targeting design elements

To protect nucleic acid cargo from degradation, clearance, and enable delivery to target cells *in vivo*, both LNP and polymer-based nanoparticles have been shown to successfully deliver the CAR-encoding nucleic acid to T cells [26–31] and macrophages [32,33]. Although inorganic nanoparticles (i.e., gold, mesoporous silica, and iron oxide) have also been used nucleic acid delivery [34], their application to the CAR T cell setting has not yet been reported. LNPs are a mixture of four component lipids: ionizable cationic lipids, helper lipids including phospholipids and cholesterol, and polyethylene glycol (PEG) conjugated lipids (Figure 2, left panel). In biological fluids, LNPs acquire a protein corona that adsorbs to the surface, including proteins like apolipoprotein E (ApoE) [35], and are taken up by cells via endocytosis, releasing the mRNA cargo into the cytosol for translation. The LNPs can be decorated with targeting antibodies (i.e., anti-CD4 or CD5) using SATA–maleimide chemistry to specifically enhance binding to and endocytosis by T cells [28,29]. Biodegradable poly(β-amino ester) (PBAE) polymer formulations are cationic and self-assemble via electrostatic interactions with anionic nucleic acid (DNA or mRNA) and undergo pH-dependent endosomal escape. To target these particles to T cells, polyglutamic acid is conjugated to an antibody (i.e., anti-CD8 or CD3) and adsorbed to the particles [26,27]. For both types of nanoparticles, receptor-mediated endocytosis enhances the otherwise inefficient endocytosis pathways of T cells, albeit to variable levels depending on the receptor [36] (see Box 2 for case studies).

To enable transient redirection of T cell functionality, *in vitro* transcribed mRNA has been encapsulated in the nanocarriers described earlier. The mRNA cassette is an attractive platform for transient *in vivo* expression of the CAR as it can be easily designed and mass-produced cost-effectively in a cell-free manner. Therapeutic synthetic mRNA mimics endogenous mRNA, incorporating a 5′ cap, a 5′ **untranslated region (UTR)**, the open reading frame encoding the CAR, a 3′ UTR, and a poly(A) tail, yet benefits from codon optimization and incorporation of the modified nucleosides (i.e., N1-methylpseudouridine) to minimize **pattern recognition receptor (PRR)** activation and maximize translation [37,38]. While the preferred UTRs that have been used for vaccine and *in vivo* CAR applications are generally derived from α and β globin genes due to their high-level expression across cell broad types [39], UTR design elements are under investigation for various applications [40–42] and UTR engineering provides an opportunity to either

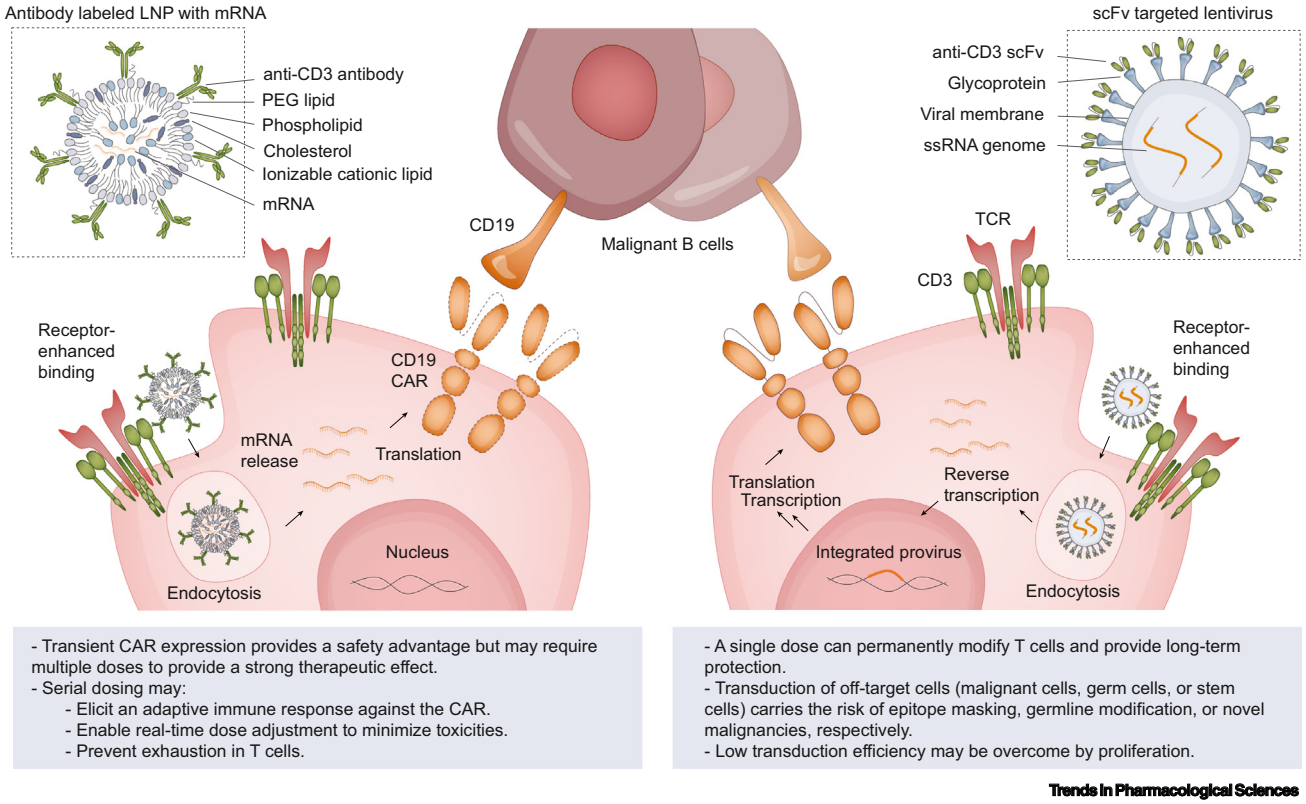

Figure 2. Anatomy of *in vivo* cell therapy platforms and cell redirection mechanisms. (Left) A lipid nanoparticle (LNP) is depicted in the top corner and is targeted to T cells using an anti-CD3 antibody. LNP binding to T cells is enhanced by interaction with CD3, and the LNP is internalized by endocytosis where it is proposed that interaction between the cationic ionizable lipids in the LNPs and the anionic lipids in the endosomal membrane facilitates release of the cargo [104]. Translation of the mRNA sequence by ribosomes (not shown) leads to the expression of the chimeric antigen receptor (CAR) molecule and transient functional redirection of the T cell through the synthetic receptor (border line for the CAR is dashed to emphasize the temporary expression). (Right) A lentiviral particle is depicted in the top corner where it is pseudotyped with a glycoprotein (derived from various viral origins) that is linked to an anti-CD3 single chain variable fragment (scFv). Following scFv–CD3 interaction, the virion is internalized by membrane fusion or endocytosis (depending on the glycoprotein used) and the genetic material is reverse transcribed, trafficked to the nucleus, and integrated into the host genome, resulting in permanent modification. The CAR transgene is then transcribed and translated as any other cellular host gene. These compositions and targeting specificities are examples only and T cells can be targeted using other reactivities beyond CD3. Likewise, a CD19 CAR is depicted which confers reactivity to B cells, however, any CAR reactivity could be used in place.

enhance the magnitude and duration of expression or detarget expression in T cells and off-target cell types, respectively. LNPs are amenable to coformulation with both mRNA and siRNA, and with the goal of both redirecting T cells and mitigating negative regulatory signals, CD19 CAR mRNA, and PD-1 siRNA [43] have been codelivered. Surprisingly, although as previously reported for coformulated RNA species [44], the interaction between the two RNA cargos increased the expression and knockdown characteristics compared with either RNA alone [43]. Plasmid DNA encoding the CAR has also been reported to redirect CAR T cells using *in vitro* and *in vivo* approaches in LNP [31] and polymer nanocarriers [26]. To enhance the persistence of the CAR, the CAR gene has been flanked with **piggyBac** inverted terminal repeats (ITRs) as a **transposon** and coencapsulated with a plasmid encoding a hyperactive form of the iPB7 transposase, thus resulting in stable integration of the CAR into the genome [26].

Advantages and challenges of nanocarriers and nucleic acids

Non-viral strategies, and particularly mRNA-LNP approaches (no polymer-based mRNA delivery system has received regulatory approval), capitalize on established severe acute respiratory

Box 2. Case studies for retargeting of T cells using nonviral delivery methods

Using the PBAE polymer nanoparticle system, Smith *et al.* demonstrated that CAR expression *in vitro* can be prolonged by coformulation of plasmids encoding the CAR and the PiggyBac transposase system, from a matter of days to sustained expression in more than half of transfected T cells for the duration of the experiment. Biodistribution revealed that intravenous administration of CD3-targeted nanoparticles to immunocompetent mice resulted in preferential transfection of $CD3^+$ T cells, with approximately 75% of the transfected cells being T cells (remaining immune cells included neutrophils, myeloid cells and B cells, and phagocytic cells of the reticuloendothelial system). PiggyBac reprogramming nanoparticles yielded a peak expansion (5.5-fold) of CD19 CAR T cells around day 12 postinjection and a single dose of 3×10^{11} lymphocyte-targeting nanoparticles conferred a similar level of control of Eμ-ALL01 leukemia compared to treatment with cyclophosphamide and 5×10^6 *ex vivo* CAR T cells over the course of ~100 days [26].

Again, using the PBAE polymer nanoparticle system, but formulated with mRNA, Parayath *et al.* demonstrated *in vitro* peak expression of the CAR molecule 2 days post-transfection with virtual extinction of expression by day 10. Using an NSG mouse model of Raji lymphoma that was reconstituted with 10^7 human T cells, mean peak transfection efficiency was ~10% of the T cells and administration of 6 weekly doses of CD3-targeted CD19 CAR mRNA nanoparticles (50μg/dose) resulted in comparable efficacy to the conventional *ex vivo* CAR T cell treatment [27].

Using a model of cardiac injury, Rurik *et al.* used CD5-targeted LNPs to deliver FAP CAR mRNA to eliminate activated fibroblasts and thereby mitigate fibrosis. In AngII/PE-injured mice, injection of 10 μg of LNPs resulted in a consistent population of FAP CAR T cells (17.5–24.7%) with all splenic T cell populations (both CD4 and CD8 subsets, as well as regulatory T cells) displaying measurable levels of transfection. Nonetheless, CAR expression was transient and undetectable 1 week after injection. Like *ex vivo*-generated CAR T cells, cardiac function was improved in injured mice, as measured by echocardiography, and *in vivo*-generated FAP CAR T cells were found to accumulate adjacent to activated fibroblasts in the heart [28].

syndrome coronavirus 2 (SARS-CoV-2) vaccine manufacturing capacity, cold-chain protocols, and proven safety profiles. Furthermore, nanoparticles and mRNA have the advantage of being produced in a cell-free manner and possess defined physicochemical properties, thus minimizing the variability of the product. Finally, mRNA administration poses no risk of insertional mutagenesis as the cargo is delivered to and translated in the cytoplasm.

To date, most of the work to generate CAR T cells *in vivo* using nanoparticles (both LNPs and other nanocarriers) has used labeling with a targeting ligand. However, conjugation is done using coupling procedures which complicates manufacturing, reduces the yield and stability of the particle and precludes long-term storage of a frozen product. Alternatively, changes in LNP lipid composition can enhance transfection of various cell types including T cells *in vitro* [45,46] or tissues to be targeted *in vivo* [47,48]. In combination with modification of the mRNA UTRs that can provide a second layer of regulation to expression in target cell types, it is possible that together LNP lipid composition and mRNA design could obviate the need for antibody targeting that poses a hurdle to scalability.

It is not yet clear how long the CAR must be expressed to provide therapeutic benefit; this will depend on the nature of the indication, the proportion of T cells transfected, and the depth of reduction of the target cell population that is required. It may be necessary to administer multiple doses of the nanomedicine or use additional methods to sustain the expression of the CAR. For LNP systems, multiple design elements can potentially address this hurdle. Optimizing the lipid composition can improve circulation lifetime and gene expression, and changing formulation parameters of the LNP can enhance the mRNA integrity and increase transfection efficiency [49]. For example, we have shown that formulation of LNPs in the presence of high concentrations of pH 4 buffers led to the formation of mRNA-rich bleb structures that are protective for the mRNA and thus enhance transfection [49]. While not yet reported in the literature for *in vivo* CAR applications, other RNA species, such as self-amplifying or circular RNAs have been demonstrated to prolong the expression of genes of interest in a vaccine setting [50,51] and may also enhance the therapeutic output of a single dose of RNA. Other approaches, such as coencapsulation of

DNA plasmids encoding the CAR flanked by ITRs and the PiggyBac transposase to generate a population of CAR T cells [26] should be approached with caution as a small fraction of CAR T cells manufactured *ex vivo* using a similar approach has been reported to lead to T cell lymphomas [52].

LNPs have been shown to act as adjuvant components of mRNA vaccines [53,54] and different ionizable lipids have distinct immunostimulatory properties [53,55,56]. Although this feature can be an advantage for LNPs used in the vaccine setting, it is possible that it may not be a benefit in repeat dosing scenarios for cargos against which an adaptive immune response is not desirable (i.e., against the CAR). Initial results look favorable, however, as repeat dosing in mice of an LNP mRNA coding for CRISPR/Cas 9 demonstrates cumulative benefits for Duchenne muscular dystrophy [57] as does repeat dosing of an LNP mRNA coding for arginase 1 with cumulative benefits for arginase deficiency [58]. Serial dosing of LNP mRNA coding for human ornithine transcarbamylase (hOTC) shows successive expression in nonhuman primates [59]. Additional indications that demonstrate that repeat dosing is viable are summarized elsewhere [60]. A remaining issue concerns complement activation-related pseudoallergy (CARPA) [61], however, this is readily controlled by a preconditioning regimen with steroids, antihistamines and antipyretics as used prior to **Onpattro**, an LNP siRNA drug that has now been administered to patients every 3 weeks for 5 years or more.

T cell-targeted viral vectors can deliver CAR genetic cargo *in vivo*

Viral vector, cargo, and targeting design elements

Two classes of viral vectors have been explored for *in vivo* CAR T cell therapy, **Adeno-associated virus (AAV)** and replication-defective lentivirus or retrovirus, with lentiviruses being the most common platform **(**Figure 2, right panel). When T cells are transduced *ex vivo* to prepare CAR T cells, lentiviruses are usually **pseudotyped** with the vesicular stomatitis virus glycoprotein (VSV G) which binds to the low-density lipoprotein receptor (LDLR) [62]. The distribution of expression of LDLR is broad, and for the engineering of purified cell populations, this is efficient. However, for *in vivo* applications where the virus is administered intravenously, many cell types would be susceptible to transduction. Administration of VSV G pseudotyped lentivirus encoding a CD19 CAR to wild-type mice can enable the transduction of a small proportion of T cells that gain a selective advantage and proliferate to reach a maximum of 13% of all T cells 3–4 weeks after administration and lead to complete B cell loss [63]. To maximize the transduction of T cells, alternative pseudotyping strategies with modified envelope proteins have been developed. For this purpose, several viral envelopes have been used, including those from measles virus [64], Nipah virus [65–67], Sindbis virus [68], and cocal fusion glycoprotein [69] (another vesiculovirus with similarity to VSV G). In these cases, the lentivirus is also engineered to express a targeting ligand, either fused to or independent of the envelope, to promote T cell tropism (see Box 3 for case studies). The ligand, in the form of an scFv, nanobody, or **designed ankyrin repeat protein (DARPin)**, mediates T cell binding by targeting a T cell surface molecule (i.e., CD3, CD4, or CD8), while the pseudotyped glycoprotein machinery enables membrane fusion once the target is engaged. Although the measles virus F and H envelope pseudotyped lentiviruses suffer from low yields, the Nipah virus glycoproteins have been shown to generate superior viral titers, and due to the low seroprevalence of the virus, are not widely neutralized by serum antibodies [70]. Instead of relying on ligand-targeted transduction, the biology of retroviruses has also been leveraged to selectively transduce T cells. Using implanted multifunctional scaffolds loaded with immobilized anti-CD3 and anti-CD28 antibodies and retroviral particles encoding the CAR transgene, T cells could simultaneously be activated and transduced *in situ* [71]. In contrast to lentiviruses which are membrane-encapsulated viruses, AAVs have a capsid, which can nonetheless be modified to display either an scFv [72] or a DARPin [64].

Box 3. Case studies for retargeting of T cells using viral vectors

Pfiffer *et al.* demonstrate *in vivo* transduction to generate human CD19 CAR T cells using a CD8-scFv fused Nipah virus glycoprotein pseudotyped lentivirus. In mice reconstituted with human peripheral blood mononuclear cells (PBMCs) and transplanted with Raji lymphoma cells, 1 week after intraperitoneal administration of only 2×10^6 transduction units (tu), 10–35% of CD8 T cells were CAR positive, and CD19 expressing B cells were undetectable. A humanized mouse model demonstrated comparable levels of transduction and also revealed the production of inflammation-related cytokines where the animals with the greatest expansion of CAR T cells exhibited symptoms of CRS [65].

Michaels *et al.* describe a cocal glycoprotein and anti-CD3 redirected lentivirus (VivoVec) which encodes a multicistronic payload that encodes a CD19 CAR and a rapamycin-activated synthetic cytokine receptor. The CD3 scFv mediates T cell activation, and the administration of rapamycin simultaneously suppresses endogenous T cell populations while delivering an IL-2/IL-15 signal to the transduced T cells. While activation and rapamycin exposure enhanced expansion *in vitro*, in humanized mice, administration of 10^7 transduction units (tu) was associated with detectable CAR T cells in the blood, and a near complete loss of circulating B cells by day 11 postinjection. Treatment of NSG mice reconstituted with human PBMCs and treated with lentivirus in the range of 2.7×10^7 to 2.7×10^8 tu led to a dose-dependent expansion of CAR T cells (up to 30 CAR T/μL blood on day 11) and near complete tumor control for close to 50 days [69].

Advantages and challenges of viral vectors

Lentiviruses and AAVs have now been used extensively for therapeutic applications and clinical-grade manufacturing protocols and reagents are well established. Lentiviral vectors lead to stable integration and expression of the CAR transgene, and although AAV does not integrate, episomal expression can be sustained for weeks to months to years, therefore generating a potentially long-lived cell product. Both lentivirus and AAV transduce dividing and non-dividing cells. Lastly, vector engineering of the lentiviral genome is well characterized and capable of accommodating multicistronic cassettes, therefore allowing for not only redirection, but the simultaneous provision of genes that enhance T cell function. One such example is the codelivery of a CD19 CAR as well as a synthetic cytokine receptor that is regulated by exogenous rapamycin and mimics interleukin (IL)-2 and IL-15 growth signals [69].

The permanent nature of lentiviral gene transfer will however require a very high level of stringency over the cell types that will be transduced *in vivo*. Gene transfer to germ cells carries the risk of germline transformation and must be entirely avoided in individuals who are reproductively competent, or who may be so in future. In somatic cells, insertion of the transgene can disrupt the expression of a host gene in which integration occurs, and the expression of other, distal genes, can also be altered if a transgene integrates into their regulatory DNA, thereby facilitating transformation. Historically, first-generation murine leukemia virus (MLV)-based vectors used to treat X-linked severe combined immunodeficiency (X-SCID) led to catastrophic outcomes with a number of patients developing T cell lymphomas associated with integration near a proto-oncogene locus and dysregulation of LMO2 by promoter activity inherent to the viral long terminal repeats (LTRs) [73]. Subsequent redesign of viral vectors, and the use of self-inactivated (SIN) γ-retroviral and lentiviral vectors in which the promoter elements of the LTRs are eliminated appear to have eradicated insertional oncogenesis [74]. The risk of T secondary malignancy due to virally induced transformation events in *ex vivo* produced cells has received significant attention recently but is thought to be extremely low and outweighed by the benefit of CAR T cell therapy [75,76]. Two rare cases of secondary T cell malignancies that express the CAR have been reported, albeit it is not clear whether the transduction was oncogenic since clones bearing the genetic aberrations appeared to pre-exist therapy [77,78]. The oncogenic risks associated with the transduction of mature T cells *ex vivo* also exist in the *in vivo* setting, in addition to the risks of transduction of rare nontarget primitive stem cells *in vivo* which may harbor stochastic or germline genetic abnormalities. A case of *ex vivo* manufactured CAR T cells illustrates this latter example where marked clonal expansion was observed following integration into and disruption of the TET2 allele in CAR T cells where the patient carried a germline hypomorphic mutation in the second allele [79].

Although TET2 disruption was associated with a strong therapeutic response in this case, the loss of TET2 has also been shown to sensitize T cells to genomic instability and malignant transformation [80].

Indications for *in vivo* cell therapy: cancer and beyond

Reducing the cost and eliminating lymphodepletion broadens the practical realities and scope of applying CAR technology to treat infectious diseases, autoimmune diseases, or fibrosis where toxicity typical of cancer therapies would be unacceptable. Indeed, these settings may be most appropriate for testing *in vivo* CAR T cell strategies where an approved CAR T cell product does not yet exist. Beyond blood cancers, the first logical extension of the CD19 CAR platform contemplated has been to deplete autoreactive B cells that drive the pathogenesis of autoimmune diseases like rheumatoid arthritis, systemic lupus erythematosus, pemphigus, multiple sclerosis, and scleroderma, where broad immune suppressants and therapeutic targeting of B cells using monoclonal antibodies have provided only modest benefit [81]. It is thought that while monoclonal antibodies can efficiently eliminate circulating B cells, tissue-resident B cells pose a greater challenge and may be better targeted by a CAR T cell with tissue-homing capabilities. CAR T cells are now being tested clinically for the treatment of systemic lupus erythematosus (SLE) [82–84]. For these autoimmune diseases where clinical symptoms may reflect permanent organ damage (i.e., kidney damage in SLE), it would be logical to implement the *in vivo* CAR approach early in disease management. Similarly, using CAR T cells to remove senescent cells [85] or activated fibroblasts that contribute to fibrosis [28] would ideally be used early in the course of the disease when lymphodepletion would not be an acceptable side effect. These indications may also have a lower threshold for efficacy where the goal is significant target cell (i.e., normal B cell) reduction, not complete eradication of all malignant B cells. CAR T cell therapy is also being investigated for the treatment of infectious diseases including HIV and hepatitis B virus and the first patients have been treated on a trial using CAR T cells targeting the HIV gp120 glycoprotein [86]. In the context of infectious diseases, *in vivo* cell therapy has many advantages. The total number of individuals living with HIV is thought to be more than 38 million [86]: while it would not be financially or logistically possible to manufacture an autologous cell product to treat this number of patients, the SARS-CoV-2 vaccine has demonstrated that mRNA and LNPs can be used on this scale. Lymphodepletion would also interfere with mounting a strong antiviral adaptive immune response and exacerbate virus infection, thus favoring *in vivo* cell therapy technology that could be administered without preconditioning.

It will need to be determined empirically which indications are most conducive to therapy using the various formats of *in vivo* treatment (i.e., nucleic acid and nanoparticles versus viral delivery). It is likely that the required durability of CAR expression will be determined by the depth of depletion of the target cell population needed to treat the disease. For example, the use of transient *in vivo* generated anti-fibroblast activation protein (FAP) CAR T cells makes a great deal of sense where a reduction in the levels of activated fibroblasts, but not complete elimination, is sufficient to improve cardiac function [28]. In contrast, either multiple doses of transient CAR mRNA, or permanent transduction with a lentivirus, may be required to eliminate cancers that risk relapse.

Concluding remarks and future perspectives

CAR technology, and synthetic biology more broadly, have the potential to transform the way that medicine is practiced. These strategies have been pioneered using *ex vivo* cell engineering and have been met with impressive clinical outcomes, however, the practical realities of manufacturing patient-specific cell products constrain the application of the technology. Delivery platforms such as nanoparticles delivering nucleic acids and viral vectors enable cellular manipulations to be performed *in situ* to treat disease. Preclinical proof-of-concept studies have demonstrated

Outstanding questions

What fraction of T cells need to be transfected/transduced using LNPs or viral vectors (and in which locations) to achieve therapy comparable to an *ex vivo* engineered cell product? Will sustained transgene expression during T cell clonal expansion be necessary for clinical efficacy, particularly for applications in oncology?

What are the consequences (toxicity/pathology) of off-target CAR expression in different tissues and cell types?

How does the innate immunogenicity of mRNA/LNPs/viral vectors impact T cell activity, antigen-presenting cell (APC) activation, and regulate reactogenicity in serial dosing schedules?

What is the optimal design of nucleic acid (circular RNA, mRNA, self-amplifying RNA, UTRs, etc.) that will balance immunogenicity and expression kinetics?

Are the costimulatory domains established in *ex vivo* cells optimal for *in vivo* CAR T cell therapy?

What route of administration maximizes CAR expression in desired cell types and minimizes off-target expression or innate immune activation (local versus systemic)?

Which cell types do we want to transduce/transfect and does targeting with ligands with tropism for multiple immune lineages (i.e., CD7 which is expressed on both T cells and NK cells) enhance the therapeutic efficacy?

Does the *in vivo* cell therapy approach better mobilize endogenous immune cell types to prime a broad adaptive antitumor immune response?

How important is the role of lymphodepletion to remove inhibitory cell types (versus enhancing engraftment) and what effect will this have on *in vivo* generated CAR T cell function? How important is the role of lymphodepletion in removing endogenous cells competing for space and resources, and what effect will this have on the ability of in vivo generated CAR T cells to proliferate?

After the CAR mRNA is lost following transient transfection, what happens

functional equivalence to conventional *ex vivo*-produced cells in oncology and cardiac injury models. Moving forward, a deep characterization of the dynamics between immune cell types that contribute to the depth of the antitumor response, and an understanding how the immunogenicity of the platforms may affect serial dosing is needed in immune competent mouse models (see Outstanding questions). The use of immunodeficient and or humanized mice reconstituted with human T cells will also provide an orthogonal lens through which to test the human clinical candidates. Lastly, nonhuman primate models will be necessary to ultimately test the safety of the approach and establish dose-dependent pharmacodynamic and pharmacokinetic profiles. The field of nanomedicines has empowered new opportunities to treat an array of diseases, including vaccination against the SARS-CoV-2 virus. Several properties of LNP systems give them advantages over viral platforms in the *in vivo* therapy arena, including their cost, tolerability, manufacturability, and ability to dose to effect, yet the greatest challenge facing all approaches is the expression of the CAR in nontarget cell types. Nonetheless, this is an emerging field, and we will need chemists, oncologists, immunologists, bioinformaticians and regulatory scientists to come together to address the outstanding questions and shepherd these technologies into clinical realities.

to the T cells which have clonally expanded and acquired effector functions? Is there a risk of autoimmunity?

Which disease indications should be prioritized for the testing of *in vivo* CAR T cell strategies?

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Declaration of interests

PRC has financial interests in Acuitas Therapeutics, Mesentech, and NanoVation Therapeutics. LE collaborates with NanoVation Therapeutics and has received material support. RAH and LS do not have any conflicts to disclose.

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