Engineering chimeric antigen receptors (CAR) or T cell receptors (TCR) helps create disease-specific T cells for targeted therapy, but the cost and rigor associated with manufacturing engineered T cells ex vivo can be prohibitive, so programming T cells in vivo may be a viable alternative. Here we report an injectable nanocarrier that delivers in vitro-transcribed (IVT) CAR or TCR mRNA for transiently reprogramming of circulating T cells to recognize disease-relevant antigens. In mouse models of human leukemia, prostate cancer and hepatitis B-induced hepatocellular carcinoma, repeated infusions of these polymer nanocarriers induce sufficient host T cells expressing tumor-specific CARs or virus-specific TCRs to cause disease regression at levels similar to bolus infusions of ex vivo engineered lymphocytes. Given their ease of manufacturing, distribution and administration, these nanocarriers, and the associated platforms, could become a therapeutic for a wide range of diseases.
The efficacy of adoptive T-cell therapies, a powerful modality in which T cells harvested from the patient or a donor are genetically modified to target cancers or infectious agents, is supported by numerous clinical trials showing impressive outcomes. However, the complexity and high costs involved in manufacturing a bespoke T-cell product for each patient, rather than preparing a drug in bulk in a standardized form, make it difficult to compete with frontline therapy options such as small molecule drugs or monoclonal antibodies. Most CAR-T and TCR-engineered T cells are currently made by a cumbersome process involving: (i) Leukapheresis to extract T cells from a patient who is connected by two intravenous tubes to an apheresis machine for several hours. This is uncomfortable for the patient, incurs a substantial monetary cost, and ultimately may be rate-limiting for large-scale adoption of autologous T cell; (ii) Activation and transduction of T cells; (iii) Expansion of transduced T cells over an approximately two-week period in a cytokine-supplemented tissue culture medium; (iv) Washing and concentrating the T cells prior to administration. For T-cell products made at central facilities and transported to remote treatment centers, cells have to be cryopreserved; and (v) Quality control release assays are necessary for each batch of CAR-T product. The entire process has to be conducted under environmentally controlled GMP-compliant conditions, which are expensive to maintain and run. Because each CAR-T product is made from starting materials (T cells) from the patient to be treated, there are no economies of scale.

In vitro-transcribed (IVT) mRNA has emerged as a disruptive new drug class that can be used to encode therapeutically relevant proteins of interest directly in vivo. Synthetic mRNA molecules can be quickly designed and manipulated and mass-produced relatively cost-effectively. Over the past decades, scientists have learned how to optimize mRNA pharmacologically and immunologically to make it more drug-like for clinical applications.

Here, we explore mRNA as an injectable drug to genetically reprogram circulating T lymphocytes to transiently express disease-specific receptors, thereby bypassing the need to extract and culture lymphocytes from patients (Fig. 1). To protect the therapeutic payload and to precisely target it to T cells, we formulated biodegradable polymeric nanocarriers. We demonstrate ex vivo that a single nanoparticle application can routinely transfect >70% of cultured T cells with the CD19-specific 1928z CAR (FDA-approved for the treatment of B-cell lymphoma) or with the HBcore18-27 TCR specific for the hepatitis B virus (HBV) core antigen (currently in a Phase I study to treat patients with HBV-related hepatocellular carcinoma; ClinicalTrials.gov Identifier: NCT03634683). Nanoparticle-transfected T cells transiently express these CAR-transgenes or TCR-transgenes on their surface for an average of 7 days. Using orthotopic xenograft mouse models of lymphoma, prostate cancer, and HBV-induced hepatocellular carcinoma, we demonstrate that, when administered periodically, CAR-encoding or TCR-encoding mRNA particles can genetically reprogram circulating T cells to induce antitumor responses with similar efficacies compared to conventional adoptively transferred T cells that have been virally transduced ex vivo.

The insertion of CARs or TCRs into lymphocytes by gene transfer currently occurs outside the patient’s body, in specialized manufacturing suites, but both the process of transporting cells to and from the clean room and the gene transfer procedure itself are labor-intense, expensive, and take valuable time. Should engineered T cell therapy reach its promise of extending to diverse populations across a variety of cancer types, the challenges of economics and manufacturing will likely grow.

We demonstrate that a mRNA nanodrug can achieve the power of effective, side-effect-free cell therapy with the convenience of an off-the-shelf drug. Just as for a conventional drug, with this new treatment modality the patient could be easily dosed for as long as medically necessary.

**Fig. 1** Schematic illustrating how we reprogram T cells in situ to express disease-specific CARs or TCRs using IVT mRNA carried by polymeric nanoparticles. These particles are coated with ligands that target them to cytotoxic T cells, so once they are infused into the patient’s circulation they can transfer the transgenes they carry into the lymphocytes and transiently program the cells to express the disease-specific CARs or TCRs on their surfaces.
Results

**MRNA nanocarriers transfect T cells with CAR- or TCR transgenes.** To deliver IVT mRNA encoding disease-specific receptor genes into human lymphocytes, we used a biodegradable poly(β-amino ester) (PBAE) polymer formulation as a carrier matrix (Fig. 2a). The PBAE-447 polymer we are using in our study to condense mRNA into nanoparticles was originally developed in the Jordan Green laboratory at Johns Hopkins University. Over the past decade, his group and others have extensively characterized the key properties of PBAEs. PBAEs enable endosomal escape by undergoing protonation at the lower pH of the endosomal compartment, leading to osmotic pressure buildup due to buffering, which causes endosomal disruption. High-throughput combinatorial library screens of PBAEs for nucleic acid delivery have shown that the presence of tertiary amines improves buffering capacity at low pH and facilitate endosomal escape. The ester bonds in the backbone structure of PBAEs undergo hydrolysis in aqueous conditions, making PBAEs less...
toxic than other nondegradable cationic polymers, such as PEI,
which has been broadly investigated as a nucleic acid delivery
vehicle. Cationic PBAE self-assembles into nanocomplexes with
anionic nucleic acids via electrostatic interactions (Fig. 2b). The
particles were made cell-targeting by coupling an anti-CD8
antibody to polyglutamic acid (PGA), forming a conjugate that
was electrostatically adsorbed to the particles. The resulting
mRNA nanocarriers can be lyophilized for long-term storage.
Prior to use, particles hydrate within seconds following addition
of sterile water to restore their original concentration. We used
Particle Tracking Analysis (NanoSight NS300, Malvern Panaly-
tical) to characterize the particles manufactured in ten indepen-
dent batches (Fig. 2c). We found the mean diameter of PbAE/
PGA-anti-CD8 nanoparticles to be 106.9 ± 7.2 nm. The ζ
potential was 4 ± 2, and mRNA encapsulation, measured by a
Qubit RNA HS assay kit, was 90.9 ± 6.2% at the 60:1 PbAE:
mRNA ratio we used in our nanoparticle formulation.

We first determined whether adding targeted mRNA nanocarriers to a culture of human lymphocytes can give robust
transfection of the cells. To test our technology in clinically
relevant system, we loaded nanoparticles with IVT mRNA
encoding the leukemia-specific 1928z CAR (Fig. 3a–e). CD19-
targeted receptors are the most investigated CAR-T cell product
today, with nearly 30 ongoing clinical trials internationally, and
three already FDA-approved cancer therapies. As a second
element, we delivered IVT mRNA encoding a high-affinity HBV-
specific TCR (Fig. 3f–j). T-cell therapy of chronic hepatitis B is a
novel approach to restore antiviral immunity and cure the
infection. The HBcore18–27 TCR specific for the HBV core
antigen was isolated from an HLA-A 02.01 donor with resolved
HBV infection. For both the 1928z CAR and the HBcore18–27 TCR
constructs, we first used real-time quantitative PCR and flow
cytometry to measure their expression levels in human T cells
following a single nanoparticle transfection. We found that
transgene expression peaked 24 h after nanoparticle exposure,
followed by a gradual decline (Fig. 3a, f). Notably, only
nanoparticles functionalized with T-cell-specific (antiCD8
or antiCD3) antibodies efficiently delivered the transgene, whereas
isotype control-functionalized nanoparticles yielded gene
expression close to background levels (Supplementary Fig. 1).
This translated into high levels of CAR or TCR surface expression,
with a maximum on day 2 (75 ± 11% of T cells expressed the
1928z CAR, Fig. 3b, c; and an average of 89 ± 4% of T cells
expressed the HBcore18–27 TCR, Fig. 3g, h). As expected, receptor
expression was transient, and was reduced to 28 ± 6% for the
CAR and 26 ± 9% for the TCR after 8 days in culture. We next
compared function (killing and cytokine production) of
nanoparticle-transfected T cells against T cells engineered with
these receptors using viral methods. To demonstrate specificity
for tumor antigens we included control groups of T cells
transduced either with tumor-irrelevant CAR genes (P28z,
targeting the Prostate-Specific Membrane Antigen24), or TCR
genes (MSLN-TCR, specific for Mesothelin22). Using real-time
Incucyte® live cell assays, we could not measure significant

Fig. 2 Design and manufacture of lymphocyte-programming nanoparticles. a Schematic of the T cell-targeted IVT mRNA nanocarrier used in our experiments. To create a reagent that can genetically modify primary T lymphocytes (which are refractory to nonviral transfection methods) simply by contact, we bioengineered polymeric nanoparticles comprised of four functional components: (i) surface-anchored targeting ligands that selectively bind the nanoparticles to T cells and initiate rapid receptor-induced endocytosis to internalize them. In our experiments we used anti-CD8 antibodies; (ii) a negatively charged coating that shields the nanoparticles to minimize off-target binding by reducing their surface charge. Because it is already widely used in drug delivery platforms, we selected polyglutamic acid (PGA) to accomplish this; (iii) a carrier matrix that condenses and protects the nucleic acids from enzymatic degradation while they are in the endosome, but releases them once the particles are transported into the cytoplasm, thereby enabling translation of the encoded protein. For this, we used a biodegradable poly(β-amino ester) (PBAE) polymer formulation that has a half-life between 1 and 7 h in aqueous conditions; and (iv) nucleic acids (IVT mRNA) that are encapsulated within the carrier and produce transient expression of the disease-specific CAR or TCR. b Diagram describing how we fabricated the nanoparticles. c Size distributions, measured using a NanoSight NS300 instrument. The mean diameter ± 5D, ζ potential, and mRNA encapsulation ± 5D are indicated on the top. N = 3 independently manufactured nanoparticle batches.
nanoparticles efficiently reprogrammed circulating T cells to recognize leukemia cells (mean 10% CAR$^+$ among CD8$^+$ ± 4.3%, Fig. 4d, e). As expected with mRNA delivery systems, these CARs were transiently expressed for up to 1 week (0.8 ± 0.4% CAR$^+$ CD8$^+$ T cells on day 7). Notably, repeat doses of nanoparticles were as effective as the first injection and achieved an average of 10.7 ± 3.6% gene transfer into host T cells (Fig. 4e). This suggests that, despite its transient nature, IVT mRNA can serve as a platform to achieve persistent in situ CAR expression in host lymphocytes.

Therapeutic responses in fully immunocompetent hosts. To examine how exclusively targeting can confine nanoparticle interactions to circulating T cells and how it affects their fates, we employed the fully immunocompetent Ai14 reporter mouse. In this genetically modified model, all cells contain a loxP-flanked STOP cassette preventing transcription of a CAG promoter–driven tdTomato protein. Only cells that are successfully transfected with mRNA encoding Cre recombinase (Cre) would excise the loxP-flanked STOP cassette, resulting in permanent tdTomato transcription and subsequent strong, amplified tdTomato.
expression. We first measured whole-organ fluorescence in A14 mice following injection of CD3-targeted (or isotype control-functionalized) nanoparticles carrying Cre mRNA. The highest gene expression mediated by nontargeted particles were found in the liver, whilst lymphocyte-targeted nanocarriers induced gene transfer mainly in the spleen, lymph nodes, and thymus (Fig. 5a, b). A detailed flow cytometry analysis of the spleen (Fig. 5c) revealed that CD3-targeted nanocarriers preferentially transduced T cells (8.1 ± 1.9%), without compromising viability (Supplementary Fig. 2). Much lower levels of dTomato signals were detected in other CD45+ (immune) subtypes, such as macrophages (3.2 ± 1.5%), B cells (1.1 ± 0.9%), neutrophils (0.3 ± 0.2%), and dendritic cells (1.9 ± 0.8%).

Based on these distribution studies, we next tested whether the quantities of mRNA nanoparticle-redirected T cells we measured are sufficient to reduce established cancers in fully immunocompetent hosts. To this end, we infused luciferase-expressing EquALL01 leukemia cells into albino C57BL/6 mice (to model B cell acute lymphoblastic leukemia in an immunocompetent mouse system). To serially monitor tumor burden by bioluminescence imaging, tumor cells were genetically tagged with Firefly luciferase (Fluc). Following orthotopic transplantation, all mice reproducibly developed lesions within three weeks (Fig. 7c, right panel), were reconstituted with human 10 × 10^6 CD3+ human T cells, and randomly assigned to the various treatment or control groups (Fig. 7d). We first measured the therapeutic efficacy of systemically injecting tumor-bearing mice with 10^6 ex vivo-transduced CAR+ T cells specific for the tumor antigen ROR1. We found that even though antiROR1 CAR-T cells did not achieve tumor clearance, the survival rates of the treated mice more than doubled (69 vs. 32 days in the no-treatment control group; Fig. 7d). To determine whether our “off-the-shelf” nanoreagent can achieve similar therapeutic effects, we systemically injected mice weekly with ROR1 CAR transgene-loaded nanoparticles (50 μg mRNA/dose; Fig. 7e). Particle-induced CAR programming extended survival by an average of 40 days compared to untreated controls, which is similar to the survival benefit achieved with conventional adoptive T-cell therapy (Δ mean survival = 3 d, N.s., P = 0.23; Fig. 7d, f). Appropriate localization and persistence of T cells is a prerequisite for activity against solid tumors, so we next assessed the frequency of infiltrating ROR1 CAR-T cells into prostate tumors over time. Flow cytometry of LNCaP C42 prostate tumors resected 4 days, 7 days, and 11 days after T-cell transfer revealed that intravenously infused T cells traffic to tumor sites (average 892 ± 295 CAR+ T cells/mg tumor tissue) but do not thrive (only 1.04-fold overall expansion between day 4 and day 11; Fig. 7g, h). Also, in situ-programmed CAR-T cells infiltrate tumors efficiently (average 648 ± 240 CAR+ T cells/mg tumor tissue) and maintain high levels of the CAR transgene before downregulating the receptor (average 91 ± 7 CAR+ T cells/mg tumor tissue on day 7, Fig. 7h).

To determine the cause of failure of both the adoptively transferred T cells and infused mRNA nanocarriers to completely clear the disease, we phenotyped the antigen profile of relapsing prostate tumors by flow cytometry. One of the most common escape strategies seen in cancer is a reduction of target antigen expression because of the selective pressure the CARs create.27,28.

Therapeutic responses in solid tumors. To confirm that this technology has relevance not only for the treatment of hematological malignancies, but also for solid tumors, we next investigated the ability of nanoparticles designed to introduce prostate tumor-specific CAR genes into circulating host T cells to induce regression of prostate tumors in mice. Unlike leukemia cells, which express high levels of the CD19 antigen and can easily be accessed by circulating lymphocytes, solid malignancies are heterogeneous and protected.26. This means that a portion of the tumor cells will evade recognition by the targeting CAR and will be surrounded by immune-suppressing defenses that can render T cells dysfunctional. In fact, co-author Dr. Peter Nelson used whole-genome/transcriptional profiling of 140 prostate cancer metastases to establish that prostate tumor lesions exhibit heterogeneous expression of three key cell-surface proteins (prostate-specific membrane antigen (PSMA), prostate stem cell antigen (PSCA), and receptor tyrosine kinase-like orphan receptor 1 (ROR1)) among patients (Fig. 7a). To recapitulate human disease, we orthotopically transplanted LNCaP C42 prostate carcinoma cells, which exhibit heterogeneous expression of these key cell-surface proteins (Fig. 7b), into the dorsal lobe of the prostate gland of NSG mice (Fig. 7c). To serially monitor tumor burden by bioluminescence imaging, tumor cells were genetically tagged with Firefly luciferase (Fluc). Following orthotopic transplantation, all mice reproducibly developed lesions within three weeks (Fig. 7c, right panel), were reconstituted with human 10 × 10^6 CD3+ human T cells, and randomly assigned to the various treatment or control groups (Fig. 7d). We first measured the therapeutic efficacy of systemically injecting tumor-bearing mice with 10^6 ex vivo-transduced CAR+ T cells specific for the tumor antigen ROR1. We found that even though antiROR1 CAR-T cells did not achieve tumor clearance, the survival rates of the treated mice more than doubled (69 vs. 32 days in the no-treatment control group; Fig. 7d). To determine whether our “off-the-shelf” nanoreagent can achieve similar therapeutic effects, we systemically injected mice weekly with ROR1 CAR transgene-loaded nanoparticles (50 μg mRNA/dose; Fig. 7e). Particle-induced CAR programming extended survival by an average of 40 days compared to untreated controls, which is similar to the survival benefit achieved with conventional adoptive T-cell therapy (Δ mean survival = 3 d, N.s., P = 0.23; Fig. 7d, f). Appropriate localization and persistence of T cells is a prerequisite for activity against solid tumors, so we next assessed the frequency of infiltrating ROR1 CAR-T cells into prostate tumors over time. Flow cytometry of LNCaP C42 prostate tumors resected 4 days, 7 days, and 11 days after T-cell transfer revealed that intravenously infused T cells traffic to tumor sites (average 892 ± 295 CAR+ T cells/mg tumor tissue) but do not thrive (only 1.04-fold overall expansion between day 4 and day 11; Fig. 7g, h). Also, in situ-programmed CAR-T cells infiltrate tumors efficiently (average 648 ± 240 CAR+ T cells/mg tumor tissue) and maintain high levels of the CAR transgene before downregulating the receptor (average 91 ± 7 CAR+ T cells/mg tumor tissue on day 7, Fig. 7h). Following an intravenous booster dose of ROR1-CAR encoding mRNA nanoparticles the same day, the tumor lesions were again infiltrated by freshly reprogrammed peripheral blood T cells (average 1066 ± 225 CAR+ T cells/mg tumor on day 11; Fig. 7h), which recapitulates the oscillating kinetics of mRNA nanoparticle-induced T-cell reprogramming we already observed in our leukemia studies (Fig. 4e).

To determine the cause of failure of both the adoptively transferred T cells and infused mRNA nanocarriers to completely clear the disease, we phenotyped the antigen profile of relapsing prostate tumors by flow cytometry. One of the most common escape strategies seen in cancer is a reduction of target antigen expression because of the selective pressure the CARs create.27,28.

![Fig. 3 IVT mRNA nanocarriers efficiently transfect human T cells with CAR or TCR transgenes.](https://example.com/fig3.png) Isolated human CD8+ T cells were stimulated with beads that are coated with antibodies against TCR/CD3 and co-stimulatory CD28 receptors. Twenty-four hours later, beads were removed and CD8-targeted nanoparticles (NPs) containing either mRNA encoding the leukemia-specific 1928z CAR (a-e) or the HBCore1B27 TCR (f-j) were mixed into the cell suspension at a concentration of 3 μg of mRNA/10^6 cells. a qPCR measurements of relative 1928z CAR mRNA expression over time after T cells were exposed to 1928z CAR NPs. Shown are mean values ± SD. N = 9 biologically independent samples. b Flow cytometry of T cells at the indicated time points after incubation with NPs bearing 1928 CAR-encoding mRNA. c Summary plot of in vitro gene transfer efficiencies. Shown are mean values ± SD. N = 9 biologically independent samples. d In vitro assay comparing cytotoxicity of nanoparticle-transfected vs. retrovirus-transfected T cells against Raji lymphoma cells. T cells were co-cultured with Raji tumor cells at a 5:1 ratio. We used the IncuCyte Live Cell Analysis System to quantify immune cell killing of Raji NucLight Red cells by 1928z-CAR or control (P28z-CAR)-transfected T cells over time. Data are representative of two independent experiments. Each point represents the mean ± s.e.m. pooled from two independent experiments conducted in triplicate. e ELISA measurements of IL-2 (at 24 h) and TNF-α and IFN-γ (at 48 h) secretion by transfected cells. Shown are mean values ± SD; two tailed unpaired Student’s t-test. N = 9 biologically independent samples. f qPCR measurements of relative HBCore1B27 TCR mRNA expression over time after T cells were exposed to HBCore1B27 CAR NPs. Shown are mean values ± SD. N = 9 biologically independent samples. g, h Gene transfer efficiencies. i Cell killing of HepG2-cure NucLight Red cells by HBCore1B27 or control (MSLN-) TCR-transfected T cells over time. T cells were co-cultured with HepG2 tumor cells at a 5:1 ratio. N = 9 biologically independent samples. j ELISA measurements of cytokine secretion by transfected cells. Shown are mean values ± SD; two tailed unpaired Student’s t-test.
This phenomenon has been reported as a cause of failure in both preclinical and clinical studies, when adoptively transferred T cells specific for only single antigens were used to treat heterogeneous tumors (such as metastatic prostate cancer). We found that, in comparison to untreated LNCaP C42 prostate tumors, which express the ROR1 tumor antigen at various levels, CAR-targeted tumors in both treatment groups (adoptively transferred T cells or nanoparticle-programmed T cells) eventually developed ROR1 low/negative immune-escape variants (Fig. 7i).

In situ programming of HBV-specific T cells. Gene transfer of IVT mRNA-encoding CARs can only target T cells to antigens located on the surface of cells, so the many tumor-antigens or viral-antigens that are intracellular are inaccessible to these receptors. We already demonstrated in vitro that lymphocyte-targeted IVT mRNA nanoparticles can reprogram T cells with engineered TCRs that recognize the intracellular HBV core antigen (HBcAg) in the context of HLA (Fig. 3f–j). Given that over 300 million people worldwide are chronically infected with HBV, with a significant number of these patients developing...
cirrhosis and liver cancer, customizing T-cell products for each patient individually is clearly not feasible. As a first step to implement our IVT mRNA technology for the treatment of this disease, we established a mouse xenograft tumor model of HBV-induced hepatocellular carcinoma (HCC). One million HepG2 cells stably transduced with HBcAg and luciferase were intrahepatically injected after laparotomy. All mice reproducibly developed multifocal lesions within 7 days (Fig. 8a), at which point they were reconstituted with unstimulated human 10 × 10⁶ CD3+ T cells and received two weekly infusions of nanoparticles loaded with mRNA (50 μg/dose) encoding the HBcAg–luciferase transgenes into sufficient HBcAg antigen-specific T cells to induce disease regression and can achieve similar therapeutic effects compared to ex vivo-engineered lymphocytes (13-fold vs. 18.9-fold reduced photon count, respectively, compared to no-treatment control, Fig. 8b, c). Flow cytometry of the dissected livers confirmed equal densities of HBcAg18–27 TCR-expressing T cells. We found that nanoparticles injections programmed sufficient HBcAg antigen-specific T cells to induce disease regression and can achieve similar therapeutic effects compared to ex vivo-engineered lymphocytes (13-fold vs. 18.9-fold reduced photon count, respectively, compared to no-treatment control, Fig. 8b, c). Flow cytometry of the dissected livers confirmed equal densities of HBcAg18–27 TCR T cells in animals treated with ex vivo-engineered T cells versus in situ programming nanoparticles (Fig. 8d, e).

In conclusion, our results demonstrate that repeated infusions of T-cell-targeted polymer nanocarriers can deliver tumor-specific CARs or virus-specific TCR transgenes into sufficient quantities of host T cells to induce disease regression at levels similar to bolus infusions of ex vivo-engineered lymphocytes.

T-cell-programming nanoparticles are biocompatible. As a first step toward advancing in situ programming of disease-specific T cells to clinical application, we worked with the Nanoparticle Characterization Laboratory (NCL) at the National Cancer Institute (https://nanolab.cancer.gov/). Systemic administration of nanomedicines has the potential to cause infusion reactions in patients, an adverse response which often delays or halts clinical translation. These reactions can manifest as fevers, chills, rigors, rashes, chest, or back pain, or difficulty in breathing, and, in rare instances, they can be fatal. Identifying the risk of infusion reactions early in the drug development process can help mitigate potential safety concerns once the product reaches clinical trials, saving developers both time and money—and saving patients from potentially dangerous complications.

We used Assay Cascade Protocols, developed by the NCL, which can be indicative of infusion reactions. Specifically, we analyzed the effect of nanoparticles on complement activation (NCL Method ITA-5.2), their hemolytic properties (ITA-1), and their effect on oxidative stress in T-cells (ITA-32). To study these effects in clinically relevant concentrations of nanoparticles, we first calculated the Theoretical Plasma Concentration (TPC) which is the efficacious mouse dose (in our experiments: 50 μg).
mRNA/dose) scaled to the equivalent human dose (=2.03 µg mRNA/mL blood; see Fig. 9a). To assess the impact of T-cell-targeted mRNA nanoparticles on erythrocytes, we performed hemolysis tests by spectrophotometric measurement of hemoglobin release after exposure to various concentrations of particles. The performance of the hemolysis assay was tested by negative (PBS) and positive (Triton-X) controls. We found that the hemolysis rates for particles at the TPC were lower than 2% (mean 1.21 ± 0.26%, compared to 0.7 ± 0.11% in the PBS control; Fig. 9b), which is defined as non-hemolytic37. Nanoparticles also did not induce activation of complement iC3b or Bb, while C4d was slightly above the 2-fold assay threshold at the TPC concentration (mean 2.3 ± 0.13%, compared to 1 ± 0.003% in the PBS control; Fig. 9c). Lastly, we measured mitochondrial oxidative stress as a key determinant of nanoparticle-induced injury, because excessive production of reactive oxygen species (ROS) causes damage to cellular organelles and DNA, eventually leading to cell death38. Moreover, another consequence of excessive ROS generation is activation of cell signaling pathways that stimulate expression of proinflammatory and fibrotic cytokines39. We found that T-cell-programming nanoparticles induced only a very modest increase in oxidative stress (mean 3.6 ± 0.2-fold), compared to the PBS control (Fig. 9d).

Guided by the in vitro assessment of T-cell-programming mRNA nanoparticles for their potential to cause infusion reactions, we next conducted a comprehensive toxicity assessment in rodents. The rat is the preferred rodent species to predict human health toxicity outcomes of nucleic acid-based molecular therapies because its metabolic physiology (in particular renal and hepatic function) is closer to that of humans than is the mouse’s40–42. Sprague Dawley rats (6–8 weeks old) were injected with one dose of nanoparticles carrying 100 µg mRNA, which is the rat equivalent of 50 µg mRNA in mice, based on normalization of dose to body surface area43. These experiments were conducted using 1928z CAR nanoparticles. The 1928z CAR recognizes human CD19 but does not cross-react with rat CD19,
Fig. 7 IVT-mRNA nanocarriers encoding prostate tumor-specific CARs can improve survival of mice with established disease. a Heat map of PSCA, PSMA, and ROR1 antigen expression across a panel of 140 prostate cancer metastases showing the diversity of antigen expression. b Heat map representation of flow cytometry data showing variability in PSCA, PSMA, and ROR1 expression by LNCaP C42 prostate carcinoma cells. The colors indicate expression levels in 350 randomly chosen cells. c Three weeks of postimplantation, LNCap C42 prostate tumors were visualized by in vivo bioluminescent imaging. A representative photo of established tumors in the dorsal lobes of the prostates (white arrows) is shown on the right. d Sequential bioimaging of firefly luciferase-expressing LNCap C42 prostate carcinoma cells orthotopically transplanted into the prostate of NGS mice. Four representative mice from each cohort (n = 8) are shown. e Time line and nanoparticle dosing regimen. f Survival of animals following therapy, depicted as Kaplan–Meier curves. Shown are eight mice per treatment group pooled from three independent experiments. ms, median survival. Statistical analysis between the treated experimental and the untreated control group was performed using the Log-rank test; P < 0.05 was considered significant. N.s. nonsignificant. g Multicolor flow cytometry of cells recovered from prostate tumors 11 days after treatment start. Adoptively transferred or in situ-programmed ROR1 CAR+ T cells were identified by positive labeling for CD45 and a c-myc tag incorporated in the receptor. h Absolute numbers of ROR1-CAR+ T cells that localized to tumors isolated on day 4, day 7, and day 11 after treatment start. Total cell counts of viable (trypan blue-negative) cells were multiplied by the percentage that was both R01-CAR and CD45 positive. Shown are mean values ± SD; two tailed unpaired Student’s t-test. N = 8 biologically independent samples pooled from two independent experiments. i Flow cytometry quantification of ROR1 antigen expression on LNCaP C42 prostate tumor cells following CAR-T cell therapy or ROR1 4-1BBz CAR NP therapy. Shown are 350 randomly chosen cells pooled from five tumors.
to ensure that changes in the parameters we measured could be attributed to the nanoparticles and not their reprogramming activity. Controls were either infused with 25 mM sodium acetate buffer (vehicle control) or received no injection. Animals were euthanized after 48 h, blood was collected to measure clinical biochemistry parameters, and we also performed a complete gross necropsy. The following tissues were evaluated by a board-certified staff pathologist: lung, liver, heart, brain, kidney, spleen, bone marrow, and duodenum. There were no histologic lesions that could be attributed to nanoparticle drug treatment (Fig. 10a). The few lesions noted were minimal to mild and considered incidental and unrelated to the study. All groups had two of five rats with minimal inflammatory infiltrates in the liver. These minimal infiltrates were considered to be background lesions and unrelated to treatment. Likewise, all groups contained individuals with minimal to mild chronic inflammation in the renal pelvis. The chronicity of the lesions was not consistent with an acute treatment effect, and therefore considered incidental. The complete blood count platelet values were slightly lower in the treatment effect, and therefore considered incidental. The chronicity of the lesions was not consistent with an acute inflammation in the renal pelvis.

**Discussion**

While CAR- and TCR-modified T cells have been transformational for a handful of blood cancers, it is clear that their current clinical applications represent only a sliver of the spectrum of possibilities that this technology might offer. In theory, both malignancies and chronic infections with targetable antigens could be treated with therapeutic T cells, as demonstrated in a large number of preclinical reports. However, current methods to generate disease-specific T cells in vitro are elaborate and cannot support the treatment of sizable patient populations, because a new lymphocyte cohort must be produced for each patient. In an effort to make T-cell products more accessible to patients, the field has turned to allogeneic technologies to provide better scale and lower costs. Several clinical-stage T-cell companies have begun testing CAR T cells manufactured as “off-the-shelf” products from healthy unrelated donors, rather than the patient. While this approach allows treatment of cancer patients for whom autologous T cells cannot be manufactured due to the patient’s low lymphocyte counts or poor T-cell quality, it requires several additional cell-engineering steps to prevent donor cells from attacking the host, and conversely to prevent a patient’s own T cells from rejecting the infused product. This is most often accomplished using multiplex gene editing to eliminate native TCR and HLA molecules from the T-cell product. But these additional manipulations add complexity, time and expense to the manufacturing process while reducing cell yield and viability. To keep rejection at bay, patients receiving universal CAR T-cells are first heavily immunosuppressed through lymphodepleting chemotherapy, which requires time and exposes the patients to additional toxicity. It is therefore unlikely that ex vivo-engineered allogeneic cell products will dramatically expand the number of patients treated with T cells, especially those with infectious diseases which require swift interventions that leave the endogenous immune system intact.

Our group previously described an injectable DNA-based nanoreagent that can program circulating T cells with leukemia-specific CAR transgenes. To overcome the inherently low gene transfer of plasmid DNA, which must enter the cell nucleus to be
transcribed into mRNA, we loaded nanoparticles with a transposon/transposase system encoding the CAR, which randomly inserted into target cells’ genomes. While that study provided proof-of-concept that in situ programming of CAR T cells using an injectable nanoreagent is possible, translation of this DNA nanomedicine into the clinic would have been challenging for the following reasons: (i) Unpredictable genotoxicity and expression kinetics. These nanoparticles stably integrate their therapeutic CAR transgenes into target cells, resulting in permanent genomic alterations and unpredictable genotoxicity in a variety of cell types. Furthermore, once nanoparticles are infused into patients, the physician has no control over the kinetics of in vivo CAR expression; (ii) Low copy numbers of relevant CAR genes per nanoparticle. The number of CAR genes we could load into these DNA nanoparticles was limited by the large size of the backbone and promoter sequences of the plasmids as well as the requirement to co-deliver a transposase expression vector for stable integration. This substantially limits in situ gene transfer efficiencies, especially when trying to deliver large transgenes encoding TCR alpha and beta chains; and (iii) The need for abundant tumor antigen to expand the small population of in situ-transfected CAR T cells to therapeutically relevant numbers. This expansion period takes time, which is a disadvantage in patients with rapidly progressive disease or defined solid tumors.

Here, we explored the use of IVT mRNA to quickly and specifically program antigen-recognizing capabilities into circulating T cells as a strategy to treat cancer and infectious disease. In contrast to DNA nanocarriers, synthetic mRNA molecules are directly translated into therapeutic target proteins without the need to enter the nucleus, ensuring high transfection rates and...

![Theoretical plasma concentration (TPC) calculations:](#)

Maximal mouse dose administered per day = 50 µg/25 g mouse = 2 mg/kg

| Parameter | Calculation | Result |
|-----------|-------------|--------|
| Human dose | mouse dose / 12.3 | 2 mg/kg / 12.3 = 0.1626 mg/kg |
| Human blood volume | Human dose / Human blood volume | 70 kg x 0.1626 mg/kg / 5.6 L = 2.03 µg/mL |

**Fig. 9 In vitro analysis of possible infusion reactions.**

- **a** Calculation of the theoretical plasma concentration.
- **b** Hemolytic activity of T-cell-targeted mRNA nanoparticles (NPs).
- **c** Quantitative determination of complement activation by an Enzyme Immunoassay. A 2-fold change relative to the negative PBS control was defined as the assay threshold (dashed line).
- **d** Mitochondrial oxidative stress in lymphocytes following NP transfection. In all panels of this figure, N = 9 biologically independent blood samples were analyzed. Also shown are the mean values ± SD. Pairwise differences between groups were analyzed by two tailed unpaired Student’s t-test.
rapid therapeutic effects. Their trim size (in our study, the actual CAR-coding or TCR-coding sequence + only 276 bases for the 5' UTR and polyA tract) results in a high copy number per nanoparticle. Also, uncontrolled insertional mutation and promoter dependency is avoided because the delivered mRNA exerts its function in the cytoplasm. We demonstrated that simple injection of rationally designed mRNA nanoparticles can selectively deliver CAR-genes or TCR-genes into host T cells and program them in quantities that are sufficient to bring about disease regression with efficiencies that are similar to adoptive methods. Several ongoing clinical trials are testing repeated infusions of ex vivo-engineered mRNA CAR T cells in cancer patients (ClinicalTrials.gov: NCT01355965, NCT01897415, NCT02277522 and NCT02624258), and the first data suggest that transient CAR expression after cell infusion is sufficient to trigger antitumor responses.36–38

Three important reasons why IVT mRNA has rapidly emerged as a new tool for adoptive T-cell therapy are its inherent safety, its highly efficient recombinant protein translation, and the ability to control pharmacokinetic properties of the therapy, similar to a conventional small molecule drug. Indeed, the kinetics of CAR-expressing T cells in mice that we measured following multiple dosing resemble the profile of a drug with a well-defined half-life (Fig. 4e). This is in sharp contrast to the rather unpredictable T-cell kinetics following adoptive transfer of engineered T cells, where the concentration of cells rises to a maximum in blood followed by a decline over a variable period of days to several months.39–62 While in situ programming gains the ability to control pharmacokinetic properties of the therapy and to periodically reprogram fresh populations of host lymphocytes—thus potentially bypassing some of the major barriers to wider deployment of T-cell therapies (e.g., T-cell exhaustion and dysfunction, and also long-term toxicities)—the technology still has some limitations: (1) It relies on the presence of a sufficient number of functional T cells in the patients. Lymphopenia is frequent in patients with advanced cancers who have been heavily pretreated with chemotherapy agents. It is therefore likely that patient blood will need to be pre-screened ahead of enrollment into clinical trials of our off-the-shelf nanoreagent. (2) The efficacy of the drug could be blunted by elicited immune responses. Since T-cell-programming nanoparticles are administered periodically into patients with intact immunity, antidrug antibodies could form. For clinical translation of this technology, it will be important to choose fully humanized CD8-targeting ligands, deliver CAR/TCR constructs that carry a low immunological risk, and synthesize mRNA with pseudouridine (or the recently
described N1-methyl-pseudouridine and 5-methylcytosine to reduce innate immune responses. To redirect circulating T cells to resident tumor cells in situ, several biotech drugmakers have developed bispecific antibodies, including BiTEs, DARTs, and diabodies. Among these, blatumomab (a CD19-specific BiTE) has shown encouraging results in clinical studies for patients with hematological malignancies. However, BiTEs must be administered as a continuous infusion, which can produce systemic toxicities. Furthermore, like conventional monoclonal antibodies, BiTEs do not undergo active biodistribution or self-amplification following infusion. By contrast, the nanoparticle-based gene-modification system described here can generate de novo tumor-specific T cells, which as a “living drug” actively localize to the target, increase in number, and serially destroy cancer cells. Interest in CAR T-cell therapy remains strong, with more than 300 ongoing trials around the world, so we believe it is timely to launch an additional developmental and regulatory considerations compared with conventional medicines. Only a few facilities with the requisite degree of expertise are currently operational in the United States. Methods Cell lines. Raji lymphoma cells were obtained from ATCC (Cat# CCL86) and cultured in RPMI 1640 containing 10% fetal bovine serum (FBS), 0.8 mM l-glutamine, 25 mM HEPES buffer, and 1% penicillin–streptomycin. The human prostate adenocarcinoma cell line PC3 C42 was provided by Dr. Michel Sadelain (Memorial Sloan-Kettering Cancer Center). These cells were cultured in RPMI 1640 supplemented with 10% FBS, 2 mM l-glutamine, 1.0 mM sodium pyruvate, and 1% penicillin–streptomycin. The human hepatoma cell line HepG2 was stably transduced with HBCAg, was provided by Dr. Antonio Bertolotti (Duke-NUS Medical School). The base medium for this cell line is Eagle’s Minimum Essential Medium (ATCC Cat# 30-2003). The EJ–ALL01 cell line (a gift from Dr. Michel Sadelain; Memorial Sloan-Kettering Cancer Center, New York, NY) was cultured in complete RPMI 1640 medium with 10% heat-inactivated fetal bovine serum (FBS), 2 mM l-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, and 0.05 mM 2-mercaptoethanol. For bio- lumescent imaging, all cell lines were retrovirally transduced with firefly luciferase (F-luc). All cell lines tested negative for mycoplasma using a DNA-based PCR test (DDC Medical). Retroviral vectors and virus production. A retroviral vector expressing the anti-CD19-28z CAR was kindly provided by Dr. Michel Sadelain (Memorial Sloan-Kettering Cancer Center, New York). The anti-CD19-28z CAR was custom- designed by Creative Biosilas (Shirley, NY). It consists of a single-chain antibody targeting human CD19 linked via a c-myc tag to a synthetic receptor skeleton comprising of the CD8 hinge, the CD28 transmembrane and signaling domain, and the signaling domain from CD3ζ. This construct was then cloned by VectorBuilder (Santa Clara, CA) into their MMLV Retrovirus Gene Expression Vector. A MMLV retroviral vector containing the Vα and Vβ chains of the HBcCore18–27– specific TCR sequences was cloned by VectorBuilder (vectorbuilder.com). To generate the 28z CAR, we transfected the Plat-A retroviral packaging cell line (cellibiosas.com) according to the manufacturer’s instructions and harvested retroviral supernatant 48 h later. Retroviral transduction of primary human T cells. To generate CAR-transduced or TCR-transduced T cells, human CD8+ T cells (obtained from the Hema- topoietic Cell Processing and Repository Core at the Fred Hutchinson Cancer Research Center) were stimulated with antiCD3/CD28-coated Dynabeads (ThermoFisher Scientific) for 48 h at a bead-to-cell ratio of 1:1 in the presence of 30 U/ mL IL-2. Beads were then magnetically removed and transferred to retrominecotin-coated plates (Takara) with virus, then spin-infected for 1 h at 3000g at 32°C. Following a second spinnulation in retrovirual supernatant the next day, the cells were cultured for 24 h in the presence of IL-2 before using them as a tumor therapeutic. PBAE synthesis. We combined 1,4-butanediol diacrylate with 4-amino-1-butanol in a 1:1 molar ratio of diacrylate to amine monomers. Acrylate-terminated poly(ethylene oxide)-b-poly(ethylene glycol) (PEO–PEG) was synthesized and purified by the tetrahydrofuran (THF). To form the pipervaine-capped 447 polymer, 786 mg of 1-(3-amino-propyl)-4-methylpyrllazine in 13 mL THF was added to the polymer/THF solution and stirred at room temperature (RT) for 2 h. The capped polymer was precipitated with five volumes of diethyl ether, washed with two volumes of fresh ether, and dried under vacuum for 1 day. Neat polymer was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 100 mg/mL and stored at −20°C. Preparation of CD8α-targeting antibodies. AntiCD8α antibody (clone OKT-8) was purchased from BioXcell (Cat# B8E004-2). Before use, Fc-glycans were deglycosylated using deGlyCt spin columns containing IgGZERO enzyme (Genovis, Cat# A1b-V6-10), according to manufacturer instructions. AntiCD3 murine IgG2a LALA-PG and nonbinder control (used only for in vivo experiments in immuneocompetent mice/Fig. 6). The antiCD3 scFv design was based on the bispecific antibody construct EpCam x 2C1 scFv (GenBank: SJL88240.1). Detailed sequence information can be found in the Supplemental Info PDF. Antibody conjugation to PGA. Fifteen kilodalton of poly-glutamic acid (from Alzheimers Polymers, Cat# PLE100) was dissolved in water to form 20 mg/mL and sonicated for 10 min. An equal volume of 4 mg/mL 1-ethyl-3-(3-dimethylamino- propyl) carbodiimide hydrochloride (Thermo Fisher) in water was added, and the solution was mixed for 5 min at RT. The resulting activated PGA was then combined with antibodies at a 4:1 molar ratio in phosphate buffered saline (PBS) and incubated for 2 h at RT. To remove unbound PGA, the solution was diluted through Amicon Ultra Centrifugal Filters (50 K MWCO). Antibody concentrations were determined by absorbance at 280 nm. mRNA synthesis. Codon-optimized mRNA for eGFP, the antiCD19-28z CAR, the antiCD19-28z CAR (Creative Biosilas Cat# CAR-T-1-M324-2Z), and the HBcCore18–27-specific TCR72 were manufactured by TriLink Biotechnologies and were capped with the anti-CD18 anti-CD8 (Arc-1) oligonucleotide (ARCA), and fully substituted with the modified ribonucleotides pseudouridine (Ψ) and 5-methylcytidine (m5C). Nanoparticle preparation. mRNA stocks were diluted to 100 µg/mL in 25 mM nucleosme-free sodium acetate buffer, pH 5.2 (NaOAc). PBAE-447 polymer in DMSO was diluted to 6 mg/mL in NaOAc, and added to mRNA at a 60:1 (w/w) ratio. After the resulting mixture was vortexed for 15 s at medium speed, it was incubated for 5 min at RT so nanoparticle (NPs) could form. To add targeting elements to the NPs, PGA-linked antibodies were diluted to 250 µg/mL in NaOAc and added at a 2.5:1 (w/w) ratio to the mRNA. The resulting mixture was vortexed for 15 s at medium speed, and then incubated for 5 min at RT to permit binding of PBAE-AD to the NPs. The NPs were lyophilized by mixing them with 60 mg/mL D-succrose as a cryoprotectant, and flash-freezing them in liquid nitrogen, before processing them in a FreeZone 2.5 L Freeze Dry System (Labconco). The lyophi- lized NPs were stored at −80°C until use. For application, lyophilized NPs were re- suspended in a volume of sterile water to restore their original concentration. Characterization of nanoparticle size distribution, concentration, ζ-potential, and mRNA encapsulation. The physicochemical properties of NPs (including hydrodynamic radius, polydispersity, ζ-potential, and stability) were characterized using a Zetapals instrument (Brookhaven Instrument Corporation) at 25°C. To measure the hydrodynamic radius and polydispersity based on dynamic light scattering, NPs were diluted 5-fold in 25 mM NaOAc (pH 5.2). To measure the ζ-potential, NPs were diluted 10-fold in 10 mM PBS (pH 7.0). To assess the stability and concentration of NPs, freshly prepared particles were diluted in 10 mM PBS buffer (pH 7.4). The hydrodynamic radius and polydispersity of NPs were measured every 10 min for 5 h, and their sizes and particle concentrations were derived from Particle Tracking Analysis using a NanoSight 300 instrument (Malvern). A Qubit RNA HS assay kit (ThermoFisher Scientific, Cat# Q32852) was used to determine mRNA quantification. It contains a proprietary cyanine dye that specifically binds to the nucleic acid. NP samples were diluted 20-fold in 385 mM HFP+ 14.5 mM TEA in
5% (v/v) MeOH. Calibration mRNA stock sample was prepared and mixed at a ratio of 1RF:3:1KkB = 25:8 with a final concentration at 8 µg/mL in 385 mM HEPES+14.5 mM TEA in 5% (v/v) MeOH. Calibration standards were prepared by diluting stock in 385 mM HEPES+14.5 mM TEA at 5% (v/v) MeOH accordingly (0.2–4.0 µg/mL range). Both NP samples and standard samples were incubated at 60°C for 60 min. Each sample (10 µL) was mixed immediately with 190 µL of assay working solution and vortexed for 2–3 s, then incubated at RT for 2 min before measuring.

**Flow cytometry.** Data were acquired using a BD LSRFortessa or FacsCanto II cell analyzer running FACSDIVA software, and analyzed with FlowJo v10.1. Antibodies and other staining reagents used in flow cytometry are summarized below. Data were collected using a BD LSRFortessa analyzer running FACS DIVA software (Beckton Dickinson).

**Real-time tumor cell killing assay.** T-cell activity was analyzed using the MitoSox reactive level of C4d (inducive of complement activation via classical or lectin pathway), Bb (indicative of complement activation via alternative pathway) and iC3b (indicative of C3 component of complement activation pathways) according to ITA-2. Briefly, fresh plasma was isolated from whole blood from three different donors. PBAE-C18-α-β mRNA nanoparticles were freshly prepared as described above. PBAE-C18-α-β RNA standards were incubated in 100 µL of serum-free DMEM and plasma at 5× TPC, TPC/5, TPC/25 for 30 min at 37°C after vortexing. Human micromol of aliquots were frozen at −20°C to determine the levels of C4d, Bb and iC3b using Micro Vue (Quidel Corp.) ELA kits (A008, A027, A006, respectively) as per the manufacturer’s instructions. Cytokine factor was used as assay control for iC3b levels, and HAGG/Doxil was used as a positive control for C4d levels. 1x phosphate buffer was used as a negative control and 25 mM sodium acetate was used as a vehicle control. Percent hemolysis was determined as follows:

\[
\text{% Hemolysis} = \left( \frac{\text{Int} - \text{Neg}}{\text{Int} - \text{Pos}} \right) \times 100
\]

*Mice and in vivo tumor models.* Animals were housed in the rodent barrier facility of Fred Hutchinson Cancer Research Center, and used in the context of an animal protocol approved by the Center’s Institutional Animal Care and Use Committee (Protocol ID: PROTO00005782, Protocol Title: G0782, Adoptive Cell Therapy bioengineering). A 14-h light/10-h dark cycle and temperatures of 65–75 °F (~18–23 °C) with 40–60% humidity were used. Experimental and control animals were co-housed.

**Cytokine secretion assays.** T-cell cytokine release was measured with ELISA (R&D Systems) 24 h (IL-2) or 48 h (IFN-γ and TNF-α) after stimulation on irradiated Raji lymphoma cells (Fig. 3e) or HepG2-HbeAg hepatocellular carcinoma cells (Fig. 3f).

**Cytochrome c secretion assays.** Cells were harvested and analyzed for cytochrome c levels by ELISA 24 h after treatment with nanoparticles.

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**Raji tumor model: NOD/SCID/IL2r−/− (NSG) mice were bred in house and engravled via tail vein with 1 × 106 Raji cells expressing green fluorescent luciferase. Once disease established, mice were carefully monitored for signs of progressive hindlimb paralysis and body weight loss as a consequence of tumor spread in the blood stream. Tumors were subcutaneously monitored using bioluminescence imaging, and animals exceeding a whole animal bioluminescent tumor photon count of >20 × 106 s−1 cm−2 sr−1 (defined as experimental endpoint) were euthanized by CO2.**

**Ep-AL01 leukemia model: Ep-AL01 leukemia cells were injected into the tail vein of 4–6 week-old male albino B6 (C57BL/6-6yr-Tcr-/-) mice (Jackson Laboratory, Stock No. 0000552) and allowed to develop for five weeks. Following tail vein injection of Ep-AL01 leukemia cells, the mice were randomly assigned to experimental cohorts. Mice were serially monitored using bioluminescence imaging to closely monitor tumor progression. We defined a whole animal bioluminescent tumor photon count of >20 × 106 s−1 cm−2 sr−1 as experimental endpoint, at which point animals were euthanized by CO2. Animals that did not reach this endpoint were euthanized by CO2 at day 54.

**Oral cytotoxicity** (LNCaP C42 prostate tumor model: For orthotopic prostate tumor cell implantation, a longitudinal incision was made in the lower abdomen of anesthetized male NSG mice. The bladder, seminal vesicles, and prostate were partially extirpated from the abdomen to expose the dorsal surface of the prostate. Using a 27-gauge needle, 2 × 106 LNCaP C42 tumor cells were injected into each lobe in 25 µL of PBS. The abdominal cavity, muscle and skin were closed in two layers of 5/8 with a layer of 7/16. The animal protocol approved by the Center’s Institutional Animal Care and Use Committee (Protocol ID: PROTO00005782, Protocol Title: G0782, Adoptive Cell Therapy bioengineering). A 14-h light/10-h dark cycle and temperatures of 65–75 °F (~18–23 °C) with 40–60% humidity were used. Experimental and control animals were co-housed.

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with Vicryl Rapide polyglin 910 P-1 11 mm absorbable sutures and 7 mm stainless steel wound clips, respectively. Tumors developed over the course of ~21 days post implantation, and were serially monitored using bioluminescent imaging. We defined a whole-animal bioluminescent tumor photon count of $>30 \times 10^6 s^{-1} cm^{-2} sr^{-1}$ as experimental endpoint, at which point animals were euthanized by CO₂.

Mouse xenograft tumor model of HBV-induced hepatocellular carcinoma (HCC): HepG2 cells stably transduced with HBcAg and luciferase were surgically injected into the liver of NOD mice. Specifically, $10^8$ tumor cells were resuspended in 250 μL of serum-free medium. Directly before injection, we mixed the cell suspension at a 1:1 (vol/vol) ratio with Matrigel to increase the viscosity of the injected cell suspension. Small 0.5–0.5 mL syringes with 28-gauge needles were used to inject the cell/Matrigel suspension to avoid leakage of tumor cells from the injection site—which might lead to local spread and “seeding” metastasis in the peritoneal cavity—we limited the injected volume to 20–30 μL. A steady and slow injection was performed to prevent leakage of the injected cell suspension, and to minimize damage to the surrounding liver tissue. After removal of the needle, the liver surface at the site of the needle tract was covered with Gelfoam for 5 min to minimize bleeding and potential backflow. Nine weeks following tumor implantation was defined as experimental endpoint, at which point all animals were euthanized by CO₂ to isolate organs for analysis.

In vivo bioluminescence imaging. We used n-Luciferin (Xenogen) in PBS (15 mg/mL) as a substrate for F-luc (imaging of Raji-luc lymphoma cells, LNCap C42-luc, and HepG2-luc hepatocellular carcinoma cells). Bioluminescence images were collected with a Xenogen IVIS Spectrum Imaging System (Xenogen, Alameda, CA). Living Image software version 4.3.1 (Caliper Life Sciences) was used to acquire data (and later quantify the data) the data 10 min after intraperitoneal injection of n-luciferin into animals anesthetized with 150 mg/kg of 2% isoflurane (Forane, Baxter Healthcare). Acquisition times ranged from 10 s to 5 min.

Toxicity analysis in rats. To measure potential in vivo toxicities of infusing T cell-targeting mRNA nanoparticles, we injected female, 6–8-week-old Sprague-Dawley rats (5/group) intravenously with a single dose of CD8-targeted nanoparticles, carrying 100 μg mRNA encoding the 1928s CAR. Controls were either infused with 50 mM sodium acetate buffer (vehicle control) or received no injection. Forty-eight hours after the infusion, animals were anesthetized and blood was collected by cardiac bleed to determine the complete blood counts. Blood was also collected for serum chemistry and cytokine profile analyses (performed by Phoenix Central Laboratories, Mukilteo, WA). Animals were then euthanized with CO₂ to retrieve organs, which were washed with deionized water before fixation in 4% paraformaldehyde for 24 h, followed by processing routine paraffin embedding and sections stained with hematoxylin and eosin. The specimens were interpreted by Dr. Amanda Koehne, DVM, DACVP, a board-certified veterinary pathologist, in a blinded fashion.

Statistical analysis. The statistical significance of observed differences was analyzed using the unpaired, two-tailed Student’s t-test or the unpaired, two-tailed one-way ANOVA test. The P values for each measurement are listed in the figures or figure legends. We characterized survival data using the Log-rank test. All statistical analyses were performed using either GraphPad Prism software version 7.0b or R software.

Study approval. Blood samples were obtained from healthy donors. Donors provided written informed consent for research protocols approved by the Institutional Review Board of the FHRCR. The care and use of mice in this study was approved by the Institutional Animal Care Use Committee (Dr. George Georges, MD, IACUC chair) at the Fred Hutchinson Cancer Research Center and complied with all relevant ethical regulations for animal testing and research (Assurance #A3226–01, IACUC Protocol Number 50782).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. All data supporting the findings of this study are available within the article and its supplementary information files and directly from M. Stephan upon reasonable request. A reporting summary for this article is available as a Supplementary Information file. Source data are provided with this paper.

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Author contributions
N.N.P. helped conceive the study and designed and performed the experiments. S.B.S. provided polymers and antibody-PGA conjugates. A.L.K. performed and analyzed the in vivo safety/toxicity studies. P.S.N. provided antigen expression profiles for human prostate cancer metastases showing the diversity of antigen expression. M.T.S. conceived the study, helped design the experiments, and wrote the manuscript.

Competing interests
M.T.S. is a consultant of Tidal Therapeutics and holds stocks in the company. The remaining authors declare no competing interests.

Additional information
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