Supplementary Materials for

Nano-optogenetic engineering of CAR T-cells for precision immunotherapy with enhanced safety

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Supplementary Note 1 – LiCAR engineering, optimization, and evaluation

Design of LiCAR. In a typical design, Component I of LiCAR shares several similar features with CAR, which bears a signal peptide from the T cell surface glycoprotein CD8 alpha chain, a Myc tag to aid extracellular staining, an anti-CD19 scFv, CD8α hinge, the CD8 transmembrane domain, the co-stimulatory 4-1BB domain, and one part of the optical dimerizer pair, and GFP was tagged to the C-terminus to aid the visualization and detection of protein expression. The cell surface expression and membrane topology of Component I was confirmed by the non-permeabilized staining of the transfected cells with an anti-Myc antibody (inset, Extended Data Fig. 1a). The prototypical Component II construct comprised a co-stimulatory 4-1BB domain, the T cell activating ITAM fragment of the CD3ζ subunit, the complementary part of the optical dimerizer pair, and the mCherry (mCh) tag to aid in visualization and flow cytometry analysis of protein expression.

Optimization of LiCAR. By using HeLa cells as a model cellular system, we first examined the subcellular distribution of the designed constructs. We found that the first-generation Component I showed poor trafficking toward the plasma membrane (PM), whereas Component II tended to accumulate within the nuclei (constructs A0+B2.0 or C0+D2.0; Extended Data Fig. 1b-c). To overcome these issues, we inserted ER trafficking and export signal peptides, derived from an inward rectifier potassium channel1,2, into Component I to enhance PM targeting (constructs A and C). In parallel, we appended a nuclear export signal (NES) from a cAMP-dependent protein kinase inhibitor (PKIα)3 into Component I in order to minimize its nuclear entry (Fig. 1b-d, and Extended Data Fig. 1d-f). When co-expressed in mammalian cells, in response to blue light illumination, these improved constructs (A+B2 or C+D2) exhibited light-dependent heteromerization, as reflected by the rapid cytosol-to-PM translocation of Component II (Fig. 1c-e). This process can be repeated multiple times by toggling the light switch (Fig. 1f-h, and Supplementary Videos 1-2). While the dissociation of the LOV2-based LiCAR complex occurred with a relatively fast half-life ($t_{1/2, off} = 33-37$ s), the reversion of CRY2-CIBN1 binding took a much longer time ($t_{1/2, off} = 4-5$ min) (Fig. 1f-h). The varying range of deactivation half-lives makes it possible to temporally control the duration of the elicited anti-tumor immune response.

We next sought to screen combinations that would permit the light-induced functional assembly of LiCARs in T cells. We used the NFAT-dependent luciferase (NFAT-Luc) reporter assay as a convenient method to assess the degree of T cell activation4. In human Jurkat-Luc T cells co-transduced with retroviruses encoding the split CAR components, we were able to achieve a transduction efficiency of 70-80% for the CRY2-based LiCAR (A+B) and over 97% for the LOV2-based LiCAR (C+D; GFP as indicator for Component I and mCherry for Component II; Supplementary Fig. 1a). The co-expression of both components at the protein level in T cells was further confirmed by immunoblotting (Supplementary Fig. 1b). NFAT-Luc Jurkat T cells expressing engineered receptors were then engaged with human CD19 (hCD19)-negative K562 leukemia cells or hCD19-positive Raji lymphoma cells (Supplementary Fig. 2a-c). In the dual presence of cognate tumor antigen (hCD19+ Raji lymphoma cells) and light stimulation, we detected a pronounced increase in NFAT-driven reporter bioluminescence, indicating the antigen/light-
dependent activation of engineered T cells (A+B1, A+B2, C+D1, or C+D2; Fig. 1i). However, these prototypical LiCAR T-cells did not appear to be efficiently activated, as they only produced 16-21% of the maximal response seen in WT CAR T-cells (Fig. 1i). We reasoned that targeting both components to the PM might reduce the entropic cost of driving the cytosolic Component II to couple with the PM-resident Component I. Therefore, we continued to modify Component II by adding the transmembrane domain of CD8 (Constructs B3 or D3) and/or the homodimeric DAP10 ectodomain (B4 or D4)\textsuperscript{5,6}, the latter of which was added to double the available numbers of CD3ζ-ITAMs in each assembled LiCAR complex in order to amplify the activation signals\textsuperscript{7}. We also reordered the positions of intracellular CAR components and optical dimerization modules, anticipating to identify the best configuration for maximal light-inducible reconstitution of functional CARs (Fig. 1b and Extended Data Fig. 1a). Ultimately, we identified the best combinations for both CRY2- and LOV2-based LiCARs, which led to 39% (A+B3) and 93% (C+D4; Fig. 1i) of the maximal response in the NFAT-Luc assay, respectively. As a stringent control, defective LiCAR T-cells (C+D5) lacking CD3ζ-ITAM in Component II did not show discernible antigen/light-dependent changes (right, Fig. 1i).

Nevertheless, these improved LiCAR T-cells (A+B3 or C+D4) displayed appreciable dark activity (i.e., pre-activation of NFAT-Luc in the dark; Figs. 1i and 2a-b). This is probably due to two reasons: (i) the relatively strong binding affinity of iLiD ($K_d$: 4.7 ± 0.7 μM (dark) vs 0.132 ± 0.005 μM (lit))\textsuperscript{8}; (ii) the highly sensitive nature of the synthetic NFAT-Luc reporter, which bears multiple copies of NFAT response elements in the promoter. We therefore explored two additional weaker versions of iLiD, in which wild type sspB was mutated to sspB-R73Q ($K_d$: 47 ± 13 μM (dark) vs 0.8 ± 0.1 μM (lit))\textsuperscript{9} (Fig. 1b-i, and Supplementary Video 2), or sspB-A58V ($K_d$: 56 ± 6 μM (lit))\textsuperscript{9} (Fig. 1b-i). We found that the use of sspB-R73Q and sspB-A58V led to a substantial reduction of background activation, but it also reduced the LiCAR activity to 71% (C+D4.1) and 51% (C+D4.3) of the maximal response in the NFAT-Luc assay, respectively (Fig. 1i).

**Tumor killing assays in vitro.** Both components of LiCAR were co-expressed in CD8\textsuperscript{+} T cells derived from peripheral blood mononuclear cells (PBMC), as confirmed by flow cytometry analysis and confocal imaging (Extended Data Fig. 3a-c). Subsequently, the cytotoxic activity of engineered CD8\textsuperscript{+} T cells against co-cultured K562 (hCD19\textsuperscript{−}) or Daudi (hCD19\textsuperscript{+}) cancer cells was quantified by staining target cells with the SYTOX Blue dye, which only penetrates dead cells with a compromised plasma membrane. After overnight incubation in the presence of pulsed blue light illumination, we observed a light-dependent boost of tumor cell killing, as reflected in the rightward shift of the SYTOX Blue staining peak in the flow cytometric histograms (Fig. 2g). The degree of targeted tumor cell killing was comparable to that observed with WT CAR T-cells. As a stringent control, the killing of cognate tumor cells was not observed in the defective LiCAR group (Fig. 2f-g). Noncognate tumor cells (hCD19\textsuperscript{−} K562 cells) survived throughout the assay, regardless of the presence of light. This attests to the high tumor antigen specificity of the designed LiCAR T-cells.

To better visualize the spatiotemporal features of CD8\textsuperscript{+} LiCAR T-cell mediated killing in response to light illumination, we performed time-lapse confocal imaging of LiCAR T-cells mixed with hCD19\textsuperscript{+} Daudi cells (Fig. 2h). Engineered human CD8\textsuperscript{+} T cells expressing WT CAR or defective LiCAR were used as positive and negative controls, respectively (Extended Data Fig. 3d, and Supplementary Videos 3-4). Again, SYTOX
blue dye was added to monitor dying cells with a compromised plasma membrane. Within 15 minutes of exposure to blue light, we started to observe the appearance of blue staining in Daudi cells engaged with LiCAR-expressing human CD8+ T cells (Fig. 2h, and Supplementary Videos 5), but not in those in the dark group (Fig. 2h, and Supplementary Videos 6). Collectively, LiCAR T-cells were able to inducibly mount anti-tumor cytotoxicity toward cognate target cells in the dual presence of tumor antigen and light ex vivo.

**Remarks on using weaker iLID variants.** We have demonstrated the successful design of photoswitchable CARs to deliver dual input (antigen + photon)-gated immune response using engineered therapeutic T cells. LiCAR enables light control over the therapeutic activity of CAR T-cells by simply varying the duration of light illumination in vitro. Depending on the readouts and various optical dimerizers used in our studies, we observed some minor leakage in the dark with some LiCAR constructs. This problem has been partially solved by using improved optical dimerizers with weaker affinities, and hence reduced interactions in the dark. Admittedly, the weaker versions cause less potent activation of re-assembled CAR T-cells, but have the advantage of minimized pre-activation to confer tight control over engineered CAR T-cells. Collectively, we provide herein a set of LiCAR tools tailored for different purposes. For potent activation, the combination of C + D4 is the best choice; whereas for more strict control, the C + D4.1 or C + D4.3 combinations will likely serve as better options. Worthy to note, the background activity of all three combinations was deemed as minor or negligible since they are unable to trigger cytokine secretion of T cells or its cytotoxic functions. Congruently, with these three options, we believe that stronger and weaker versions of LiCAR variants provide a more dynamic system for tunable control of engineered immune cells.

**Supplementary Note 2 – Upconversion nanomaterials.**

**UCNPs characterization.** To enhance upconversion luminescence intensity, we designed injectable hexagonal shaped upconversion nanoplates (UCNPs) with a core-shell composition of β-NaYbF₄:0.5%Tm@NaYF₄, in which Yb³⁺ serves as the sensitizer to accept excitation energy and Tm³⁺ serves as the emitter (Fig. 3c). Compared to the conventional ~30 nm spherical β-NaYF₄:30%Yb,0.5%Tm@NaYF₄ core-shell UCNPs used previously⁴, such UCNPs possessed an elevated Yb³⁺ concentration and enlarged nanoparticle size (Fig. 3d and Extended Data Fig. 4a). This nanoplate design is expected to be able to enhance upconversion luminescence. This is due to the prior knowledge that the increased amount of Yb³⁺ could enhance the energy absorption ability of the UCNPs and that surface defect-induced luminescence quench can be further significantly reduced in large nanoparticles. In particular, owing to the smaller ionic size of Yb³⁺ compared to Y³⁺, the replacement of Y³⁺ with Yb³⁺ in UCNPs synthesis suppressed the nucleation process and pronouncedly prolonged nanocrystal growth¹⁰. In this regard, the number of nanoparticles in the reaction solution will be less, but their size will be enlarged when using the same amount of lanthanide precursors. As a result, the synthesized β-NaYbF₄:0.5%Tm core possesses a hexagonal plate shape with an enlarged size (~155 nm in diameter and ~62 nm in height; Extended Data Fig. 4b). The produced β-NaYbF₄:0.5%Tm@NaYF₄ core-shell nanoplate displayed a hexagonal plate shape with a size of approximately 200 nm × 85 nm (Extended Data Fig. 4b). We observed that the resultant core/shell nanoplates via these two modifications (higher Yb³⁺ doping and larger size core/shell structure) indeed had markedly boosted upconversion luminescence brightness (Fig. 3e and Extended Data Fig. 4c). When excited at 980 nm, the synthesized UCNPs exhibited intense emission peaks in the blue light range,
with an intensity increased by 4.5-fold when compared to the conventional UCNPs with the same amount of total lanthanide ions (Extended Data Fig. 4c).

Silica-coated UCNPs. To further transfer these UCNPs from the organic solvent to an aqueous solution, a layer of silica shell was coated on the surface of the core-shell UCNPs, giving the final silica-coated core-shell nanoplate a size of \(~217\) nm (diameter) \(\times\) \(~103\) nm (height) (Fig. 3d and Extended Data Fig. 4b) or an apparent hydrodynamic radius of approximately \(220\) nm (Fig. 3d). When illuminated with a 980-nm laser, blue light emitting from the UCNP-containing cuvette in aqueous solutions can easily be seen by the naked eye, even after penetrating through multiple water-containing plastic cuvettes juxta-positioned to the UCNPs (Extended Data Fig. 4d).

Biocompatibility of silica-coated UCNPs. To examine their biocompatibility, we moved on to characterize the potential in vitro cytotoxicity and in vivo biosafety of silica-coated UCNPs. In the cytotoxicity study, B16-OVA-hCD19 cells were incubated with culture media containing UCNPs at increasing concentrations (0 \(\mu\)g/ml – 100 \(\mu\)g/ml). Using an MTT assay, we found that the cell viability was insignificantly affected by the UCNPs. With up to 100 \(\mu\)g/ml UCNPs in the cell culture solution, the cell viability remained at more than 90\%, thus indicating no overt cytotoxicity of the UCNPs (Supplementary Fig. 5a). Furthermore, we investigated the in vivo toxicity of UCNPs through histopathological studies and blood sample analyses. In both studies, tumor-bearing mice were injected with UCNPs (1 mg/ml, 150 \(\mu\)L; or an effective concentration of 35 \(\mu\)g/ml within a tumor with a diameter of 2-cm) and sacrificed at different times (1 day, 7 days and 14 days) after UCNP-injection. PBS-injected mice at the tumor sites were used as the control. By comparing H&E-stained images of the major organs from UCNP- or PBS-injected mice, we found that neither group displayed noticeable organ damage or inflammatory lesions (Supplementary Fig. 5b-c), suggesting negligible toxicity of UCNPs to major organs including heart, liver, spleen, lung, and kidney. In the blood analyses, UCNP-injected mice showed similar parameters compared to the PBS-injected group (Supplementary Table 1), with both falling within the normal range. In particular, the white blood cells of both groups were at the similar level (Supplementary Table 1), which indicates that no noticeable immune responses were elicited by the UCNPs. In addition to the tumor-bearing mice, the in vivo biosafety studies were also performed on healthy mice, which also showed no obvious toxicity induced by the UCNPs at this dosage (Supplementary Fig. 5c). Because macrophages are capable of phagocytosing foreign bodies, we further examined whether silica-coated UCNPs could cause macrophage dysfunction by comparing macrophage numbers and polarization in PBS- or UCNP-injected tumor bearing mice at 14 days after injection. We found no significant difference in the total, M1, or M2 subpopulations of macrophages (isolated from spleen or tumor) between the two tested groups (Supplementary Fig. 5d-e), indicating that UCNPs did not lead to aberrant changes in macrophage function. Together, these data clearly establish the excellent biocompatibility of our synthesized silica-coated UCNPs in living animals.

Intradermal melanoma models for anti-CD19 LiCAR-transduced T cells to eliminate antigen-specific melanoma. After 9 days of tumor establishment, the tumor sites were injected with a mixture composed of engineered WT CAR (the positive control) or LiCAR CD8\(^+\) T cells (2 \(\times\) 10\(^6\)) and UCNPs, in the presence or
the absence of pulsed NIR light stimulation for 8-9 subsequent days (980 nm at a power density of 250 mW/cm²; 2 h/day; pulses of 20 sec ON + 5 min OFF).

Element mapping showing silica-coated UCNPs being restrained in the tumor. To more rigorously rule out the potential trace leakage of the nanoparticle to the surrounding tissue and major organs, we performed a systematic element analysis of the nanoparticle distribution in living tissues by using an energy-dispersive X-ray spectroscopy (EDS) coupled SEM system. The lanthanide elements (Y and Yb) as well as Si of the silica shell were only detectable in the UCNP-injected tumor (Supplementary Fig. 7a). In all the other tissues/organs, including the tumor surrounding tissues (Supplementary Fig. 7b), heart, liver, spleen, lung, and kidney (Supplementary Fig. 7c-g), we did not detect lanthanide elements or Si derived from the injected UCNPs. The backscattered electron (BSE) images, which could differentiate heavy atoms (i.e., lanthanide elements) from light atoms, also agreed with the element mapping results: only the UCNP-injected tumor (Supplementary Fig. 7a, right), but not other surrounding tissue or major organs (Supplementary Fig. 7b-g, right), was clearly visible due to the presence of Ln³⁺. As summarized in Supplementary Table 2, the composition of elements in the UCNP-injected tumor largely agreed with the composition of silica-coated NaYbF₄:TM@NaYF₄ UCNPs. By contrast, other tissues were mainly composed of the physiologically-relevant elements, such as O, C, S, and P.

We monitored UCNPs through TEM at different time points and found that the UCNPs were clearly observable with no obvious morphological changes through 28 days (Supplementary Fig. 6a). In addition, under NIR excitation, the UCNP emissions were clearly observable (Supplementary Fig. 6b) but no other organs of the same mouse. Hence, the UCNPs remained stable in vivo, both physically and optically, for at least 28 days. To more rigorously rule out any potential leakage of the nanoparticle to the surrounding tissue and major organs, we performed an element analysis of the nanoparticle distribution in living tissues using an (Supplementary Fig. 7), whose results agreed well with the TEM and luminescence visualization of UCNPs being stably restrained in the tumor.

Supplementary Note 3 - Stv-UCNP-LiCAR T-cells for systemic delivery

Generation of Stv-UCNP-LiCAR T-cells. We first biotinylated the CAR T-cells using a well-adopted cell surface modification protocol. The biotinylation efficiency turned out to be very high, reaching over 98% (Extended Data Fig. 9b). More importantly, the surface biotinylation process did not seem to negatively affect the activation of both WT CAR or LiCAR T-cells, as judged by the NFAT-Luc reporter assay (Extended Data Fig. 9c). In parallel, the UCNPs were conjugated with streptavidin (Stv) following our previous protocol. Given the high affinity between the biotin and streptavidin (Kₐ = 10⁻¹⁴ to 10⁻¹⁵ M), Stv-UCNPs could efficiently bind to biotinylated immune cells.

Confirmation of the presence of Stv-UCNP-LiCAR T-cells in the tumor. In order to confirm the presence of Stv-UCNP-LiCAR T-cells (abbreviated as Stv-UCNP-CAR T) in the tumor site, we further sacrificed the mice at 48 hours after the second intravenous injection, with the isolated tumors and major organs subjected to NIR laser illumination. We detected eye-visible blue emission in tumors from the Stv-UCNP-LiCAR T-cells treated group (Supplementary Fig. 9a). Independently, the physical presence of lanthanides,
Yb and Y, in the tumor site was also detected by EDS imaging (Supplementary Fig. 9b, and Supplementary Table 3). By contrast, we only detected physiologically relevant elements, such as O, Na, S, and P, in the non-cancerous major organs and tissues (Supplementary Table 3). Furthermore, the successful infiltration of engineered LiCAR T-cells into the tumor site was confirmed by immunostaining of tumor cryosection samples. We observed notable GFP and mCherry signals, arising from the two LiCAR components, in the tumor sample (Supplementary Fig. 9c).

Supplementary Note 4 – Evaluation of side effects.

Design of mouse LiCAR-T (mLiCAR-T). To more rigorously evaluate the “on-target, off-tumor” side effects of LiCAR compared to the conventional CAR in a syngeneic mouse model of tumor, we designed a new set of mouse-specific LiCARs (mLiCARs; Fig. 5a), in which the hCD19-recognizing scFv was replaced by a mouse version derived from a mouse anti-CD19 mAb (1D3 ScFv)\(^{12}\). In order to examine whether 1D3 ScFv can recognize the mouse-specific CD19 (mCD19) antigen as well as to assess the degree of T cell activation, we again used the NFAT-Luc reporter assay (Fig. 5b). NFAT-Luc Jurkat T cells expressing engineered receptors (WT mCAR or mLiCAR) were engaged with B16-OVA melanoma cells or mCD19-positive B16-OVA-mCD19 cells (Supplementary Fig. 10a). WT mCAR T-cells only required the presence of mCD19 to be activated and the resultant bioluminescence level was comparable to that triggered by human CD19-targeting WT CAR T-cells (engaged by B16-OVA-hCD19 cells), suggesting that mCAR constructs can efficiently recognize mCD19 as the Ag (Fig. 5b). In the dual presence of the cognate tumor antigen from B16-OVA-mCD19 cells and light stimulation, we detected an increase in bioluminescence, indicating the antigen/light-dependent activation of engineered mLiCAR T cells (combination I + D4.1; Fig. 5b). Again, defective mLiCAR T-cells lacking CD3-ITAMs did not show detectable antigen/light-dependent changes (I + D5; Fig. 5b).

Tumor suppression of WT mCAR and mLiCAR in vivo. Next, similar to the human LiCAR system, we assessed the ability of anti-mCD19 WT CAR (mCAR) or LiCAR-transduced (mLiCAR) T cells to kill tumor cells in vivo using the same melanoma model, in which tumor cells bearing the non-cognate antigen (B16-OVA) or the cognate antigen (B16-OVA-mCD19) were inoculated into each flank of the same C57BL/6J mouse (Fig. 5c-d). After 9 days of tumor establishment, the tumor sites were injected with either WT mCAR T-cells/UCNPs (Fig. 5c) or mLiCAR CD8+ T cells/UCNPs (Fig. 5d). Pulsed NIR light stimulation for 10 days was applied to the mLiCAR group only. Both WT mCAR and mLiCAR T-cells exhibited tumor antigen-specificity by suppressing the growth of tumors composed of B16-OVA-mCD19 cells, but not the B16-OVA cells (Fig. 5c-d), thus validating the on-target effects of mLiCAR T-cells.

LiCAR system mitigates cytokine release syndrome (CRS). To evaluate the extent of CRS in our system, we adapted a well-established xenograft model of CAR T-cell-induced CRS in SCID-Beige mice recently developed by Giavridis et al\(^{13}\), which has been shown to recapitulate major CRS hallmarks seen in the clinic. We injected (i.p.) large numbers of Raji tumor cells (3x10^6) and allowed tumor growth for 3 weeks. Thereafter, a large amount of WT CAR-T cells or LiCAR T/UCNPs cells (3x10^7 cells) were implanted into the tumor sites to elicit CRS\(^{13}\), with acute weight loss and IL-6 production as two independent readouts (Fig. 6a). LiCAR-treated mice were subjected to pulsed NIR light stimulation for 3 days (980 nm at a power
density of 250 mW/cm²; pulse of 20 sec ON, 5 minutes OFF; 2 h/day). In addition to monitoring the weights for 3 consecutive days, sera were collected on day 0 and day 3 and subjected to serum cytokine analysis (mIL-6). The results showed that after 3 days, the mice treated with WT CAR T cells experienced significant weight lost (Fig. 6b). Furthermore, the level of mIL6 was higher in the tumor-bearing mice injected with WT CAR T-cells compared to those injected with LiCAR T/UCNPs under pulsed NIR light (Fig. 6c), suggesting that the LiCAR system indeed mitigated the cytokine release syndrome.

**Supplementary Note 5. Comparison between LiCAR and other inducible CAR systems.**

Our wireless NIR induced optogenetics provides a new non-invasive tool to circumvent the limitations of conventional and chemical-based methods. Such NIR light-inducible approaches show multiple advances in terms of real-time spatial and temporal capacity, tunability, and noninvasiveness. For instance, a rapamycin analog-induced FRB-FKPB heterodimerization system was used to enable chemogenetic control over therapeutic T cells. Nonetheless, the use of conventional chemicals does not allow reversible real-time spatiotemporal control over CAR T-cell function as offered by our method. Moreover, such chemical methods will also likely introduce potential undesirable effects in biological systems such as cytotoxicity, and perturbation to host physiology due to possible drug-induced off-target effects, including impaired testicular size and function, kidney impairment, impaired metabolism and neurological disorders in humans. Furthermore, rapamycin analog does not have ideal pharmacokinetic properties and has a short half-life in plasma (about 4 hours), therefore requiring a frequent injection of the drug into treated subjects. Finally, to date, the tumor model used in such chemical-based study was observed for only 40 hours (compared to our 2-to-4-week treatment), which does not represent the therapeutic function of such CAR T-cells on clearing tumors.

In addition, although a couple of light-induced expression of the CAR gene has been attempted, but they are primarily demonstrated ex vivo and cannot conduct real-time switch (Supplementary Table 4). In particular, first in the published studies, blue light was simply used to inducibly drive the expression of CAR transgene or others. This represents a non-reversible system since once CAR is expressed, CAR will present on the T cell surface to cause constitutive activation of T cells. By contrast, our LiCAR system is fully tunable as a two-component system – the expression of either component will not activate engineered T cells. Functional CAR will only be reassembled in the dual presence of light and antigens to mount anti-tumor immune response. Second, the existing methods use blue light with very limited tissue penetration (less than 1-2 mm), which is known to be a general limitation for in vivo optogenetic applications as we also observed in our control experiment (Extended Data Fig. 8). Moreover, we have successfully demonstrated that the UCNP/LiCAR T-cell hybrid system can be administered either locally (intratumoral delivery) or systemically (intravenous injection) to achieve NIR light-inducible tumor regression.
**Supplementary Figure 1 | Expression of engineered CAR components (A+B or C+D combinations) in human Jurkat T cells. (related to Figure 1)**

**a,** Quantification of transduction efficiency in Jurkat T cells. Jurkat cells were retrovirally transduced with the indicated A+B or C+D combinations to assemble functional LiCARs. The expression of A/C (GFP⁺) or B/D (mCherry⁺) components was determined by flow cytometry.

**b,** Immunoblot analysis of LiCAR components expression in Jurkat T cells. T cells were transduced with retroviruses encoding GFP-tagged WT CAR or LiCAR components (A-GFP + B-mCherry; or C-GFP + D-mCherry). Component I was probed with an anti-GFP antibody whereas Component II was probed with an anti-mCh antibody. GAPDH was used as a loading control. The experiment was independently repeated two times with similar results.
Supplementary Figure 2 | Optimizing the ratio of effector T cells to target tumor cells (E/T ratio) to evaluate the function of engineered CAR T-cells. (related to Figure 2)

a, Quantification of CD19 expression in target B cells. K562 cells showed negligible human CD19 (hCD19) expression whereas Raji or Daudi lymphoma cells showed over 98% positive staining for hCD19.

b, NFAT-Luc activity of conventional CAR T-cells when co-cultured with different amounts of either hCD19- K562 cells (open box) or hCD19⁺ Raji cells (red box). An E/T ratio of 1:3 was able to elicit potent NFAT-dependent activity. n = 3 independent biological replicates (mean ± s.e.m.).

c, NFAT-Luc activity of WT CAR T-cells incubated with Raji cells at a ratio of 1:3 compared with that of non-transduced (negative control) T cells. n = 3 independent biological replicates (mean ± s.e.m.).
Supplementary Figure 3 | Expression of engineered CARs in mouse primary CD8+ T cells. *(related to Figure 3)*

**a,** Quantification of CD19 expression in melanoma cells (B16-OVA) and melanoma cells exogenously expressing human CD19 (B16-OVA-hCD19).

**b,** Evaluation of the purity of CD8+ T cells isolated from mouse spleens. Isolated T cells were stained with an anti-mouse CD8a eFluor 450 antibody. Non-stained CD8+ T cells were used as negative control to aid the gating.

**c,** Quantification of WT CAR (GFP-tagged), LiCAR (C-GFP + D4-mCh) or defective LiCAR (C-GFP + D5-mCh) expression in transduced mouse CD8+ T cells. GFP-positive (for the WT CAR group) or double positive cells (for the LiCAR and the defective LiCAR groups) were used for functional assays.

**d,** Assessing the viability of murine melanoma cells expressing hCD19 (B16-OVA-hCD19) after co-culture with engineered mouse CD8+ T-cells. Mouse CD8 T cells expressing WT CAR, LiCAR or defective LiCAR were co-cultured with 1,000 pre-seeded B16-OVA-hCD19 cells at the indicated E:T ratios and incubated in the dark or exposed to blue light. Floating dead cells and CAR T-cells were washed away and viable tumor cells, which remained attached to the plate bottom, were made visible by DAPI staining under a confocal microscope. LiCAR T-cells effectively killed tumor cells under blue light. As the positive control, WT CAR T-cells showed light-independent killing of tumor cells. By contrast, defective LiCAR T-cells did not trigger tumor killing even under photo-illumination.
Supplementary Figure 4 | The growth rates of B16-OVA and B16-OVA-hCD19 cells in vitro and in vivo. (related to Figure 3)

a, In vitro cell proliferation of B16-OVA-hCD19 and B16-OVA cells quantified using the WST-1 colorimetric assay. Absorbance at 450 nm was used as readout. n = 6 independent biological replicates (mean ± s.e.m.). P values were calculated using two-sided unpaired Student’s t-tests.

b, The growth curves of 5x10^5 B16-OVA-hCD19 and B16-OVA melanoma cells after intradermal injection into the left and right flanks, respectively, of C57BL/6J mice. Tumor sizes at the indicated time points were measured by a digital caliper with the tumor areas calculated in mm^2 (length x width). No significant difference in tumor sizes were noted. P = 0.686 when compared to the hCD19-negative B16-OVA group at day 19 (two-sided unpaired Student’s t-tests; n = 9 biologically independent mice; mean ± s.e.m.)
Supplementary Figure 5 | In vivo biosafety and biocompatibility evaluation of silica-coated UCNPs. Scale bar, 100 µm. (related to Figures 3-6)

a, B16-OVA-mCD19 cell viability assessed by an MTT assay upon incubation with different concentrations of UCNPs. n = 5 independent biological replicates (mean ± s.e.m.). P value was calculated by one-way ANOVA test.

b-c, Typical H&E staining images of major organs (heart, liver, spleen, lung, kidney and tumor) isolated from UCNPs (1 mg/ml, 150 µL) or PBS-injected (150 µL at tumor sites) tumor-bearing mice (b) and healthy mice (c). The experiment was independently repeated four times with similar results.

d-e, The percentage of total, M1 and M2 macrophages in the tumor (d) and spleen (e) isolated from UCNP- or PBS-injected mice. n = 2 independent biological replicates (mean ± range).
Supplementary Figure 6 | Evaluation of the stability of UCNPs in vivo up to 28 days.

a, TEM images showing the morphology of UCNPs at different time points after injection in tumor-bearing mice (Day 1, 7 and 14 [end point when tumor reaching the maximally allowed size by IACUC]) or the leg muscle of healthy mice (Day 28). Three images per each time point were taken.

b, UCNPs showing bright emission under 980 nm laser excitation at 28 days after being injected into the leg muscle of mice. Other organs without UCNPs were used as the controls.
a UCNPs implanted in tumor

b Tumor-surrounding tissues
Heart from a UCNP-implanted mouse

Liver from a UCNP-implanted mouse
e  Spleen from a UCNP-implanted mouse

f  Lung from a UCNP-implanted mouse
Supplementary Figure 7 | Element analysis of the nanoparticle distribution in living tissues using an energy-dispersive X-ray spectroscopy (EDS) coupled SEM system. (Related to Figure 3)

**a-g.** The element analysis of the UCNP injected tumor, tumor-surrounding tissues, as well as other major organs (heart, liver, spleen, lung and kidney; as shown in Extended Data Fig. 7) from the UCNP-injected mouse. Top, SEM images of the indicated tissues (left); the element mapping showing the major three elements of the sample (middle); and the corresponding backscattered electron images (right; Note: heavy atoms such as lanthanides gave strong signals, while light atoms remained dim in the image); Bottom, the energy-dispersive X-ray spectrum of the indicated tissue samples.
Supplementary Figure 8 | LiCAR T-cells retention and expansion in vivo. (related to Figure 3g-h)

a, Schematic of the in vivo experimental setup for LiCAR T-cell isolation from mice bearing melanoma tumors. Left, B16-OVA-hCD19 inoculation, without LiCAR T-cells + UCNPs and NIR treatment (as mock control); Right, B16-OVA hCD19 (left flank) and B16-OVA inoculation (right flank), with LiCAR T-cells + UCNPs and NIR treatment.

b, FACS analysis of LiCAR T-cells (GFP+/mCherry+) within the isolated tumor masses at day 19 (shown in Extended Data Fig. 6). LiCAR T-cells were mostly detected in the B16-OVA-hCD19 tumor but were not readily detectable in the mock and B16-OVA tumors (n= 5 biologically independent mice).

c, Monitoring the off-tumor distribution of LiCAR T-cells by flow cytometry. Nominal GFP+/mCherry+ signals were detected in the spleen and peripheral blood isolated from the B16-OVA-hCD19 group.

d, FACS analysis of LiCAR T-cells within the isolated tumor masses at day 18 (shown in Extended Data Fig. 6). LiCAR T-cells were more abundantly detected in the tumors exposed to NIR light (from four mice) when compared to the mock and the non-NIR treated tumors (n = five biologically independent mice), indicating more robust activation/expansion of LiCAR T-cells after NIR-inducible reconstitution of functional CARs.
Supplementary Figure 9 | The presence of UCNPs and LiCAR T-cells in the tumor after intravenous injection of Stv-UCNP-LiCAR T-cells. (related to Figure 4)

a, NIR excitation showed that UCNPs accumulated in the tumor site 48 h-post intravenous administration of Stv-UCNP-LiCAR T-cells.

b, Element analysis of the nanoparticle distribution within the tumor site at 48 h after intravenous administration of Stv-UCNP-LiCAR T cells using an energy dispersive X-ray spectroscopy (EDS) coupled SEM system (Inset, the element mapping of metal ions).

c, Immunostaining to confirm the persistent presence of LiCAR T-cells (expressing Part I-GFP (green), and Part II-mCh (red)) in the tumor microenvironment at the endpoint (Day 21). Blue, nuclear DAPI staining. Three images were taken for each condition. Scale bar, 10 µm.
Supplementary Figure 10 | Validation of melanoma cells expressing mouse CD19 (mCD19) antigen and quantification of B cell population in mCAR or mLiCAR-treated mice by flow cytometry. *(related to Figure 5)*

**a,** Quantification of mCD19 expression in melanoma cells (B16-OVA) and melanoma cells exogenously expressing mouse CD19 (B16-OVA-mCD19).

**b,** Representative peripheral blood B cell populations from WT mCAR T-cells/UCNPs *(Figure 5c)* or mLiCAR T-cells/UCNs/NIR pulses *(Figure 5d)*-treated mice bearing B16-OVA/B16-OVA-mCD19 tumors on day 0 and day 3. B cells from peripheral blood of healthy mice were used as a control for normal B cell counts. B cells isolated from spleens were stained with anti-CD19-APC (positive control) and non-stained B cells (negative control) were used to aid in the gating of the cell populations (top panels).
**Supplementary Table 1.** Blood biochemistry and complete blood panel analysis of mice injected with PBS or UCNPs.

|                | Normal range | Tumor bearing mice | Healthy mice |
|----------------|--------------|--------------------|--------------|
|                | Day 1        | Day 7              | Day 14       | Day 1        | Day 7              | Day 14       |
| WBC (K/µL)     | 1.8-10.7     | 3.39               | 2.85         | 3.17         | 3.21               | 4.14         | 3.87         | 3.56         | 2.93         | 3.73         | 3.86         | 4.6          | 4.82         |
| LY (K/µL)      | 0.9-9.3      | 2.96               | 2.32         | 2.72         | 2.78               | 3.02         | 2.48         | 3.25         | 2.24         | 3.29         | 3.28         | 4.1          | 4.21         |
| MO (K/µL)      | 0.0-0.4      | 0.09               | 0.11         | 0.11         | 0.09               | 0.22         | 0.22         | 0.12         | 0.23         | 0.13         | 0.07         | 0.09         | 0.14         |
| NE (K/µL)      | 0.1-2.4      | 0.34               | 0.42         | 0.34         | 0.35               | 0.9          | 1.17         | 0.2          | 0.46         | 0.31         | 0.51         | 0.41         | 0.47         |
| RBC (M/µL)     | 6.36-9.42    | 10.04              | 8.13         | 8.18         | 8.41               | 10.31        | 9.38         | 9.28         | 7.95         | 9.34         | 9.56         | 8.57         | 9.41         |
| HGB (g/dL)     | 11.0-15.1    | 14.3               | 11.5         | 11.7         | 12.5               | 13.6         | 12.5         | 12.4         | 10.7         | 12.7        | 13.7         | 12.1         | 13.4         |
| HCT (%)        | 35.1-45.4    | 53.45              | 41.38        | 44.45        | 45.92               | 56.88        | 51.78        | 50.57        | 54.19        | 47.47        | 47.65        | 48.89        | 53.34        |
| MCV (fL)       | 45.4-60.3    | 53                 | 51           | 54           | 55                  | 55           | 55           | 54           | 57           | 51          | 50           | 57           | 57           |
| MCH (pg)       | 14.1-19.3    | 14.2               | 14.1         | 14.3         | 14.9               | 13.2         | 13.3         | 13.3         | 13.5         | 13.6        | 14.3         | 14.1         | 14.3         |
| MCHC (K/µL)    | 30.2-34.2    | 26.7               | 27.8         | 26.3         | 27.3               | 23.9         | 24.1         | 24.4         | 23.8         | 26.8        | 28.7         | 24.7         | 25.2         |
| RDW (K/µL)     | 12.4-27.0    | 23.5               | 24.1         | 21.2         | 21.8               | 22.2         | 22.2         | 22.6         | 22.6         | 25.9        | 22.1         | 20.5         | 20.8         |
| MPV (fL)       | 5.0-20.0     | 7.7                | 5.6          | 6.3          | 6.4                 | 6.8          | 6.8          | 7.9          | 6.8          | 6.2         | 6.3          | 6.7          | 7.6          |

**Abbreviations:** White blood cell (WBC), Neutrophils (NE), Lymphocytes (LY), Monocytes (MO), Eosinophils (EO), Basophils (BA), Red blood cell (RBC), Hemoglobin (HGB), Hematocrit (HCT), Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), Mean corpuscular hemoglobin concentration (MCHC), Red blood cell distribution width (RDW), Platelet thrombocyte (PLT), Mean platelet volume (MPV).
Supplementary Table 2. Major chemical element distributions in the indicated tissues.

| Atomic (%) composition | Tumor/injection site | Tumor surrounding tissues | Heart | Liver | Spleen | Lung | Kidney |
|------------------------|---------------------|---------------------------|-------|-------|--------|------|--------|
| Y                      | 4.02                |                           |       |       |        |      |        |
| Yb                     | 4.07                |                           |       |       |        |      |        |
| Si                     | 7.91                |                           |       |       |        |      |        |
| F                      | 46.76               |                           |       |       |        |      |        |
| Na                     | 6.19                | 0.20                      |       | 13.11 | 0.28   |      |        |
| O                      | 31.05               | 66.60                     | 69.77 | 66.72 | 64.67  | 66.59| 66.79  |
| C                      |                     | 33.09                     | 33.14 |       | 32.84  | 32.65|        |
| S                      |                     |                           | 14.29 | 0.15  | 5.15   | 0.14 | 0.19   |
| P                      |                     |                           | 7.30  |       | 17.07  | 0.14 | 0.37   |
| Zn                     |                     |                           |       |       | 8.65   |      |        |
| Mo                     |                     |                           |       |       | 0.11   |      |        |
**Supplementary Table 3.** Major chemical element distributions in the indicated tissues from a mouse receiving systemic injection of Stv-UCNP-LiCAR T-cells (at 48 hours).

| Atomic (%) composition | Tumor | Heart | Liver | Spleen | Lung | Kidney |
|------------------------|-------|-------|-------|--------|------|--------|
| Y                      | 0.93  |       |       |        |      |        |
| Yb                     | 0.37  |       |       |        |      |        |
| F                      | 3.7   |       |       |        |      |        |
| Na                     | 19.76 | 14.72 | 14.48 | 15.62  | 18.56| 14.29  |
| O                      | 57.73 | 64.95 | 64.93 | 63.51  | 62.44| 64.89  |
| S                      | 8.17  | 13.50 | 12.41 | 7.04   | 11.32| 11.38  |
| P                      | 9.34  | 6.84  | 8.18  | 13.84  | 7.68 | 9.44   |
**Supplementary Table 4.** Comparison of LiCAR with a light-inducible CAR expression system (LINTAD CAR).

| Parameters                          | LiCAR (this study)                                      | LINTAD CAR                                                                 |
|------------------------------------|---------------------------------------------------------|-----------------------------------------------------------------------------|
| PM location                        | Significantly improved                                   | N/A                                                                         |
| Tunability                         | YES                                                     | Once CAR expression is induced by light, it loses spatial and temporal control (largely irreversible) |
| Dual gate (light + antigen)        | YES                                                     | Cannot be controlled by light once CAR expression was induced               |
| Reversibility                      | YES                                                     | No                                                                          |
| Various kinetic systems            | $T_{1/2, on} = 3$-$10$ sec $T_{1/2, off} = 30$-$60$ sec | ON only, cannot be turned off; takes hours for gene expression              |
| Spatiotemporal control             | Yes. Upon light withdrawal, LiCAR T-cells will be deactivated | CAR T-cells after light induction will circulate to other sites – lack of spatial control |
| Tumor killing assessment           | YES                                                     | YES                                                                         |
| Cytokine release syndrome (CRS)    | YES. Significantly mitigated CRS in a mouse model of lymphoma | Because it cannot be turned off, CRS will remain once CAR expression was induced |
| On target off tumor effects        | YES. LICAR reduced the side effect since it is highly spatially controlled and it can be inactivated without light | This side effect will persist because of irreversibility                    |
| Depth of tissue penetration        | Up to 2-3 cm with UCNPs/NIR                              | < 0.5 mm with 470 nm                                                        |
| Light source                       | NIR or blue light                                        | Blue light                                                                  |
| Synthetic complications            | Straightforward; two components (modular)               | A complicated three component system; requiring multiple steps (nuclear translocation, dimerization and CAR gene expression) and often takes hours to take effect. Given cell-to-cell variation for multiple component transduction with viruses, the performance will be inconsistent. |
Captions for Supplementary Videos

Supplementary Video 1 | Light-induced recruitment of cytosolic Construct D2 (mCherry-tagged) toward Construct C localized in the plasma membrane. Two repeated dark-light cycles (470 nm; 40 mW/cm²) were applied to visualize the reversible cytosol-to-PM translocation of Construct D2. Related to Figure 1d. Scale bar, 5 µm.

Supplementary Video 2 | Light-induced recruitment of cytosolic Construct D4.2 (mCherry-tagged) toward Construct C localized in the plasma membrane. Two repeated dark-light cycles (470 nm; 40 mW/cm²) were applied to visualize the reversible cytosol-to-PM translocation of Construct D4.2. Related to Figure 1e. Scale bar, 5 µm.

Supplementary Video 3 | Time-lapse imaging of Daudi tumor cell killing mediated by human CD8⁺ T cells transduced with WT CAR (GFP-tagged; green) in the dark. T cells (indicated by “T”) were surrounded by co-cultured Daudi cells (indicated by “D”). SYTOX blue was used to stain dying Daudi tumor cells with compromised plasma membranes. Related to Extended Data Fig. 3d. Scale bar, 5 µm.

Supplementary Video 4 | Time-lapse imaging of Daudi (indicated by “D”) tumor cells co-cultured with human CD8⁺ T cells (indicated by “T”) expressing defective LiCAR (C-GFP + D5-mCh). The mixture was exposed to blue light illumination (470 nm; 40 mW/cm²; for 5 h). No appreciable SYTOX blue staining was observed. Related to Extended Data Fig. 3d. Scale bar, 5 µm.

Supplementary Video 5 | Time-lapse imaging of light-induced tumor cell killing mediated by human CD8⁺ T cells expressing LiCAR (C-GFP + D4-mCh). LiCAR T-cells (T) were surrounded by Daudi lymphoma cells (D) and subjected to photostimulation (470 nm; 40 mW/cm²; for 5 h). Daudi cells started to die at 15 min, as indicated by positive SYTOX blue staining. Within 5 h, we noticed the death of all three surrounding Daudi cells. Related to Figure 2h. Scale bar, 5 µm.

Supplementary Video 6 | Time-lapse imaging of Daudi cells (D) co-cultured with human CD8⁺ T cells (T) expressing LiCAR (C-GFP + D4-mCh) in the dark for more than 5 h. The LiCAR T-cells did not induce any tumor cell death in the absence of blue light, as reflected by negative SYTOX blue staining. Related to Figure 2h. Scale bar, 5 µm.
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