

APPLIED SCIENCES AND ENGINEERING

On-demand cancer immunotherapy via single-cell encapsulation of synthetic circuit-engineered cells

Yue Zhao^{1†}, Rui Li^{2†}, Yirui Han¹, Chaochen Shi¹, Kyubae Lee³, Guangjun Nie^{4*}, Yazhou Chen^{1*}

Despite the therapeutic potential of engineered immune cell therapy against metastases, it faces challenges including cytokine-driven systemic toxicity, off-target biodistribution, and host rejection. Here, we develop red/far-red light-regulated individually encapsulated (RL/FRL-EnE) cells, integrating optogenetics with biomaterial encapsulation for precise immunomodulation. This system uses a phytochrome A-based photoswitch (Δ PhyA-PCB) that enables bidirectional control. RL (660 nanometers) triggers interferon- γ , interleukin-6, and anti-CD47 expression via Δ PhyA-PCB-far-red elongated hypocotyl 1 heterodimerization, while FRL (740 nanometers) rapidly reverses production, minimizing toxicity. Single-cell nanoencapsulation prevents intercellular cross-talk and immune clearance, enabling strict light-dependent regulation and extended tumor residence. In vivo, RL/FRL-EnE cells remodeled the tumor microenvironment, reducing immunosuppressive myeloid cells (1.3- to 1.7-fold), while enhancing dendritic cell (1.4-fold) and CD8⁺ T cell (2.8-fold) infiltration. Collectively, this work establishes a paradigm for closed-loop cellular immunotherapy, where light-regulated living therapeutics achieve on-demand immune reprogramming.

INTRODUCTION

Pulmonary metastasis represents a primary determinant of cancer-related mortality, responsible for over 30% of solid tumor fatalities according to global epidemiological analyses (1, 2). Current therapeutic paradigms are substantially limited by several factors. Local therapies, such as surgery and radiotherapy, often struggle to effectively target micrometastatic deposits smaller than 2 mm in diameter due to spatial resolution constraints (3, 4). Systemic chemotherapy demonstrates broad-spectrum antineoplastic activity but suffers from poor therapeutic index due to nonselective cytotoxicity against healthy tissue (5, 6). Moreover, the alveolar-capillary barrier markedly impedes pulmonary drug delivery, with fewer than 5% of administered chemotherapeutic agents achieving therapeutic concentrations in lung tissue (7, 8).

Immunotherapy has emerged as a paradigm-shifting approach through its capacity to harness endogenous immune defenses against metastatic disease (9, 10). Contemporary approaches use various pharmacological and cellular engineering techniques to enhance innate immune responses, generate tumor-specific memory T cells, and establish sustained systemic immune surveillance (11, 12). However, the clinical implementation of immunotherapeutic modalities is constrained because uncontrolled delivery of immunotherapeutic agents may trigger excessive immune activation, leading to potentially fatal adverse effects (13). This paradox is exemplified in chimeric antigen receptor T cell therapy, where engineered lymphocytes demonstrate remarkable efficacy against hematologic malignancies but frequently provoke cytokine release syndrome (CRS) through massive cytokine secretion during tumor engagement (14, 15). Clinical data

reveal that severe CRS manifests in 15 to 20% of treated patients with associated mortality rates exceeding 10%, underscoring the urgent need for dynamic control systems in cell-based immune therapies (16–18).

Recent innovations in synthetic biology have empowered researchers to engineer mammalian cell chassis with synthetic gene circuits that function as biological computation platforms for precision medicine (19–21). The emerging field of synthetic designer cell therapeutics demonstrates that cells equipped with multi-input responsive systems can orchestrate on-demand therapeutic delivery through programmable regulation of protein expression dynamics (22, 23). This modular control strategy enables engineered cells to execute logic operations, initiating multilayered immunomodulatory responses exclusively upon detection of disease-specific biomarkers, while maintaining transcriptional quiescence in the absence of activating signals. Notably, advancements in optogenetics have established an innovative framework for precision cell therapies with tunable expression kinetics and reversible operational parameters (24, 25). Conventional systemic delivery of mammalian cells faces biological barriers that critically compromise therapeutic efficacy in metastatic diseases. Moreover, the administered cells themselves frequently demonstrate insufficient homing efficiency to target lesions. This is compounded by hemodynamic shear forces and immune clearance, which synergistically compromise functional persistence through membrane destabilization and premature exhaustion (26–28). Encapsulation of individual mammalian cells with semipermeable biocompatible materials emerges as a promising strategy to address these challenges. This approach combines steric protection against phagocytic recognition with preserved paracrine signaling capabilities (29–31).

Here, we developed a mammalian synthetic system termed red/far-red light-regulated individually encapsulated (RL/FRL-EnE) cell that enables dynamic, reversible control of immunomodulatory factor expression (Fig. 1). This system integrates a phytochrome A (PhyA)-based photoswitch module (Δ PhyA), which allows bidirectional regulation of tumor-suppressive cytokines through light wavelength-dependent dimerization states. Under 660-nm RL irradiation, the

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¹Henan Institute of Advanced Technology, School of Materials Science and Engineering, Zhengzhou University, Zhengzhou 450003, PR China. ²The First Affiliated Hospital of Zhengzhou University, Zhengzhou University, Zhengzhou 450052, PR China. ³Department of Biomedical Materials, Konyang University, Daejeon 35365 Republic of Korea. ⁴CAS Key Laboratory for Biomedical Effects of Nanomaterials & Nanosafety, CAS Center for Excellence in Nanoscience, National Center for Nanoscience and Technology, Beijing 100190, China.

*Corresponding author. Email: yzchenbio@zzu.edu.cn (Y.C.); niegj@nanoctr.cn (G.N.)

†These authors contributed equally to this work.

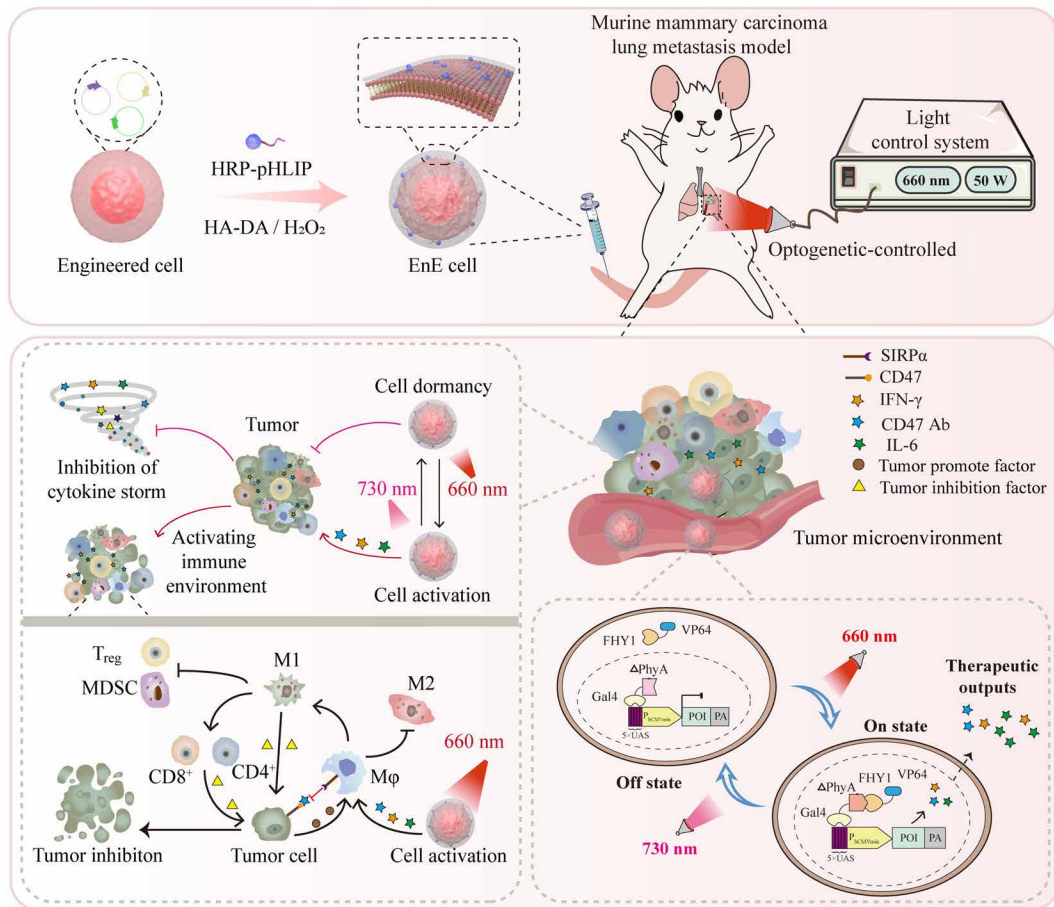


Fig. 1. The schematic diagram illustrating the optogenetic cytokine immunotherapy system mediated by engineered EnE cells. The synthetic engineered cells are individually encapsulated in a hyaluronic acid (HA)-based hydrogel, which permits free diffusion of oxygen, ions, and secretory proteins while protecting the cells from immune clearance. Following intravenous delivery, EnE cells accumulate in lung tumors via the high affinity between HA and CD44, a receptor overexpressed in cancer cells. Upon 660-nm RL irradiation, the Δ PhyA-PCB photosensor heterodimerizes with FHY1, inducing synchronized expression of IFN- γ , CD47 antibody (Ab), and IL-6. Conversely, 730-nm FRL exposure triggers PhyA chromophore photoconversion, dissociates the Δ PhyA-PCB-FHY1 complex, rapidly shutting off cytokine production. This light-switchable system enables precise cytokine homeostasis, sustaining therapeutic levels while avoiding toxicity. M ϕ , macrophage; POI, protein of interest.

truncated photoreceptor Δ PhyA forms nanoscale heterodimers with far-red elongated hypocotyl 1 (FHY1), inducing synchronized expression of interferon- γ (IFN- γ), CD47 antibody, and interleukin-6 (IL-6). Conversely, 740-nm FRL exposure triggers PhyA chromophore photoconversion, dissociates the Δ PhyA-PCB-FHY1 complex immediately, and terminates cytokine production. This RL/FRL-EnE cell system achieves precise spatiotemporal control, providing a dynamic expression range and enhanced cancer targeting ability. It allows for the maintenance of therapeutic cytokine concentrations while preventing pathological cytokine toxicity.

RESULTS

Design and validation of an RL/FRL-switchable optogenetic system

We developed an RL-inducible cytokine expression system by modifying the previously reported REDMAP optogenetic regulation module (24). The core element of this system is a truncated photoreceptor protein (Δ PhyA), which acquires light-sensing properties through covalent binding with the phycocyanobilin (PCB)

chromophore, enabling reversible conformational changes under alternating illumination with 660-nm RL and 730-nm FRL. This light-dependent conformational change facilitates specific binding and dissociation with the shuttle protein FHY1. Here, we fused the light-sensing element Δ PhyA with the yeast galactose-metabolism regulatory protein 4 (Gal4) DNA binding domain to create a hybrid DNA binding protein (Δ PhyA-Gal4). In addition, we fused the shuttle protein FHY1 with a minimal tetramer of the herpes simplex virus-derived repressor VP16 (VP64), resulting in the construction of a light-dependent transcriptional activator (FHY1-VP64). The optogenetic system operates through a light-switchable molecular mechanism (Fig. 2A). Upon RL irradiation (660 nm), Δ PhyA-Gal4 and FHY1-VP64 undergo specific heterodimerization via Δ PhyA-FHY1 domain interactions. This light-induced complex subsequently recruits the Gal4 DNA binding domain to specifically recognize and engage the 5XUAS regulatory element (yeast upstream activating sequence) within the reporter construct. The resultant DNA-protein assembly facilitates transcriptional activation of the target gene under the $P_{hCMVmin}$ promoter. Following FRL exposure (730 nm), wavelength-dependent structural reorganization disrupts the PhyA-FHY1 binding

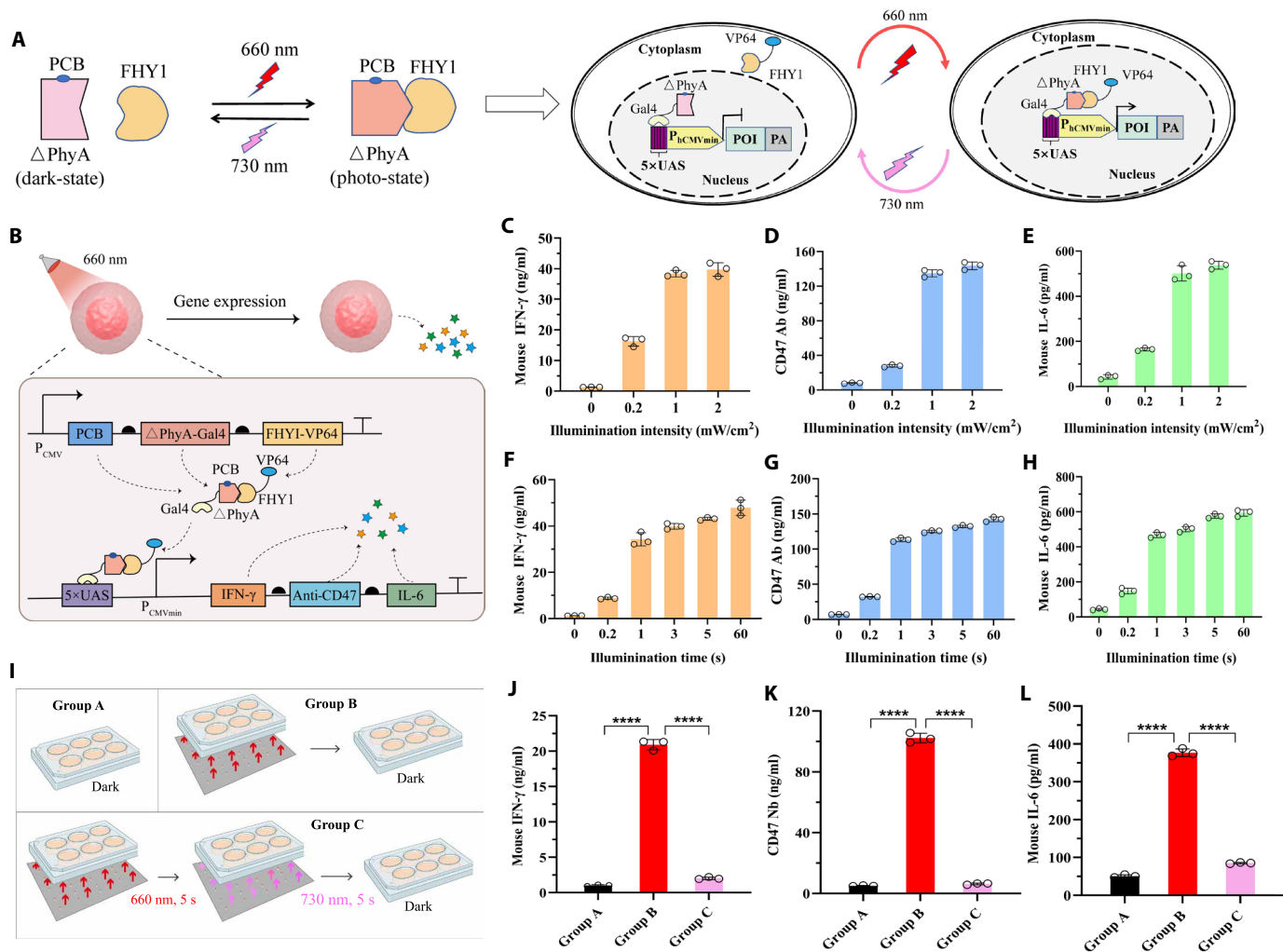


Fig. 2. Construction and performance characterization of the optogenetic control system. (A) Schematic representation of the optogenetic control system, illustrating the light-induced (660 nm) association and (730 nm) dissociation of Δ PhyA and FHY1. (B) Schematic diagram of engineered cells for RL activation. (C to E) Light intensity-dependent cytokine secretion profiles. Engineered cells were irradiated with 660-nm light at different intensities (0, 0.2, 1, and 2 mW/cm²), with each session lasting 5 s, once daily for 2 days. Culture supernatants were collected 48 hours after the first irradiation to quantify the production of (C) IFN- γ , (D) CD47 antibody, and (E) IL-6 using corresponding ELISA kits. (F to H) Time-dependent cytokine secretion profiles. Engineered cells were irradiated with 660-nm light (1 mW/cm²) for varying durations (0 to 60 s) daily for 2 days. Culture supernatants were collected 48 hours after the first irradiation to quantify the production of (F) IFN- γ , (G) CD47 antibody, and (H) IL-6 using corresponding ELISA kits. (I) Schematic for the experimental procedure of activation/deactivation performance of the optogenetic control system. Group A, dark control; group B, 660 nm, 5-s activation; group C, 660 nm + immediate 730 nm, 5 s each. (J to L) Activation and deactivation performance of (J) IFN- γ , (K) CD47 antibody, and (L) IL-6 in groups A to C. *P* values were calculated by two-tailed unpaired *t* test. *****P* < 0.0001 for all comparisons. All data are presented as the means $\pm$ SD (*n* = 3 independent experiments).

interface, triggering complex disassembly. This photoreversible dissociation effectively terminates transcriptional activation, achieving precise spatiotemporal control of transgene expression.

We next transfected this optogenetic control system (containing the photoreceptor Δ PhyA-Gal4, the transcriptional activator FHY1-VP64, the PCB biosynthesis gene pYZ484, and the immunoregulatory cytokine expression vector pYZ3 driven by the light-responsive 5xUAS- $P_{hCMVmin}$ promoter) into human embryonic kidney (HEK) 293T cells for optimized the induction profiles (fig. S1A). Functional validation of the optogenetic control system was first performed in HEK 293T cells (fig. S1, B to E). Following confirmation of light-responsive activation, we generated stable monoclonal cell lines through antibiotic-based clonal selection (fig. S2). Quantitative characterization of 20

genetically engineered clones identified clone 4 as the optimal candidate, exhibiting superior light-switchable dynamics with minimal basal leakage and maximal induction amplitude (fig. S2, A to D). The selected clone demonstrated precise spatiotemporal control over therapeutic protein production. Under dark conditions, background expression remained at baseline levels. RL illumination (660 nm) triggered robust co-expression and secretion of multiple payloads, including IFN- γ , CD47 antibody, and IL-6, as quantified by enzyme-linked immunosorbent assay (ELISA) and flow cytometry. This activation was effectively reversed upon FRL exposure (730 nm), demonstrating bidirectional optical regulation. In vitro characterization revealed that the engineered cells exhibited dose-dependent secretion profiles correlated with light exposure intensity and duration (Fig. 2, B to H). The system

maintained complete reversibility through activation-suppression cycles without observable functional attenuation (Fig. 2, I to K, and fig. S3, A to F), thereby validating the operational stability of this red/far-red-responsive platform for precision cytokine regulation.

Development of an enzyme-mediated EnE cell

To achieve single-cell encapsulation, we developed an enzyme-mediated *in situ* polymerization strategy for cell surface engineering (Fig. 3A). First, we constructed an HRP-pHLIP fusion protein expression system using genetic recombination, fusing the C terminus of horseradish peroxidase (HRP) with the N terminus of a pH-low insertion peptide (pHLIP). This fusion protein was successfully expressed in *Escherichia coli* BL21(DE3). SDS–polyacrylamide gel electrophoresis (PAGE) analysis confirmed the high-purity (>90%) isolation and purification of the target fusion protein (Fig. 3B). Under weakly acidic conditions (pH 6.5), the pHLIP domain undergoes a conformational transition to adopt a transmembrane α -helical configuration, enabling directional anchoring of the fusion protein to the plasma membrane while maintaining extracellular exposure of the HRP catalytic domain (32). To validate the membrane localization of the HRP-pHLIP fusion protein, we fluorescently labeled it with fluorescein *N*-hydroxysuccinimide ester (FAM-NHS) and analyzed its distribution via confocal microscopy. The resulting images exhibited a strong fluorescence signal exclusively at the cell periphery, demonstrating its successful anchoring to the plasma membrane (fig. S4). Subsequently, the anchored HRP catalyzed the H₂O₂-mediated cross-linking of dopamine-modified hyaluronic acid (HA-DA), whose successful synthesis was verified by ¹H nuclear magnetic resonance (NMR) (fig. S5), to rapidly form a cohesive hydrogel matrix around each cell.

To characterize the formation and protective efficacy of cell surface cross-linking frameworks, we used a combined strategy of fluorescence imaging and quantitative analysis. Fluorescent labeling with FAM-HA-DA allowed visualization of cellular encapsulation. Confocal microscopy revealed uniform green fluorescence on encapsulated 293T cell surfaces (Fig. 3C), with flow cytometry confirming FAM-labeling in $90.3 \pm 2.1\%$ of cells (Fig. 3D), demonstrating effective surface cross-linking. Scanning electron microscope (SEM) showed smooth, continuous layers on encapsulated cells (Fig. 3E), while transmission electron microscopy (TEM) revealed tightly adhered nanogel layers on plasma membranes (Fig. 3F, black arrows). Zeta potential measurements yielded intermediate values (-25.3 ± 1.2 mV) between native cells (-15.8 ± 0.9 mV) and free HA-DA (-32.6 ± 1.5 mV) (Fig. 3G), confirming successful surface modification. Biological assessments showed excellent biocompatibility. Postencapsulation cell viability was $92.4 \pm 3.1\%$, similar to untreated controls ($94.7 \pm 2.8\%$) (Fig. 3H). Protease resistance tests revealed enhanced survival rates ($85.2 \pm 2.9\%$ versus $62.3 \pm 4.1\%$) after 30-min 0.1% trypsin-EDTA treatment (Fig. 3I), indicating effective enzymatic protection. Mechanical stability under 400g centrifugation showed improved viability for encapsulated cells ($76.9 \pm 4.1\%$ versus $32.7 \pm 2.0\%$) (Fig. 3J). To assess the functional impact of cell encapsulation, we used secreted alkaline phosphatase (SEAP) as a reporter protein. HEK 293T cells were transfected with either the P_{hCMV}-SEAP-poly(A) tail (PA) plasmid (experimental group) or the P_{CDNA3.1} empty vector (control group) and encapsulated 48 hours posttransfection. Analysis of SEAP secretion at early time points (fig. S6) and 72 hours (Fig. 3K) revealed no significant difference between encapsulated and unencapsulated cells. This indicates that the hydrogel encapsulation did not impede the secretion of proteins. Given that SEAP is larger than

the other proteins expressed in this study, it is likely that their secretion was similarly unaffected.

Following single-cell encapsulation, we evaluated the ability of the encapsulated cells to express immunomodulatory factors upon RL induction (Fig. 4A). The encapsulated cells retained robust and sustained expression of IFN- γ , CD47 antibody, and IL-6 under 660-nm illumination (Fig. 4, B to D). Systematic investigation of illumination intensity (fig. S7, A to C) and time (fig. S7, D to F) revealed a dose-dependent response in the induction of these immunomodulatory factors. Furthermore, alternating exposure to RL (660 nm, 1 mW/cm²) and FRL (730 nm, 1 mW/cm²) confirmed the reversible and dynamic regulation of the expression system (Fig. 4, E to G, and fig. S7, G to I). To assess long-term performance, we subjected EnE cells to repeated RL/FRL cycles and found that they maintained robust, light-inducible expression for at least 12 days. Throughout this period, transgene expression was reliably modulated without significant functional decay (fig. S8). We also investigated the cytokine secretion profile induced by a single RL exposure. Although secretion of IFN- γ , CD47 antibody, and IL-6 began to decline after 24 hours, all three factors remained detectable for up to 96 hours (fig. S9). To quantify the efficiency of FRL-mediated suppression, we performed a sequential activation-deactivation experiment *in vitro*. A single exposure to FRL was sufficient to inhibit subsequent cytokine secretion by over 80% compared to the fully activated state (fig. S10). While not a complete shutdown, this result demonstrates that FRL provides a potent and reliable off switch, enabling rapid and substantial modulation of the therapeutic output in response to an optical signal. Collectively, these results demonstrate that the EnE cells enable robust and tunable cytokine expression in response to different optical stimuli.

In vivo biodistribution of EnE cells and their reversible cytokine release

To investigate the metabolic behavior and biodistribution of encapsulated cells *in vivo*, we administered Cyanine 5 (Cy5)-labeled HEK 293T cells intravenously to BABL/c mice, followed by longitudinal tracking using an *in vivo* imaging system (IVIS). Unencapsulated HEK 293T cells, as foreign entities, are typically rapidly cleared from circulation by host immune responses. In contrast, HA-based encapsulation can enhance targeting efficiency owing to its biocompatibility, biodegradability, and high affinity for CD44, a receptor frequently overexpressed in cancer cells. As anticipated, encapsulated cells maintained detectable fluorescence in blood for over 24 hours, while unencapsulated cells were cleared within 12 hours (fig. S11). To distinguish between active, HA-mediated targeting and nonspecific accumulation, we engineered an HA-deficient control. We encapsulated cells using DA-modified poly(γ -glutamic acid) (PGA), a nontargeting polymer with similar anionic properties to HA but no affinity for CD44. Following intravenous injection, major organs (heart, liver, spleen, tumor-bearing lungs, and kidneys) were harvested at 2 to 24 hours for *ex vivo* imaging (Fig. 5A). These PGA-encapsulated cells exhibited significantly reduced retention in metastatic lung lesions compared to their HA-encapsulated counterparts (Fig. 5B and figs. S12 and S13). This result indicates that the enhanced tumor accumulation is specifically driven by the HA-CD44 interaction and is not a general consequence of nanoencapsulation. A critical challenge for systemic cell therapies is off-target accumulation. While our EnE cells targeted the lungs, a significant population also accumulated in the liver. We therefore sought to determine whether

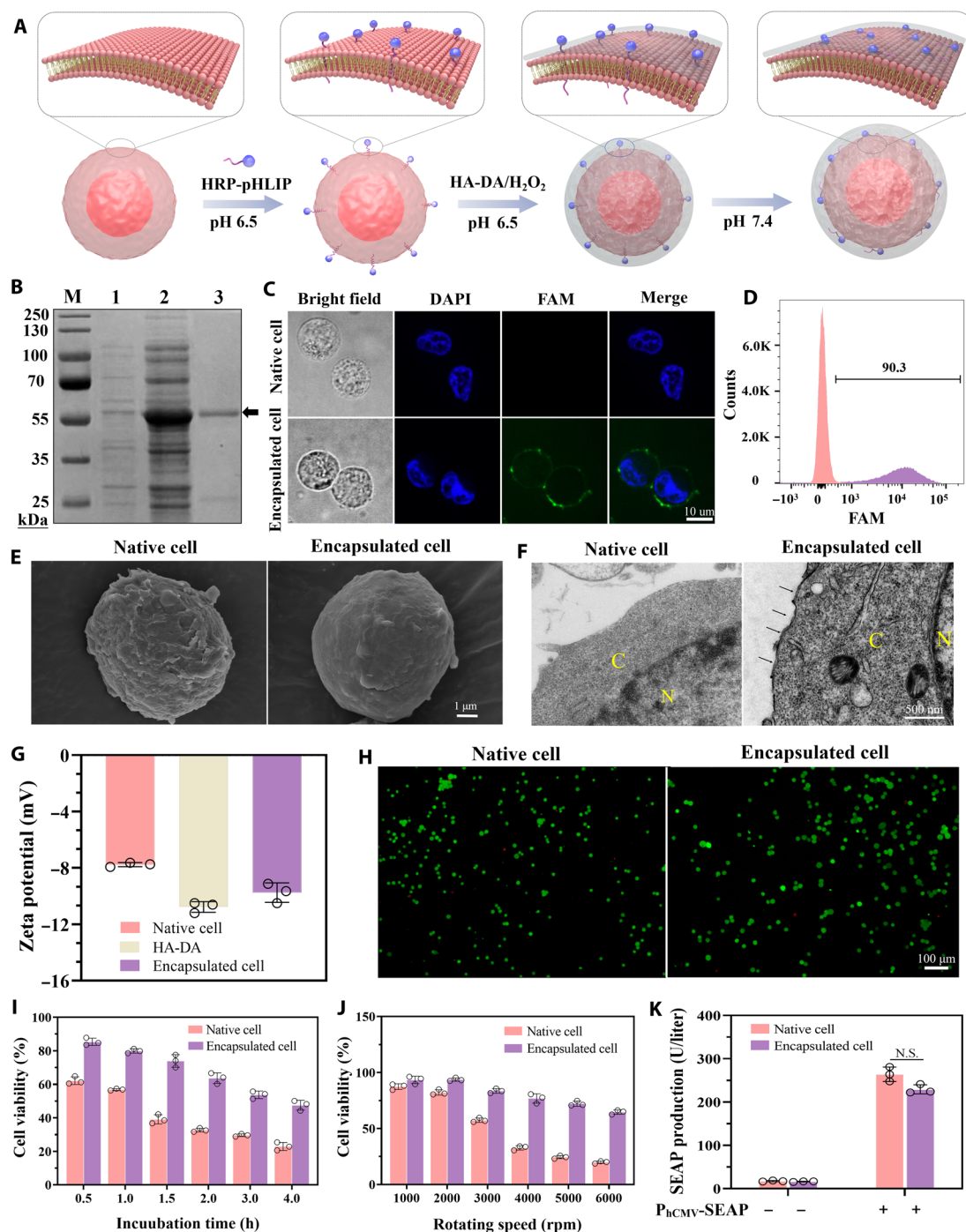


Fig. 3. Engineering and characterization of encapsulated cell. (A) Schematic illustration of cell surface encapsulation engineering. (B) Expression and purification of HRP-pHLIP. Lane M, protein molecular weight marker; lane 1, *E. coli* BL21 (DE3) cells without induction; lane 2, *E. coli* BL21 (DE3) cells after induction; lane 3, purified HRP-pHLIP. (C) Fluorescence microscopy images comparing native and encapsulated cells. Green, FAM-labeled HA-DA polymer shell; blue, 4',6-diamidino-2-phenylindole (DAPI)-stained nuclei. Scale bar, 10 μ m. (D) Flow cytometry quantification of cell surface encapsulation efficiency using FAM-HA-DA. Pink, native cells; purple, encapsulated cells. (E) SEM images of native (left) and encapsulated cells (right). Scale bar, 1 μ m. (F) TEM images revealing surface morphology of native (left) and encapsulated cells (right). Black arrows, nanogel on cell membrane. C, cytoplasm; N, cell nucleus. Scale bar, 500 nm. (G) Zeta potential measurements in phosphate-buffered saline (PBS) (1 $\times$) for native cells, HA-DA polymer, and encapsulated cells. Data represent means $\pm$ SD ($n = 3$ independent experiments). (H) Live/dead assay visualized by fluorescence microscopy. Green, calcein-AM-stained viable cells; red, propidium iodide (PI)-stained dead cells. Scale bar, 100 μ m. (I) Time-dependent cell viability assessment under trypsin-EDTA (0.1%, w/v) treatment. Data represent means $\pm$ SD ($n = 3$ independent experiments). h, hours. (J) Centrifugation stress tolerance evaluated at indicated speeds. Data are presented as means $\pm$ SD ($n = 3$ independent experiments). (K) Comparative SEAP production between native and encapsulated cells. Data are presented as means $\pm$ SD ($n = 3$ independent experiments). Statistical significance was determined by two-tailed unpaired t test. N.S., no significant difference.

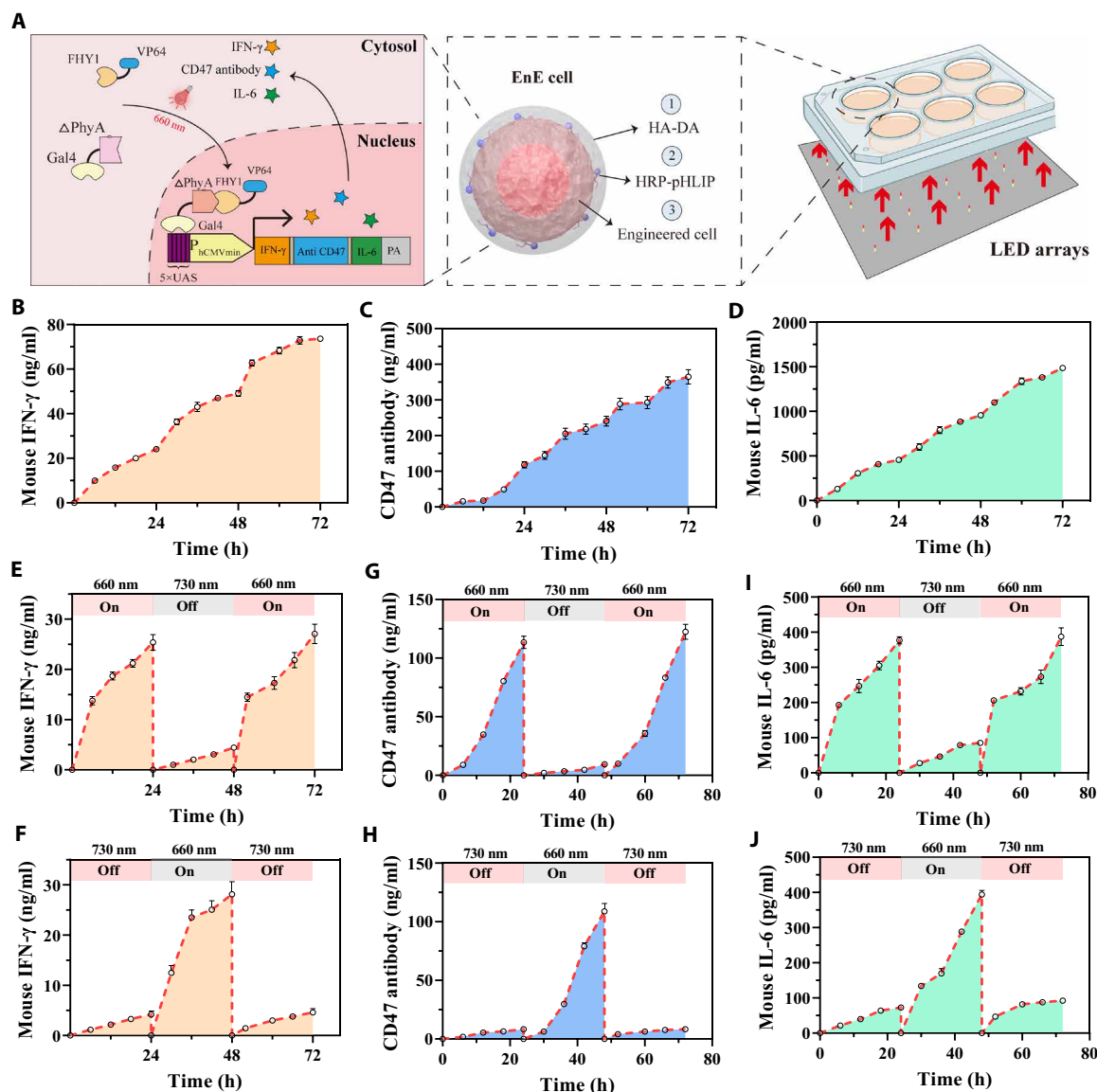


Fig. 4. Kinetic characterization of cytokine production by encapsulated engineered cells. (A) Schematic of the optical experimental procedure for encapsulated engineered cells. (B to D) Increased cytokine release of (B) IFN- γ , (C) CD47 antibody, and (D) IL-6 after 5 s of 660-nm light irradiation. (E to J) Reversible cytokine release regulation by encapsulated engineered cells. Cells were exposed to RL (660 nm, 1 mW/cm²) for 5 s (on) or FRL (730 nm, 1 mW/cm²) for 5 s (off). Cytokines production including [(E) and (F)] IFN- γ , [(G) and (H)] CD47 antibody, and [(I) and (J)] IL-6 was measured every 6 hours over 72 hours. Fresh medium was replaced every 24 hours. Data are means $\pm$ SD ($n = 3$).

focused light could provide precise spatial control over therapeutic gene expression, decoupling biodistribution from bioactivity. We engineered EnE cells to express luciferase under the control of RL. After systemic cell injection, we irradiated only the lungs of the mice (fig. S14). Despite significant cell numbers in the liver, luciferase expression was strongly induced in the lungs but remained at baseline levels in the liver and indistinguishable from that of other nonirradiated organs (Fig. 5, C and D). This result confirms that local light delivery effectively decouples the site of cell residence from the site of therapeutic action.

In vivo assessment of light-responsive cytokine release from encapsulated engineered cells was performed through serial retro-orbital blood

sampling following optogenetic stimulation. Mice were intravenously administered 1×10^6 encapsulated cells and subjected to controlled light exposure regimens (fig. S15). The results showed that encapsulated engineered cells maintained their responsiveness to light-induced cytokine release (IFN- γ , IL-6, and CD47 antibody), exhibiting reversible on/off kinetics under alternating RL and FRL (Fig. 5, E to M). To confirm that the FRL-induced decrease in cytokine levels was due to active gene suppression rather than passive decay, we introduced a control group that received RL activation on day 1, followed by a return to darkness for the subsequent days (on-then-dark). In these mice, serum cytokine levels remained substantially elevated, exhibiting a modest decline attributable to natural protein

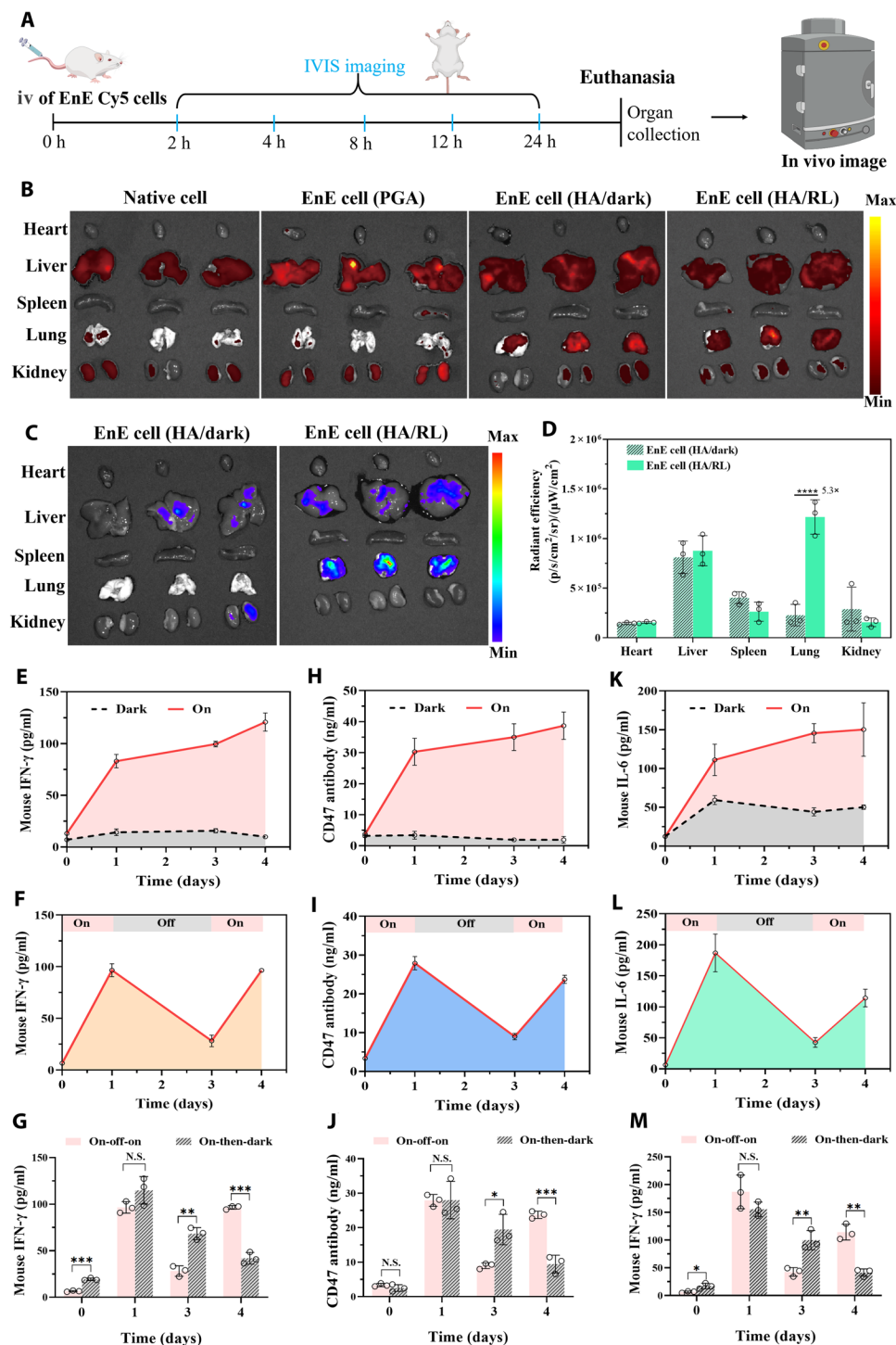


Fig. 5. In vivo biodistribution and therapeutic function of EnE cells. (A) Schematic of the experimental design for assessing in vivo cell retention and organ targeting. iv, intravenous. (B) Ex vivo analysis of cell biodistribution at 24 hours postinjection. Representative IVIS images show the accumulation of different Cy5-labeled cell formulations [native, EnE (PGA), and EnE (HA)] in harvested organs, including the heart, liver, spleen, tumor-bearing lungs, and kidneys. (C) Representative ex vivo IVIS images and (D) corresponding fluorescence quantification of organs from mice injected with EnE cells (HA). The two groups were either kept in darkness (dark) or received RL treatment. Data are presented as means $\pm$ SD ($n = 3$ mice per group). (E to M) Light-controlled systemic release of therapeutic proteins. Mice were intravenously injected with 1×10^6 EnE cells and assigned to three treatment groups. Dark, no light treatment; on, activated with RL (660 nm) twice daily; on/off, activated with RL on days 1 and 4 and deactivated with FRL (730 nm) on days 2 and 3; on-then-dark, activated with RL on days 1 and then with dark treatment on days 2 to 4 [(G), (J), and (M)]. The serum level of [(E) to (G)] IFN- γ , [(H) to (J)] CD47 antibody, and [(K) to (M)] IL-6 was quantified by ELISA at specified time points. Data are presented as means $\pm$ SD ($n = 3$ mice per group). P values were calculated using a two-tailed unpaired t test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

clearance (Fig. 5, G, J, and M, and fig. S16). In contrast, the FRL-treated group displayed a rapid and significant drop in cytokine levels. This comparison confirms that the removal of RL stimulation alone is insufficient to halt secretion and that FRL is necessary for the active suppression of transgene expression.

Photo-controlled cellular immunotherapy achieves efficacy-toxicity decoupling

To evaluate the antitumor efficacy of encapsulated engineered cells *in vivo*, we established a lung metastasis model by intravenously injecting 4T1 cells stably expressing luciferase (4T1-luc) into female BALB/c mice. The treatment regimen is illustrated in Fig. 6A. On day 1, bioluminescence imaging (IVIS) confirmed consistent baseline tumor burden across all groups, as quantified by the bioluminescent signal intensity of 4T1-luc cells (Fig. 6B). Tumor progression was dynamically monitored via *in vivo* imaging on days 3, 7, 11, and 15 (Fig. 6C).

Encapsulated engineered cells (RL/FRL, G6) exhibited the most potent suppression of lung metastases compared to the control groups (G1, G2, and G3). Although constitutive cytokine expressing cells (G4) and nonencapsulated engineered cells (G5) showed moderate antitumor activity, their efficacy remained suboptimal, likely because of uncontrolled cytokine release and insufficient tumor targeting. On day 15, bioluminescence imaging revealed varying degrees of tumor growth inhibition among the treatment groups, with G6 showing the lowest fluorescence intensity, significantly different from the other groups (Fig. 6D). In addition, the constitutive cytokine expression cells induced leukocytosis and thrombocytopenia, suggestive of immune hyperactivation and potential cytokine storm–related toxicity (Fig. 6, E and F) (33). Collectively, these results demonstrate that RL/FRL-EnE cell therapy effectively mitigates cytokine storm risk while maintaining durable antitumor activity. Additional safety evaluations through body weight tracking (fig. S17) Hematological analysis (fig. S18), hepatic and renal function analysis (figs. S19 and S20), and histopathological evaluation of hematoxylin and eosin (H&E)–stained organ sections (fig. S21) consistently support the safety of RL/FRL-unencapsulated engineered (UnEnE) cell treatment.

In vivo evaluation immune response induced by EnE cells

To monitor the dynamic cytokine responses, we collected peripheral blood samples from experimental mice on days 7 and 14 posttreatment for ELISA analysis. Quantitative assessment revealed significantly elevated systemic levels of IFN- γ , CD47 antibody, and IL-6 in the RL/FRL-EnE cell treatment group compared to negative controls (Fig. 7, A to C), indicating appropriate cytokine release kinetics during the therapeutic intervention. For assessment of systemic immune activation, serum cytokine profiling was further performed. RL/FRL-EnE cell treatment induced distinct immunomodulation, characterized by elevated IL-17 and reduced IL-1 β , IL-10, monocyte chemoattractant protein-1 (MCP-1), and vascular endothelial growth factor (VEGF) levels compared to negative controls (Fig. 7, D to H). This cytokine profile suggests a coordinated immunomodulatory cascade initiated by elevated IFN- γ , CD47 antibody, and IL-6 concentrations produced from EnE cells.

To systematically evaluate the antitumor lung metastasis efficacy after different treatment, we conducted histopathological and immunological analyses of lung tissues from each group. Histopathological examination confirmed the potent antitumor effect of the light-controlled therapy. Lungs from mice treated with EnE cells (RL/FRL)

were largely free of metastatic nodules, whereas all other groups displayed numerous, distinct tumor foci (Fig. 7I). This observation was corroborated by a semiquantitative analysis of the metastatic area, which further validated the superior efficacy of the light-controlled treatment (fig. S22). To investigate the immunological mechanism underlying this therapeutic response, we assessed immune cell infiltration by immunofluorescence staining. Quantitative analysis revealed a significant increase in the infiltration of both CD80⁺ antigen-presenting cells and CD8⁺ cytotoxic T cells in the lung tissues of the active treatment groups [constitutive, non-EnE (RL/FRL), and EnE (RL/FRL)] compared to the control groups [phosphate-buffered saline (PBS), normal cells, and EnE (dark)] (Fig. 7J and fig. S23).

Furthermore, quantitative analysis of lung tissue cytokines post-treatment showed that the expression levels of IFN- γ , CD47 antibody, and IL-6 were significantly elevated in the encapsulated engineered cell (RL/FRL) group compared to the control group. Notably, the cytokine levels in the nonencapsulated engineered cell (RL/FRL) group were markedly lower than those in the encapsulated engineered cell groups (G4 and G6), suggesting that encapsulation effectively enhances the functional performance of the engineered cells *in vivo* (Fig. 7, K to M). We further examined how EnE cells (RL/FRL) modulate the tumor microenvironment (TME) by analyzing immune cell populations in spleen and lung tissues using flow cytometry on day 15 posttreatment (fig. S24). Compared to native cells, EnE cells (RL/FRL) significantly reduced the number of immunosuppressive SIRP α ⁺ macrophages (CD11b⁺ and F4/80⁺), M2 macrophages (CD206⁺, CD11b⁺, and F4/80⁺), and myeloid-derived suppressor cells (MDSCs; CD45⁺ CD11b⁺ GR1⁺) in lung tissue, with 1.7-, 1.4-, and 1.3-fold reduction, respectively (Fig. 8, A to C). Conversely, dendritic cell (DC) populations (CD45⁺CD11c⁺F4/80[−]CD11b⁺) showed a 1.4-fold increase, suggesting enhanced antigen-presenting capacity within the TME (Fig. 8, A to D). Furthermore, the EnE cell group demonstrated a 2.8-fold increase in CD8⁺ T cells (CD45⁺CD8⁺) and a 1.8-fold increase in CD4⁺ T cells (CD45⁺CD4⁺), accompanied by a 1.6-fold reduction in regulatory T cells (T_{reg} cells; CD4⁺Foxp3⁺) (Fig. 8, E to G). This shift in T cell subsets may be attributed to the elevated antigen-presenting cell activity, which likely promotes the expansion of antitumor T cell populations. Consistent with these findings, we observed parallels in immune cell population changes within the spleen, the largest secondary lymphoid organ (fig. S25). EnE cells elicited a comparable pattern of immune modulation in splenic tissue, characterized by increased infiltration of CD8⁺ and CD4⁺ T cells, a reduction in the frequency of T_{reg} cells, and increased antigen-presenting cell activity. This systemic immune response indicates that EnE cell-mediated immunomodulation extends beyond the TME to peripheral lymphoid organs.

DISCUSSION

Advances in synthetic biology have facilitated the development of sophisticated engineered transgene switches, enabling precise regulation of therapeutic protein expression and cellular functions (34–36). The design of synthetic therapeutic cells has thus emerged as a transformative strategy in biomedicine, particularly in cancer immunotherapy (37–39). Here, we developed an engineered cell delivery platform that uses single-cell encapsulation technology combined with RL/FRL-regulated immunomodulatory synthetic designer cells to achieve spatiotemporally controlled cytokine release in metastatic tumor models. Our encapsulation strategy incorporates several distinct

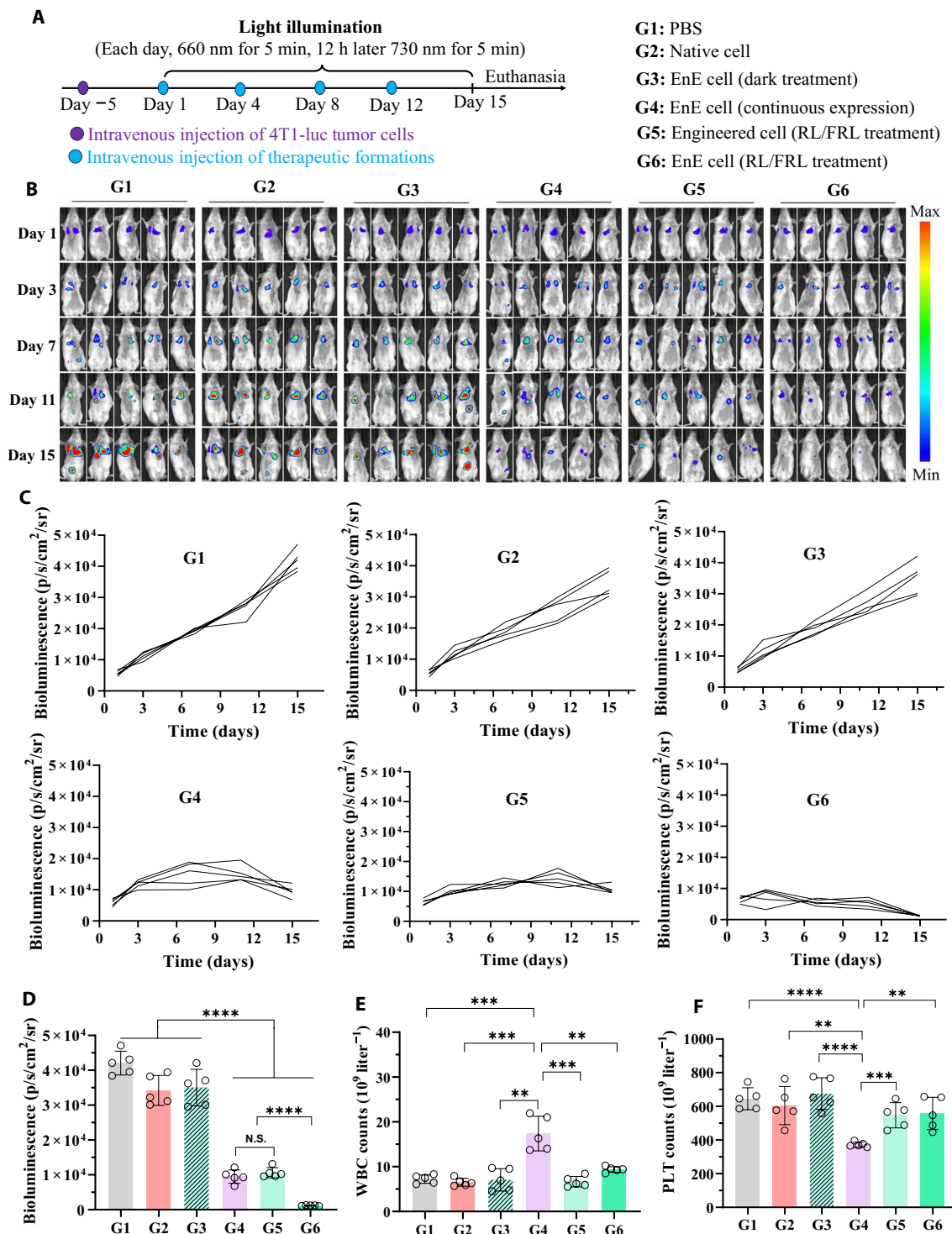


Fig. 6. Therapeutic efficacy of encapsulated engineered cells in a lung metastasis model. (A) Schematic illustration of the experimental procedure and timeline. (B) In vivo progression of lung metastatic tumors, monitored by serial bioluminescence signals from 4T1-luc cells, under various treatments: G1, PBS; G2, native cells; G3, encapsulated engineered cells (dark); G4, encapsulated engineered cells (continuous expression); G5, engineered cells (RL/FRL); G6, encapsulated engineered cells (RL/FRL). (C) Quantified bioluminescence intensity for different treatment groups. (D) Bioluminescence intensity of treated mice on day 15 ($n = 5$ mice). (E and F) Quantification of (E) white blood cells (WBCs) and (F) platelets (PLTs) in peripheral blood. Data are presented as means $\pm$ SD ($n = 5$ mice). P values were calculated using a two-tailed unpaired t test. $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

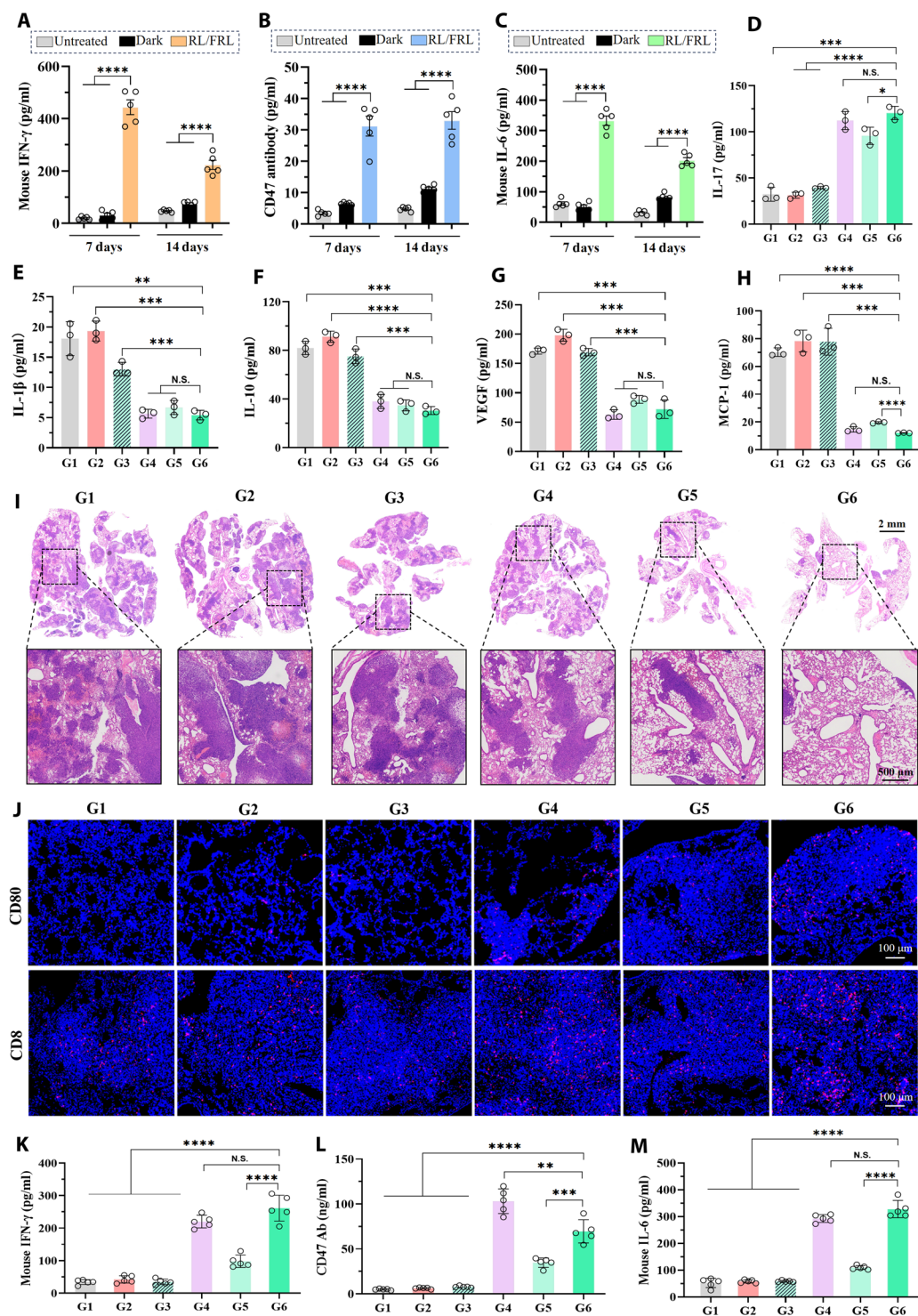


Fig. 7. In vivo antimetastatic efficacy and immune response evaluation. (A to C) Cytokine production profiles showing (A) IFN- γ , (B) CD47 antibody, and (C) IL-6 levels quantified by ELISA at days 7 and 14. Data represent means $\pm$ standard error of the mean ($n = 5$ independent experiments). P values were calculated using a two-tailed unpaired t test. **** $P < 0.0001$. (D to H) Analysis of serum cytokine levels as indicators of immune responses in mice. The levels of (D) IL-17, (E) IL-1 β , (F) IL-10, (G) VEGF, and (H) MCP-1 were measured using ELISA kits. Data are presented as means $\pm$ SD ($n = 3$ independent experiments). P values were calculated using a two-tailed unpaired t test. ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$. (I) H&E staining images of lungs from mice subjected to various treatments. (J) CD80 and CD8 immunofluorescence staining images of lungs from mice after various treatments. (K to M) Expression levels of (K) IFN- γ , (L) CD47 antibody, and (M) IL-6 in the lungs of mice after various treatments. Data are presented as means $\pm$ SD ($n = 5$ independent experiments). P values were calculated using a two-tailed unpaired t test. ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

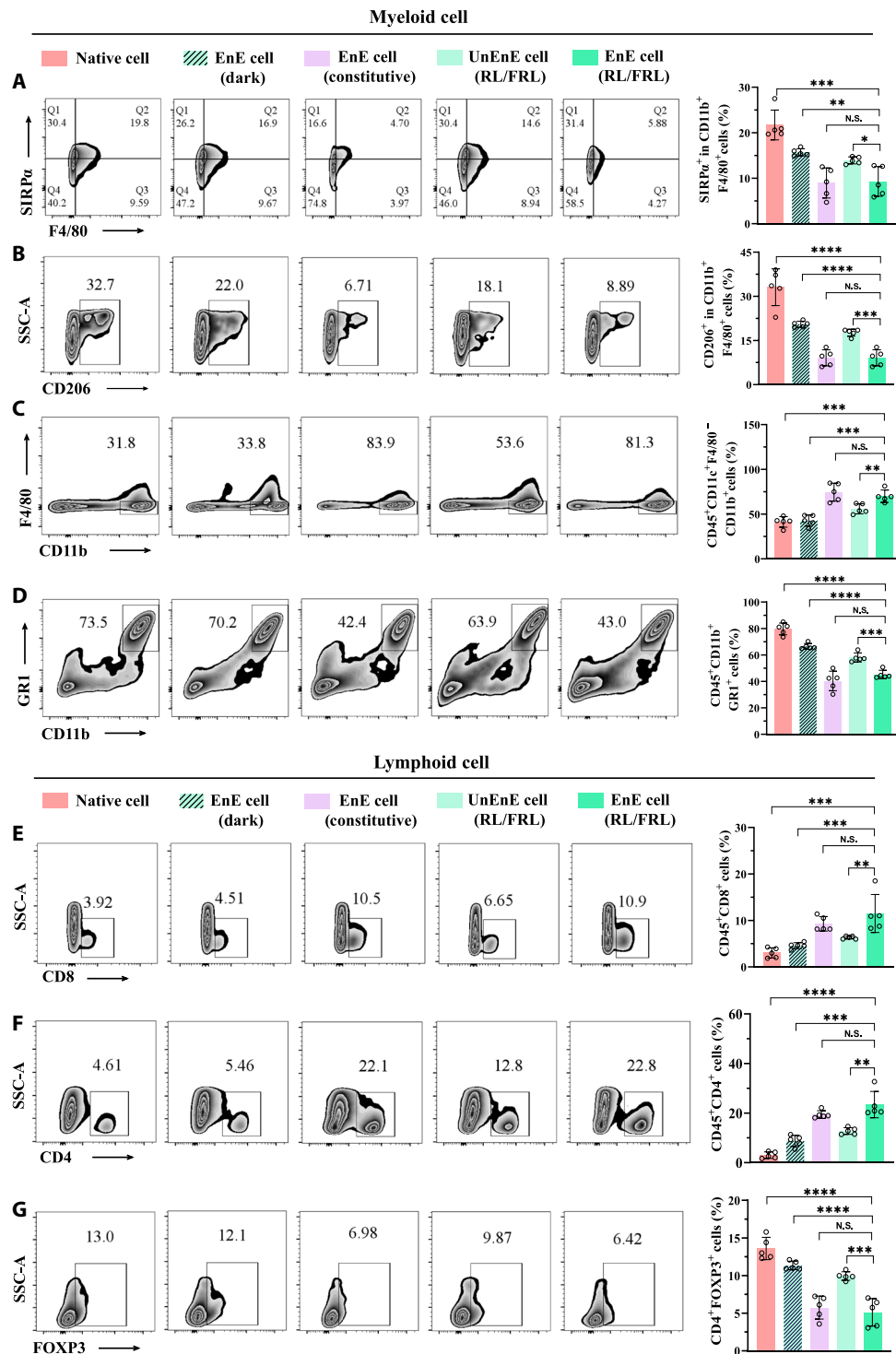


Fig. 8. Antitumor immune response mediated by cytokine-releasing encapsulated engineered cells. (A to D) Flow cytometry analysis of lung myeloid cell effector phenotypes from mice euthanized on day 15. Left: Representative flow cytometry plots. Right: Quantification of (A) SIRPα⁺CD11b⁺F4/80⁺, (B) CD206⁺CD11b⁺F4/80⁺, (C) CD45⁺CD11c⁺F4/80⁺CD11b⁺, and (D) CD45⁺CD11b⁺GRI⁺ cell populations. (E to G) Flow cytometry analysis of lung lymphoid cell effector phenotypes. Left: Representative flow cytometry plots. Right: Quantification of (E) CD45⁺CD8⁺, (F) CD45⁺CD4⁺, and (G) CD4⁺FOXP3⁺ cell populations. Data are presented as means ± SD (*n* = 5 independent experiments). SSC-A, side scatter-area. *P* values were calculated using a two-tailed unpaired *t* test. ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.

features that distinguish it from conventional methods. A key innovation lies in the use of a pHLP to form a transient and reversible scaffold on the cell surface (40). In contrast to techniques such as layer-by-layer deposition (41), which often rely on covalent bonds or strong electrostatic interactions, our method avoids permanent alteration of the plasma membrane. Another key advantage is that our entire encapsulation process is conducted under strictly physiological conditions. Moreover, the pHLP anchor is engineered to dissociate from the membrane at neutral pH, enabling restoration of native membrane fluidity and integrity. By contrast, many existing surface modification strategies cause marked reductions in membrane fluidity, leading to increased cell fragility and susceptibility to rupture (42, 43). Our use of HA as the encapsulation material was based on its clinical translational benefits. As a native component of the extracellular matrix, HA is inherently biocompatible and biodegradable (44, 45), which not only supports fundamental cell function but also simplifies the path to clinical translation compared to synthetic polymers. Controlled by RL/FRL, the system provides on-demand, tunable secretion of IFN- γ , CD47 antibody, and IL-6. This single-cell encapsulation strategy isolates cells to prevent signaling cross-talk and shields them from immune clearance, thereby extending their therapeutic window in vivo. In contrast to conventional therapies plagued by systemic toxicity, our approach offers tight, localized, and dynamic control over potent immunomodulators (46, 47). While encapsulation could potentially hinder the secretion of larger proteins, the permeability of our hydrogel shell is a highly tunable parameter. It is governed by cross-linking density and resulting pore size, which can be precisely modulated by adjusting the concentrations of the HA conjugate, H₂O₂, and HRP (48). This tunability, combined with the shell's minimal nanometer-scale thickness (49), is intentionally designed to minimize any diffusion barrier. Our experimental results confirmed that no significant difference in the output of the reporter protein SEAP between encapsulated and native cells at either early (fig. S5) or late (Fig. 3K) time points. Since SEAP is larger and more structurally complex than the other therapeutic proteins in our study, it is likely that their secretion was also unhindered by the hydrogel layer. However, the shell's permeability to even larger or more complex proteins requires further study.

In metastatic models, this light-tunable platform significantly suppressed tumor progression without inducing systemic toxicity. After administering these cells intravenously to mice, our data (Fig. 5, C and D) confirm that low-level off-target activation occurs in the liver. To address this limitation and improve the therapeutic window, we propose using interventional radiology techniques, such as direct administration into the bronchial artery (50). This approach would bypass systemic circulation, thereby minimizing liver accumulation and providing a straightforward strategy to prevent off-target activation. Our results demonstrate that the RL-triggered release of CD47 antibody operates synergistically with IFN- γ and IL-6 to establish a potent antitumor immune cascade. IL-6 potentiates macrophage antigen presentation via signal transducers and activators of transcription 1 pathway activation (51, 52), and IFN- γ induces exposure of “eat-me” signals (e.g., surface calreticulin) on tumor cells (53), while CD47 antibody blockade of the CD47-signal regulatory protein alpha (SIRP α) axis reverses macrophage “do-not-eat-me” suppression (54, 55). This tripartite coordination not only enhanced macrophage phagocytic rates but also promoted tumor antigen cross-presentation, driving CD8⁺ T cell-mediated eradication of metastatic lesions in our murine models. The RL/FRL-EnE cell system

simultaneously activated local innate immunity while inducing systemic adaptive immune responses via enhanced antigen presentation pathways. In metastatic models, treated mice exhibited significantly increased proportions of antigen-specific T cells in the spleen, establishing durable antimetastatic immunity. While our light-controlled cell therapy demonstrated potent antitumor efficacy, the incomplete tumor regression observed after 15 days highlights the formidable challenges of eradicating established solid tumors. We attribute this residual disease to an interplay between the finite persistence of the therapeutic agent and the intrinsic resistance of the tumor. The limited in vivo lifespan of the engineered cells leads to a gradually diminishing local cytokine dose, which may eventually fall below the threshold required for complete tumor clearance. Furthermore, this waning therapeutic pressure must overcome powerful intrinsic tumor resistance mechanisms (56, 57). These include tumor heterogeneity, which allows for the survival of less-sensitive cancer cell subclones, and the profoundly immunosuppressive TME, which can actively neutralize the induced immune response (58). Technically, the platform faces challenges related to the limited tissue penetrance of FRL, necessitating optimization for treating deep-seated metastases in osseous or cerebral microenvironments. Future development could integrate ultrasound genetic systems (59) or semiconductor-based synthetic biology tools to overcome penetration limitations (60). Patient-specific immune landscapes demand customizable cytokine release kinetics and combinatorial profiles. Future iterations could use biosensor-integrated closed-loop circuits to achieve real-time, microenvironment-responsive therapeutic adjustment.

In conclusion, we present an optogenetically controlled single-cell system that integrates RL/FRL-responsive gene circuits, multimodal immunomodulators (IFN- γ /CD47 antibody/IL-6), and cytoprotective nanohydrogel matrices. This system converts optical inputs into precisely timed immune responses, demonstrating potent suppression of pulmonary metastases through dual complementary mechanisms. Light-triggered cytokine release directly activates tumor-associated macrophages, while CD47 antibody-mediated blockade enhances phagocytic capacity by disrupting the “don't eat me” signal. The semi-permeable encapsulation matrix prolongs tumor residence time by maintaining cellular viability within the TME while preventing systemic dissemination, thereby establishing localized “living drug factories” capable of programmable therapeutic activity. This work proves the concept of closed-loop controllable immunotherapy, where engineered living drug factories enable on-demand immune reprogramming. The combination of optogenetics and biomaterial delivery represents an innovative therapeutic strategy for developing noninvasive, patient-specific therapies.

MATERIALS AND METHODS

Animals

The experiments involving animals were approved by the Institutional Animal Care and Use Committee of Zhengzhou University and followed institutional guidelines for animal welfare. The protocol (IACUC approval no. 2021-KY-0028) was approved by the Animal Care and Use Committee of Zhengzhou University. Female, 6- to 7-week-old BALB/c mice (Animal Center of Zhengzhou University) were maintained under specific pathogen-free conditions at 20° to 22°C with 30 to 70% humidity and 12:12-hour light-dark cycles, with ad libitum access to food and water. Humane end points were strictly observed throughout the study.

Plasmid construction

The plasmids were assembled using the Gibson assembly method in accordance with the manufacturer's protocol (MultiS One Step Cloning Kit, catalog number C113-01, Vazyme Inc.). cDNAs encoding IFN- γ , CD47 antibody, and IL-6 were chemically synthesized by Genewiz Inc. (Suzhou, China), and all gene segments were subsequently confirmed through Sanger sequencing by Genewiz Inc. Tables S1 and S2 provides an overview of how all expression vectors were designed and constructed.

Cell culture and transfection

HEK 293T cells (human renal epithelial cell line; Wuhan Pricella Biotechnology Co. Ltd.) were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; catalog no. C11995500BT, Gibco) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (catalog no. A5256701, Gibco) and 1% (v/v) penicillin/streptomycin (catalog no. PB180120, Pricella Inc.). The luciferase-expressing murine breast cancer cell line 4T1-luc (catalog no. CC9022L, Cellcook Inc.) was maintained in RPMI 1640 (catalog no. 11875093, Gibco) containing identical supplements. All cell lines were incubated at 37°C in a 5% CO₂-humidified atmosphere and routinely screened for mycoplasma contamination using polymerase chain reaction-based detection.

For transient transfection, HEK 293T cells were seeded in 24-well plates at a density of 1×10^5 cells per well and allowed to adhere overnight, achieving 70 to 80% confluence before transfection. Lipofection was performed according to the manufacturer's protocol (Lipo293, C0521, Beyotime Inc.), using 2 μ l of transfection reagent and 1 μ g of plasmid DNA mixture per well. Following 24-hour incubation, transfection efficiency was assessed via fluorescence microscopy (for green fluorescent protein-tagged constructs) or luciferase assay (for reporter systems).

Generation of stable cell lines

To establish a stable cell line capable of light-inducible coexpression of mouse IFN- γ , IL-6, and CD47 antibody, HEK 293T cells were cotransfected with the following plasmids at a 4:3:1 molar ratio, including pYZ484 (carrying the optogenetic induction system, ITR-P_{hCMV}- Δ PhyA-Gal4-P2A-FHY1-VP64-pA::P_{hCMV}-PcyA-P2A-HO-P2A-Fd-P2A-FNR-pA::P_{mpak}-Puro-pA-ITR), pYZ3 (encoding the cytokine/antibody expression cassette, ITR-5 $\times$ UAS-P_{hCMVmin}-IFN- γ -P2A-anti-CD47-HisTag-P2A-IL-6-T2A-EGFP-pA::P_{mSV40}-BleoR-pA-ITR), and the Sleeping Beauty transposase expression vector P_{hCMV}-SB100 $\times$ (P_{hCMV}-SB100 $\times$ -pA). Transfected cells were selected using puromycin (1.5 μ g/ml; ST551, Beyotime) and bleomycin (200 μ g/ml; ST1450, Beyotime) for 3 weeks. Afterward, monoclonal populations were isolated and functionally validated using a custom light-emitting diode (LED) array (660/730 nm, 1 mW/cm²). Light-responsive clones were identified through correlative cytokine ELISA. Monoclonal cell lines demonstrating optimal optogenetic induction kinetics were selected and expanded for downstream experimental applications.

Quantification of cytokine and antibody levels

Cytokine concentrations (IFN- γ and IL-6) and CD47 antibody levels in cell culture supernatants and murine serum samples were quantified using commercial ELISA kits according to the manufacturers' protocols. Specifically, IFN- γ (E-EL-M0048, Elabscience), IL-6 (E-EL-M0044, Elabscience), and CD47 antibody (L00436, GenScript) were

measured with absorbance readings performed on a Synergy H1 hybrid multimode microplate reader (BioTek) controlled by Gen5 software (v2.04). All assays included appropriate standard curves and quality controls to ensure measurement accuracy, with samples analyzed in technical duplicates.

Optogenetic regulation of cytokine secretion

To quantify light-controlled cytokine release, engineered cells or EnE cells (8×10^5 cells per well) were plated in six-well culture dishes and incubated for 24 hours. Cells were then exposed daily for 2 consecutive days to 660-nm illumination at varying intensities (0, 0.2, 1, or 2 mW/cm²) or durations (0, 0.2, 1, 3, 5, or 60 s) using a custom 4 $\times$ 6 LED array. Culture supernatants were collected 48 hours after initial irradiation for cytokine quantification via ELISA. To assess wavelength-dependent regulation, cells received alternating 5-s pulses of 660 nm ("on" state) and 730 nm ("off" state) light at an intensity of 1 mW/cm². Following each 24-hour stimulation cycle (preceded by medium replacement), cytokine secretion profiles were monitored at 6-hour intervals over 72 hours.

The duration of cytokine production was assessed in vitro by exposing EnE cells (8×10^5 cells per well) to a 5-s pulse of RL (660 nm, 1 mW/cm²), followed by dark incubation for 96 hours. Periodic monitoring of IFN- γ , anti-CD47 antibody, and IL-6 levels was performed every 12 hours. To maintain cell viability and accurately measure cytokine accumulation over each interval, the culture medium was replaced with fresh medium immediately following each measurement. To assess optogenetic cytokine suppression, EnE cells were irradiated with RL (660 nm, 1 mW/cm²) and then cultured for 24 hours, after which the supernatant was collected for quantification of IFN- γ , CD47 antibody, and IL-6. Following a medium change, the cells received a 5-s pulse of FRL (730 nm, 1 mW/cm²) and were cultured for a further 24 hours before supernatant collection for the same analytes. The inhibition rate was calculated as follows: Inhibition Rate (%) = $[1 - (48\text{-hour value} / 24\text{-hour value})] \times 100\%$.

Synthesis of HA-DA

HA (12 kDa; Lifecore Biomedical, Chaska, USA) was initially dissolved in PBS under gentle agitation. To activate the carboxyl groups of HA, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (5 mM) and NHS (10 mM) were added to the solution. The pH was adjusted to 5.0 and maintained for 6 hours under continuous stirring. Subsequently, dopamine (10 mM; Aladdin Biochemical Technology, Shanghai, China) was introduced, and the reaction was allowed to proceed for 24 hours at room temperature under a nitrogen atmosphere with constant stirring to prevent oxidation. Upon completion, the crude product was purified by dialysis (14-kDa molecular weight cutoff membrane) against deionized water for 72 hours to remove unreacted reagents and by-products. The purified solution was then lyophilized to obtain the final HA-DA conjugate. Chemical structure confirmation was performed using ¹H NMR spectroscopy.

Gene cloning, protein expression, and purification

The target genes encoding HRP (National Center for Biotechnology Information accession: HW399451.1) and the HRP-pHLIP fusion protein were custom synthesized by Genewiz Inc. (Suzhou, China), with the pHLIP domain containing the optimized nucleotide sequence: 5'-GCGGAGCAGAACCCGATTTATTGGGCGCGCTATGCGGATTGGCTGTTTACCACCCCGCTGTTACTGCTG-GATCTGGCGCTGCTGGTGGATGCGGATGAAGGCACC-3'.

These gene fragments were subsequently subcloned into the pET28a(+) expression vector using restriction enzyme-based cloning, generating the recombinant construct pET28a-HRP-pHLIP. The verified plasmids were transformed into *E. coli* BL21(DE3) competent cells (Thermo Fisher Scientific) via heat shock treatment (42°C for 45 s). Transformed colonies were selected on Luria-Bertani (LB) agar plates containing ampicillin (60 µg/ml) after overnight incubation at 37°C. For protein expression, single colonies were inoculated into 5 ml of LB broth supplemented with ampicillin (60 µg/ml) and cultured aerobically at 37°C with shaking (200 rpm) for 12 to 16 hours. A 1% (v/v) inoculum was transferred into 30 ml of fresh LB medium in a 250-ml Erlenmeyer flask and grown to mid-log phase (optical density at 600 nm $\approx$ 0.8) under identical conditions. Protein expression was induced by adding 0.5 mM isopropyl β -D-thiogalactopyranoside, followed by 8 hours of low-temperature induction at 16°C with continuous shaking (220 rpm).

Harvested cells were pelleted by centrifugation (8000g for 10 min at 4°C) and resuspended with lysis buffer [50 mM NaH₂PO₄ and 300 mM NaCl (pH 8.0)]. Bacterial cell disruption was achieved through ultrasonic treatment (30% amplitude, 5-s pulse/5-s rest cycles for 15 min) using an ice-water bath to prevent overheating. The lysate was clarified by centrifugation (12,000g for 30 min at 4°C), and the supernatant containing soluble His-tagged pHLIP-HRP fusion protein was subjected to Ni-nitrilotriacetic acid affinity chromatography. Eluted fractions were analyzed by 12% SDS-PAGE under reducing conditions. Purified proteins were lyophilized and stored at -80°C until further use.

Single-cell encapsulation

HEK 293T cells were harvested and resuspended in PBS (pH 6.5) containing HRP-pHLIP, followed by a 30-min incubation at room temperature. The mixtures were then centrifuged at 1000 rpm for 5 min and washed three times with PBS to remove any unbound HRP-pHLIP. For encapsulation, the labeled cells were mixed into a 2% (w/v) HA-DA hydrogel solution containing 2 mM H₂O₂ and gently stirred for an additional 30 min. After stirring, the cells were resuspended and washed three times with PBS (pH 7.4) to remove any residual reagents.

Cell viability assessment

Cell viability was assessed using a dual-fluorescence Live/Dead assay (Beyotime Biotechnology, China) according to the manufacturer's protocol. Briefly, both encapsulated cells and untreated controls ($n = 3$ per group) were incubated in serum-free DMEM containing 2 µM calcein acetoxymethyl ester (calcein-AM) (for viable cell staining) and 4 µM propidium iodide (PI; for nonviable cell detection) at 37°C in 5% CO₂ for 30 min. Following incubation, cells were rinsed twice with PBS to remove excess dye. Fluorescent images were acquired using an inverted fluorescence microscope (Axio Observer 7, Zeiss, Germany) with standard fluorescein isothiocyanate [excitation/emission (ex/em), 495/515 nm] and tetramethyl rhodamine isothiocyanate (ex/em, 535/617 nm) filter sets for calcein-AM (green fluorescence, viable cells) and PI (red fluorescence, nonviable cells), respectively.

Characterization of encapsulated cell

The zeta potential of encapsulated cells was measured in triplicate using a Zetasizer Nano ZS90 (Malvern Instruments, UK) at 25°C in PBS (pH 7.4) with an applied voltage of 150 mV. For visualization, HA-DA was covalently labeled with FAM-NHS (5 µM) before

encapsulation. Postencapsulation, cells were stained with Hoechst 33342 (10 µg/ml for 10 min) to label nuclei, followed by three PBS washes. Cellular distribution and hydrogel morphology were analyzed by confocal laser scanning microscopy (LSM 880, Zeiss, Germany) using 488-nm (FAM) and 405-nm (Hoechst) excitation lasers.

Encapsulated cells were collected by centrifugation (300g for 5 min) and then exposed to 0.1% (w/v) trypsin-EDTA in 96-well plates (5×10^3 cells per well). At predetermined intervals (0.5, 1, 1.5, 2, 3, and 4 hours), enzymatic activity was neutralized with complete medium. Cell viability was quantified using Cell Counting Kit-8 (CCK-8; Beyotime Biotechnology, China) following the manufacturer's protocol, with absorbance measured at 450 nm. Nonencapsulated cells underwent identical treatment as controls. To evaluate physical stability, encapsulated cells were subjected to centrifugal stress (1000 to 6000 rpm) for 5 min at 4°C. Postcentrifugation viability was assessed via CCK-8 as described above, with parallel controls of native cells ($n = 3$).

TEM analysis

Native and encapsulated cells were collected and immediately fixed in 2.5% glutaraldehyde in 0.1 M PBS (pH 7.4) for 2 hours at 4°C. Afterward, samples were washed three times with 0.1 M PBS (pH 7.4) and postfixed with 1% osmium tetroxide in the same buffer for 1 hour at 4°C. Fixed samples were progressively dehydrated in an ethanol series (30, 50, 70, 80, 90, 95, and 100%, v/v) at 4°C, with 15-min intervals at each concentration. Complete dehydration was achieved through three exchanges of absolute ethanol. Samples were then transitioned to acetone and subjected to resin infiltration. Polymerized blocks were sectioned to 70 nm in thickness using a Leica UC7 ultramicrotome with a diamond knife. Sections were collected on 200-mesh copper grids and doubly stained with uranyl acetate (saturated solution in 50% ethanol for 10 min), followed by lead citrate to enhance contrast. Grids were examined using a Hitachi H-7800 TEM operated at 80 kV.

SEM analysis

The harvested samples were immediately fixed in 2.5% glutaraldehyde (in 0.1 M phosphate buffer, pH 7.4) at 4°C for 24 hours to optimally preserve cellular ultrastructure. Following fixation, samples underwent sequential ethanol dehydration as follows: 50, 70, 80, 90, and 95% (v/v) ethanol (15 min each step), followed by three 10-min washes in absolute ethanol (100%, v/v). The ethanol was then gradually replaced with isobutanol through three 10-min exchanges. Afterward, samples were freeze dried using a SCIENTZ-18 N/B *tert*-butanol freeze dryer (SCIENTZ, China) under controlled conditions to maintain structural integrity. Before imaging, samples were sputter coated with a 5-nm osmium layer using an OPC80T osmium plasma coater (Filgen, Japan) to ensure optimal conductivity and surface electron emission. SEM imaging was conducted using an ultrahigh-resolution field-emission SEM (JSM-IT800SHL, JEOL, Japan) operated at 1.0-kV accelerating voltage.

SEAP assay

Transmembrane molecular transport was evaluated using the SEAP reporter system. HEK 293T cells were transiently transfected with the P_{hCMV}-SEAP-pA plasmid to express SEAP. For SEAP activity measurement, cell culture supernatants were collected 48 hours after transfection, filtered through a 0.22-µm membrane, and heat inactivated at 65°C for 30 min to eliminate endogenous phosphatase activity. Samples were then immediately cooled on ice to terminate the heat treatment. Afterward, 80 µl of heat-treated supernatant was combined with

100 μ l of 2 $\times$ assay buffer [20 mM homoarginine, 1 mM MgCl₂, and 21% (v/v) diethanolamine (pH 9.8)] and 20 μ l of 120 mM p-nitrophenyl phosphate substrate solution. The reaction mixture was incubated at 37°C. A microplate reader (Tecan, Switzerland) was used to record the absorbance at 405 nm every min for 30 min to establish the linear rate of absorbance increase, which was used to quantify the SEAP production.

In vivo biodistribution of encapsulated cell

The harvest 293T cells were resuspended in 1 ml of PBS containing Cy5-NHS (0.25 mg/ml) and incubated in the dark at room temperature for 30 min to label the cells. The Cy5-labeled cells were then encapsulated, with unencapsulated Cy5-labeled cells serving as controls. For biodistribution studies, 8-week-old BALB/c mice received tail vein injections of PBS suspensions containing 1×10^6 cells (either encapsulated or native Cy5-labeled cells). Serial blood samples (20 μ l each) were collected via orbital venous plexus puncture at predetermined time points (0.5, 1, 2, 4, 8, 12, and 24 hours) postinjection. All blood samples and live animals were imaged using an IVIS imaging system (Xenogen Corporation, Hopkinton, MA). At the 24-hour end point, mice were humanely euthanized, followed by surgical excision of major organs (heart, liver, spleen, lungs, and kidneys) for ex vivo fluorescence quantification using the same IVIS imaging platform.

Biosafety assessment

A comprehensive biosafety evaluation was conducted on day 15 post-treatment. Following euthanasia, peripheral blood samples were collected from all experimental groups for biochemical profiling, including hepatic function markers (alanine aminotransferase, aspartate aminotransferase, and lactate dehydrogenase), renal parameters (creatinine kinase-myocardial band (CKMB), uric acid (UA), and urea (UREAL)), and complete blood counts [white blood cell (WBC), red blood cell, and platelet (PLT)]. Major organs (heart, liver, spleen, lungs, and kidneys) were then harvested, rinsed with physiological saline (0.9% NaCl), and fixed in 4% neutral buffered formalin (25°C for 48 hours). After decalcification (48 hours), tissues underwent standard histological processing: graded ethanol dehydration (70 to 100%, 1 hour per step), xylene clearing, and paraffin embedding. Serial sections (6 μ m) were cut using a rotary microtome, heat-fixed (60°C for 2 hours), and stained with hematoxylin (5 min) and eosin (2 min) with intermediate differentiation in acid-alcohol (1% HCl/70% ethanol). After final dehydration and neutral resin mounting, tissue morphology was systematically evaluated by light microscopy (Carl Zeiss, Germany).

Quantification of lung metastatic burden

To quantify the metastatic burden, whole-slide images of H&E-stained lung sections were analyzed using ImageJ software [National Institutes of Health (NIH)]. For each section, the total lung tissue area and the cumulative area of all metastatic foci were manually traced and measured. The metastatic burden was then calculated as the percentage of the total lung area occupied by tumors, according to the following formula: Metastatic Burden (%) = (Total Tumor Area / Total Lung Area) $\times$ 100%.

Immunofluorescence staining and quantitative analysis

Deparaffinized and rehydrated tissue sections underwent microwave-induced antigen retrieval. Sections were then blocked with 3% bovine serum albumin and incubated with primary antibodies overnight at

4°C, followed by fluorescent secondary antibodies for 50 min at room temperature. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). For quantitative analysis, images were processed using ImageJ software (NIH). After separating the color channels, the total area of the positive signal (red fluorescence) and the total nuclear area (blue fluorescence from DAPI) were measured. The proportion of positive signal was calculated as the ratio of the red fluorescence area to the blue fluorescence area.

In vivo cytokine detection

Cytokine levels were monitored over time through serial orbital blood collections at days 7 and 14 following treatment initiation. Serum samples were analyzed for IFN- γ , CD47 antibody, and IL-6 levels using standardized ELISA protocols. Terminal blood collections at study completion enabled additional cytokine quantification, including proinflammatory markers (IL-1 β and IL-17), the anti-inflammatory cytokine IL-10, the chemokine MCP-1/C-C motif chemokine ligand 2 (CCL2), and the angiogenic factor VEGF. All assays were performed using commercially available ELISA kits: IL-1 β , IL-10, and MCP-1/CCL2 kits from Thermo Fisher Scientific (Waltham, MA, USA) and IL-17 and VEGF detection kits from MULTI SCIENCES (LIANKE) Biotech Co. Ltd. (Hangzhou, China). Sample dilutions were optimized according to the manufacturer's specifications for each analyte.

Flow cytometry

To identify the immune cell subsets responsible for the observed antitumor effects, we performed comprehensive flow cytometry analysis of seven distinct immune cell populations, including CD206⁺ macrophages, SIRP α ⁺ macrophages, CD8⁺ T cells, CD4⁺ T cells, DCs, MDSCs, and T_{reg} cells. All antibodies were purchased from BioLegend and diluted 1:200. The antibodies used for macrophage cell labeling were Alexa Fluor 647 (AF647) anti-mouse F4/80 (catalog no. 123122, clone BM8) and Brilliant Violet 605 anti-mouse/human CD11b (catalog no. 101257, clone M1/70). For labeling CD206⁺ macrophage cells, the antibodies used were AF647 anti-mouse F4/80 (catalog no. 123122, clone BM8), Brilliant Violet 605 anti-mouse/human CD11b (catalog no. 101257, clone M1/70), and phycoerythrin (PE)/Cy7 anti-mouse CD206 (catalog no. 141720, clone C068C2). The antibodies for labeling SIRP α ⁺ macrophage cells included AF647 anti-mouse F4/80 (catalog no. 123122, clone BM8), Brilliant Violet 605 anti-mouse/human CD11b (catalog no. 101257, clone M1/70), and PE anti-mouse CD172a (SIRP α) (catalog no. 144012, clone P84). To mark CD8⁺ T cells, the antibodies used were AF700 anti-mouse CD45 (catalog no. 103128, clone 30-F11) and PerCP/Cy5.5 anti-mouse CD8a (catalog no. 100734, clone 53-6.7). For CD4⁺ T cells, the antibodies used were AF700 anti-mouse CD45 (catalog no. 103128, clone 30-F11) and BV510 anti-mouse CD4 (catalog no. 100559, clone RM4-5). To label DCs, the antibodies used were AF700 anti-mouse CD45 (catalog no. 103128, clone 30-F11), PerCP/Cy5.5 anti-mouse CD11c (catalog no. 117328, clone N418), AF647 anti-mouse F4/80 (catalog no. 123122, clone BM8), and Brilliant Violet 605 anti-mouse/human CD11b (catalog no. 101257, clone M1/70). The antibodies used for MDSC cell labeling were AF700 anti-mouse CD45 (catalog no. 103128, clone 30-F11), BV510 anti-mouse Ly-6G/LY-6C (Gr1) (catalog no. 108457, clone RB6-8C5), and Brilliant Violet 605 anti-mouse/human CD11b (catalog no. 101257, clone M1/70). The antibodies for T_{reg} cells were BV510 anti-mouse CD4 (catalog no. 100559, clone RM4-5) and PE anti-mouse FOXP3 (catalog no. 126404, clone MF-14). The

stained cells were analyzed using a BD FACSCanto flow cytometer (BD Biosciences), with a minimum of 5000 events per plot. Data were analyzed using FlowJo V10 software. The percentages shown in the flow cytometry analysis images are based on this analysis. The gating strategy for flow cytometry is provided in fig. S24.

Statistical analysis

Quantitative data are presented as means $\pm$ SD. Intergroup comparisons were analyzed using Student's *t* test, while multigroup comparisons were assessed through one-way analysis of variance (ANOVA). Statistical significance was denoted as **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001, with N.S. (not significant) indicating *P* $\geq$ 0.05. All statistical procedures were implemented using GraphPad Prism software (version 8.0.2).

Supplementary Materials

The PDF file includes:

Figs. S1 to S25

Tables S1 and S2

Legends for data S1 and S2

Other Supplementary Material for this manuscript includes the following:

Data S1 and S2

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