

A DNA-Scaffolded Natural Killer Cell with Adaptable Multimodules for Enhanced Immunotherapy and Early Efficiency Reporting

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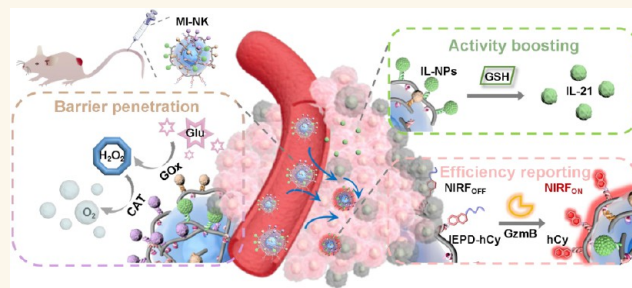
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ABSTRACT: Adoptive cell therapy is promising with many clinical trials, but its application efficiency is significantly influenced by individual immune microenvironments and the varied responses of different patients. Equipping adoptive cells with multifunctional modules for function enhancement and real-time efficacy readouts is a key solution but remains challenging due to the dynamic nature of the cell membrane and the limited accessibility of available binding sites on the membrane. Herein, we report a multimodule intelligent NK (MI-NK) cell that encapsulates NK cells within a durable DNA scaffold anchored with multiple adaptable functional modules, including a barrier penetration module (glucose oxidase-catalase cascade enzymes), an activity boosting module (IL-21 nanoparticles), and an efficiency reporting module (granzyme B-activatable hemicyanine NIR fluorescent probe). Oxygen generated from cascade enzymatic reactions drives MI-NK cells and promotes their tumor infiltration. IL-21 nanoparticles are disintegrated by GSH to release IL-21 for on-site NK cell activation and expansion. Activated NK cells secrete granzyme B, which restores the NIR fluorescence of the efficiency-reporting module to enable response reporting as early as 6 h after adoptive cell administration. The as-obtained MI-NK effectively enhances the *in vivo* immunotherapeutic efficiency. The types and proportions of adaptable modules can be expanded, offering a universal approach for adoptive cell therapy efficiency.

KEYWORDS: Adoptive cell therapy, intelligent NK cells, DNA scaffold engineering, barrier penetration, activity boosting, efficiency reporting



INTRODUCTION

Recent years have witnessed rapid growth in immunotherapy for cancer treatment,¹ and among them, adoptive cell therapy (ACT), which expands and activates immune cells *ex vivo* followed by reinfusion into patients,² has shown remarkable prospect.³ Natural killer (NK) cells have drawn significant attention as adoptive immune cell candidates due to their lower risk of neurotoxicity and cytokine release syndrome (CRS)^{4,5} and have emerged as a promising approach with many clinical trials.^{6,7}

The harsh physical barriers and immunosuppressive microenvironment of solid tumors^{8,9} affect adoptive cell penetration and proliferation; therefore, they have been *ex vivo* engineered with immune activators for efficiency enhancement.^{10,11} Considering that ACT efficiency is significantly influenced by the individual immune microenvironment of different patients, the same immune activator formulation may result in varied

therapeutic efficiency.¹² Therefore, early detecting patients' response to ACT is crucial for adjusting treatment plans and improving cancer immunotherapy. Developing a generalized platform with adaptable, engineered multiple modules for enhancing adoptive cell immunotherapy and early reporting of its therapeutic efficiency is urgently demanded.¹³ Optogenetic modulation of membrane receptor expression^{14–16} and backpacking with functional materials¹⁷ such as metal/metal oxide nanoparticles¹⁸ and enzymes¹⁹ have been reported to enhance the functions of adoptive cells and promote their

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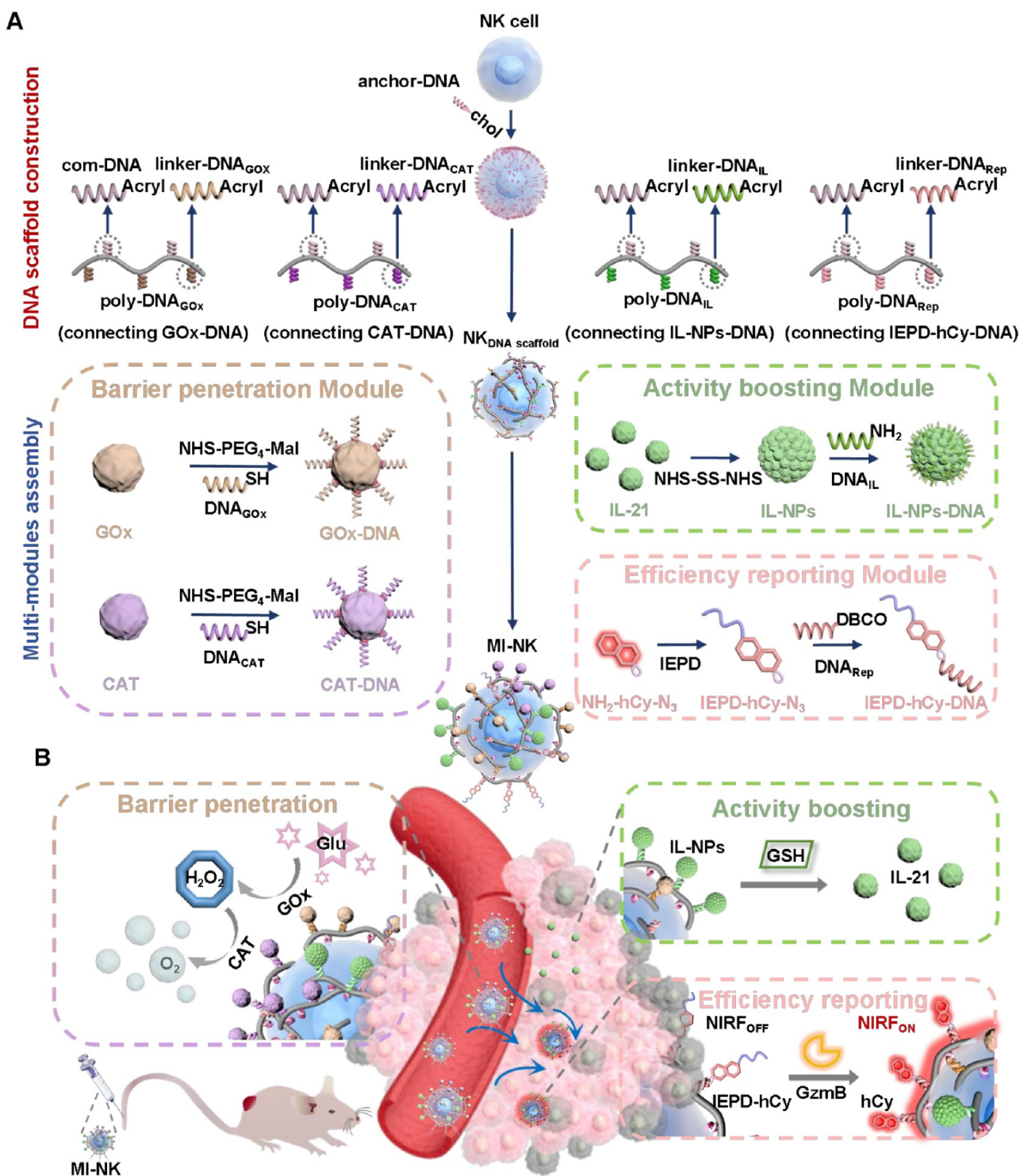
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Scheme 1. Schematic Illustration of (A) Synthesis of Multimodule Intelligent NK (MI-NK) cell and (B) Performance of three Modules at the Tumor Site to Enhance Adoptive Immunotherapy



infiltration into the tumor microenvironment.^{20–23} However, most currently reported functional adoptive immune cells employ a single-tasking mode. Equipping adoptive cells with multiple functionalities remains challenging.¹⁷ Genetic editing for the expression of multifunctional modules requires specific designs for individual modules, which increases inherent technical complexity and expenditure.^{24–26} Directly clicking or covalently conjugating multiple functional modules to the cell membrane is rarely reported due to the expression amount

limit of receptors and membrane dynamic status, which may induce dropping off or endocytosis of functional modules and impair adoptive immune cell survival and functions.^{27–29}

Taking advantage of their rigid structure and functionalization convenience, DNA scaffolds have been constructed along the cell membranes to enhance membrane rigidity,³⁰ with cell recognition probes displayed.^{31,32} Here, we develop a DNA-framed intelligent NK cell that is functionalized with adaptable multimodules including a barrier penetration module, an

activity boosting module, and a real-time efficiency-reporting module, to effectively enhance adoptive cell therapy. During DNA scaffold construction, cholesterol-functionalized anchor-DNA is inserted into the NK cell membrane via hydrophobic–hydrophobic interactions between cholesterol and lipids. A series of poly-DNA strands are synthesized by polymerizing an acrydite-modified complementary DNA (com-DNA) strand (for complementary pairing with anchor-DNA) and an acrydite-modified linking DNA (linker-DNA) strand (for linking different modules). There are four kinds of linker-DNA strands, correspondingly obtaining four kinds of poly-DNA strands: poly-DNA_{GOx} and poly-DNA_{CAT} for linking the barrier penetration module, poly-DNA_{IL} for linking the activity boosting module, and poly-DNA_{Rep} for linking the efficiency reporting module. The as-obtained poly-DNA strands are hybridized with anchor-DNA to construct DNA scaffolded NK cells (Scheme 1A, NK_{DNA scaffold} construction).

In the multimodule assembly step, four modules are prepared. For the barrier-penetration module (GOx-DNA/CAT-DNA), glucose oxidase (GOx) and catalase (CAT) are modified with the linker molecule NHS-PEG₄-Mal and conjugated to thiol-modified DNA_{GOx} and DNA_{CAT} that contain complementary sequences to linker-DNA_{GOx} and linker-DNA_{CAT}, respectively (Scheme 1A, GOx-DNA, CAT-DNA). To prepare the activity boosting module (IL-NPs-DNA), IL-21 protein is self-assembled into IL-21 nanoparticles (IL-NPs) via NHS-SS-NHS and subsequently modified with amino-modified DNA_{IL} that has a complementary sequence to linker-DNA_{IL} (Scheme 1A, IL-NPs-DNA). To prepare the efficiency reporting module (IEPD-hCy-DNA), an amino- and azide-functionalized hemicyanine fluorophore (NH₂-hCy-N₃) with near-infrared emission (700–750 nm) is conjugated to the Granzyme B (GzmB) substrate peptide IEPD via an amidation reaction, which blocks intramolecular charge transfer (ICT) and quenches fluorescence.³³ GzmB is one of the indicators of NK cell cytotoxic activity³⁴ and acts as an early reporter of ACT efficiency. The as-obtained IEPD-hCy-N₃ is then linked with DBCO-modified DNA_{Rep} that has a complementary sequence with linker-DNA_{Rep} by click chemistry (Scheme 1A, IEPD-hCy-DNA). The as-obtained barrier-penetration module GOx-DNA/CAT-DNA, activity-boosting module IL-NPs-DNA, and efficiency-reporting module IEPD-hCy-DNA are then anchored to NK_{DNA scaffold} cells by hybridizing with the linker-DNA in poly-DNA_{GOx}, poly-DNA_{CAT}, poly-DNA_{IL}, and poly-DNA_{Rep} respectively to prepare the multimodule-functionalized “three-in-one” intelligent NK cell (MI-NK) (Scheme 1A, multimodule assembly). The construction of a DNA scaffold on the NK cell membrane increases the number of anchoring sites for multiple modules and secures the functional modules on the cell membrane without detachment or endocytosis during systemic circulation.

The as-obtained MI-NK cells are then intravenously injected into A549 tumor-bearing mice. Glucose (Glu) and hydrogen peroxide that are present in the tumor microenvironment provide MI-NK cells with self-propulsion capability,^{35–38} enabling their active motility within the tumor tissue. The barrier-penetration module generates cascade enzymatic reactions, converting Glu to hydrogen peroxide via GOx and subsequently to O₂ by CAT, thereby producing propulsion forces that enhance MI-NK cell infiltration into the tumor (Scheme 1B, Barrier penetration). Taking advantage of the highly expressed GSH in the tumor microenvironment, the

activity-boosting module, IL-NPs-DNA, enables spatiotemporally controlled release of IL-21 through GSH-responsive cleavage of disulfide bonds. As a pivotal immunoregulatory cytokine for NK cells, IL-21 serves as an immunoadjuvant³⁹ to boost NK cell activity by promoting their proliferation and maturation *in vivo*⁴⁰ (Scheme 1B, Activity boosting). The efficiency-reporting module indicates NK cell cytotoxic efficacy in real time. GzmB cleaves its substrate peptide of IEPD-hCy and restores hCy NIR fluorescence via restoration of the ICT process (Scheme 1B, Efficiency reporting, NIRF_{ON}). Overall, the intelligent “three-in-one” multimodule MI-NK effectively enhances its infiltration into dense solid tumors and augments adoptive tumor immunotherapy while enabling early efficiency reporting.

RESULTS AND DISCUSSION

Preparation of NK_{DNA scaffold}

As major components for DNA scaffold construction, a series of poly-DNAs were synthesized by copolymerizing the acrydite-modified com-DNA with sequences complementary to anchor DNA and acrydite-modified linker DNAs (Figure S1). Poly-DNA_{GOx}, poly-DNA_{CAT} were prepared for linking the barrier penetration module; poly-DNA_{IL} was prepared for linking the activity boosting module; and poly-DNA_{Rep} was prepared for linking the efficiency reporting module (Scheme 1A; poly-DNA_{GOx}, poly-DNA_{CAT}, poly-DNA_{IL}, poly-DNA_{Rep}). Take poly-DNA_{GOx} for example, PAGE analysis confirmed the successful synthesis of poly-DNA, showing sharp bands with high mobility for com-DNA and linker DNA_{GOx} (Figure S2, lanes 1 and 2), whereas poly-DNA_{GOx} exhibited a pronounced decrease in mobility due to increased steric hindrance (Figure S2, lane 3). Gel permeation chromatography (GPC) analysis was further performed to confirm the synthesis of poly-DNAs. Poly-DNA_{GOx} showed a distinct polymer distribution profile with a molecular weight of 43 408 Da (Figure S3A). Based on molecular weight analysis derived from the GPC results, each poly-DNA_{GOx} chain contained ~5 DNA strands, together with an average of ~203 acrylamide repeating units. Similarly, poly-DNA_{CAT} (46 695 Da), poly-DNA_{IL} (48 833 Da), and poly-DNA_{Rep} (44 371 Da) all contained ~5 DNA strands per polymer chain, together with ~253, ~279, and ~219 acrylamide repeating units, respectively (Figure S3B–D).

As the anchoring points for constructing a DNA scaffold, cholesterol-functionalized anchor-DNA was inserted into the NK cell membrane via hydrophobic–hydrophobic interactions. The anchor-DNA incubation concentration was optimized with TAMRA-labeled anchor-DNA (anchor-DNA_{TAMRA}) and characterized by flow cytometry. Compared with cells incubated in PBS, NK cell membrane TAMRA fluorescence increased with anchor-DNA_{TAMRA} concentration and saturated at 1 μ M (Figure S4), which was chosen as the optimal incubation concentration. The anchor-DNA_{TAMRA} modified NK cells exhibited clear and evenly distributed TAMRA fluorescence on the cell membrane (Figure S5), indicating efficient modification of the cell membrane by anchor-DNA. To assess the anchoring efficiency of anchor-DNA on NK cells, TAMRA-labeled anchor-DNA (anchor-DNA_{TAMRA}) was used to modify 1×10^6 NK cells, and the as-obtained TAMRA fluorescence intensity (Figure S6A) was compared with the anchor-DNA_{TAMRA} calibration curve (Figure S6B,C) to determine the anchoring density as approximately 1.86×10^6 anchor-DNA strands per NK cell,

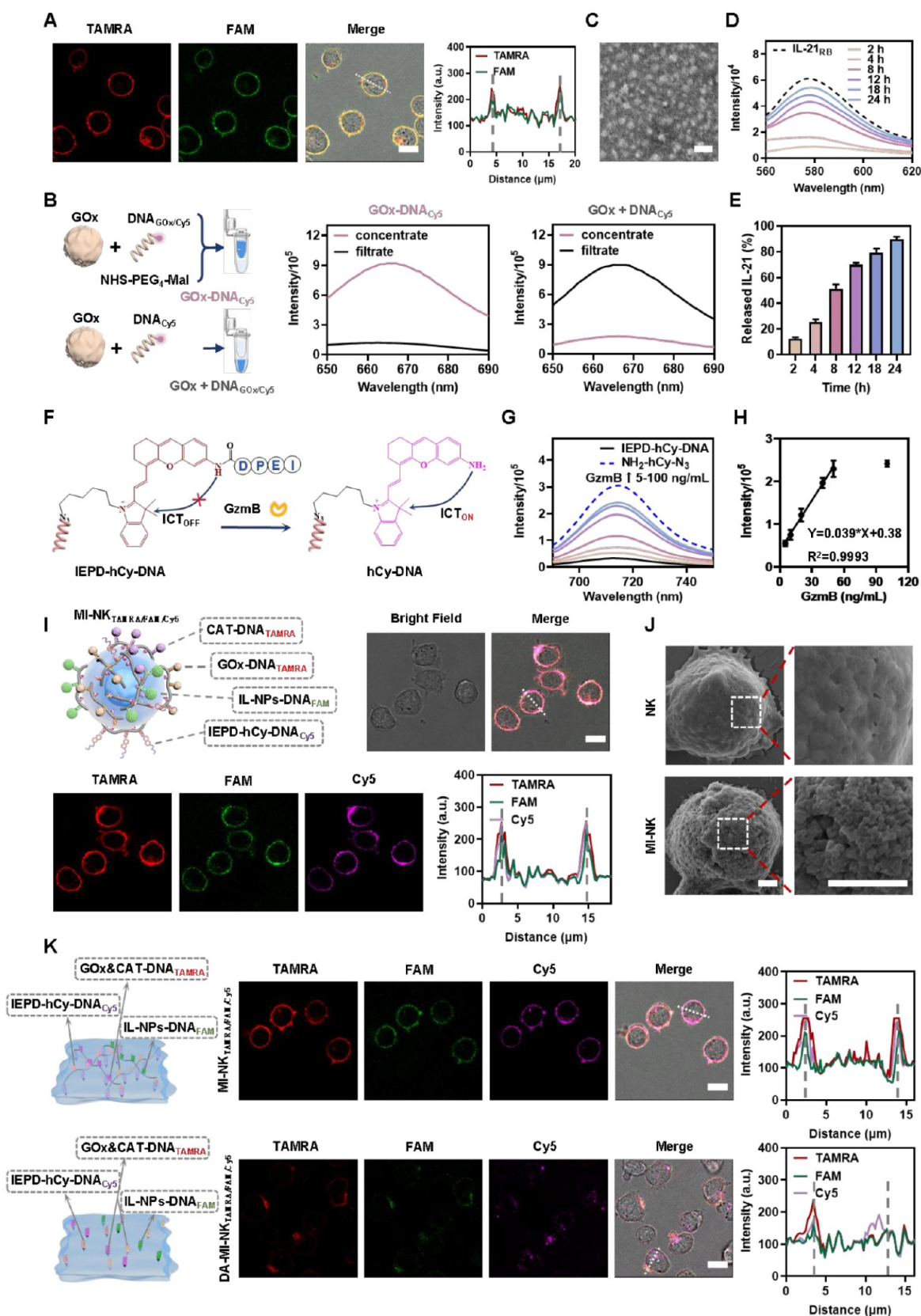


Figure 1. Preparation and characterization of NK_{DNA} scaffold and adaptable modules. (A) Confocal images of NK_{DNA} scaffold prepared with anchor-DNA_{TAMRA} (red) and poly-DNA_{GOx/FAM} (green) and the corresponding fluorescence colocalization analysis. Scale bar: 10 μm . (B) Fluorescence spectra of the concentrate and filtrate collected from as-obtained GOx-DNA_{Cy5} and the mixture of GOx and DNA_{GOx/Cy5} (GOx + DNA_{GOx/Cy5}). (C) TEM image of IL-NPs. Scale bar: 50 nm. (D) Time-dependent fluorescence spectra and (E) the corresponding IL-21_{TAMRA} release percentage for IL_{TAMRA}-NPs-DNA treated with 5 mM GSH. Data are presented as mean $\pm$ SD ($n = 3$ independent samples). (F) Schematic illustration of IEPD-hCy-DNA fluorescence recovery in response to GzmB, with (G) the corresponding fluorescence spectra.

Figure 1. continued

of IEPD-hCy-DNA in response to 5, 10, 20, 40, 50, and 100 ng/mL GzmB and (H) the linear relationship. Data are presented as mean $\pm$ SD ($n = 3$ independent samples). Confocal fluorescence images of (I) MI-NK_{TAMRA/FAM/Cy5} and (K) MI-NK_{TAMRA/FAM/Cy5} and DA-MI-NK_{TAMRA/FAM/Cy5} after 4 h of incubation, and the corresponding fluorescence colocalization analysis. Scale bar: 10 μ m. (J) SEM images of NK cells and MI-NK cells. Scale bar: 1 μ m.

providing a robust foundation for subsequent surface functionalization. The poly-DNA coating concentration on NK_{anchor-DNA} cells was optimized using FAM-labeled poly-DNA strands (poly-DNA_{FAM}) and characterized by flow cytometry. Compared with cells incubated in PBS, membrane FAM fluorescence increased with poly-DNA_{FAM} concentration and reached a plateau at 100 nM (Figure S7), which was selected as the optimal coating concentration. NK_{DNA scaffold} cells were then prepared by hybridizing the previously prepared poly-DNAs to anchor-DNA immobilized on the NK cell membrane. Take poly-DNA_{GOx} for example, poly-DNA_{GOx/FAM} was prepared with FAM-labeled linker-DNA_{GOx} (linker-DNA_{GOx/FAM}) and incubated with anchor-DNA_{TAMRA} immobilized NK cells, which demonstrated good overlap of FAM and TAMRA fluorescence around the cell membrane (Figure 1A), indicating efficient construction of the DNA scaffold on the NK cell membrane. To further quantify the amount of poly-DNAs immobilized on NK cells, fluorescence-based quantification was performed. Calibration curves were established using TAMRA-labeled poly-DNA_{GOx} (poly-DNA_{GOx/TAMRA}), Cy5-labeled linker-DNA_{IL} (poly-DNA_{IL/Cy5}) and FAM-labeled poly-DNA_{Rep} (poly-DNA_{Rep/FAM}) (Figure S8B). The fluorescence intensities of the DNA-scaffolded NK cells were then compared with the corresponding calibration curves to determine the surface density of each poly-DNA strand (Figure S8A). The results indicated that each NK cell carried approximately 2.76×10^5 strands of poly-DNA_{GOx} and poly-DNA_{CAT}, 1.44×10^5 strands of poly-DNA_{IL} and 1.34×10^5 strands of poly-DNA_{Rep}. Hybridization of poly-DNA strands fastened the anchor-DNA on the NK cell membrane, which retained $\sim 81.8\%$ of its initially inserted amount after a 24 h incubation period (Figure S9, NK_{DNA scaffold}). These quantitative results further confirmed the efficient assembly of poly-DNAs on the anchor-DNA-modified NK cell membrane and provided sufficient binding sites for subsequent DNA-mediated functional module construction.

To eliminate the possibility of anchor-DNA or poly-DNA strands self-assembling into micelles during incubation, methyl orange (MO) was added to a series of concentrations of anchor-DNA and poly-DNAs respectively, and the resulting solutions showed little to no red-shift in the characteristic MO absorbance peak at 464 nm at the anchor-DNA working concentration of 1 μ M and the poly-DNA strand working concentration of 100 nM (Figure S10), indicating that both remain monomeric during the incubation process.

Preparation of Adaptable Functional Modules

The barrier-penetration module includes two DNA-functionalized enzymes: GOx-DNA was modified with DNA_{GOx} that has a complementary sequence to linker DNA_{GOx}, and CAT-DNA was modified with DNA_{CAT} that has a complementary sequence to linker DNA_{CAT}. Thiol-functionalized DNA_{GOx} and DNA_{CAT} were conjugated to GOx and CAT, respectively, via NHS-PEG₄-Mal linker to yield the barrier penetration module GOx-DNA and CAT-DNA (Scheme 1a, barrier-penetration module). To verify the synthesis of GOx-DNA, Cy5-labeled

DNA_{GOx} (DNA_{GOx/Cy5}) was incubated with GOx and NHS-PEG₄-Mal, showing strong Cy5 fluorescence from the concentrate and negligible Cy5 fluorescence signal from the filtrate (Figure 1B, GOx-DNA_{Cy5}). In contrast, mixing DNA_{GOx/Cy5} and GOx in the absence of NHS-PEG₄-Mal showed Cy5 fluorescence from the filtrate instead of the concentrate (Figure 1B, GOx + DNA_{GOx/Cy5}). These results confirmed the stable conjugation of DNA_{GOx} to GOx instead of nonspecific adsorption, which guaranteed efficient subsequent conjugation of GOx to NK_{DNA scaffold}. The GOx-DNA_{Cy5} production yield was determined to be 88.8% by comparing the Cy5 fluorescence intensity of GOx-DNA_{Cy5} with that of the reactant DNA_{GOx/Cy5}. CAT was incubated with DNA_{CAT/Cy5} and NHS-PEG₄-Mal according to the same procedure, which also showed strong Cy5 fluorescence for the as-obtained CAT-DNA_{Cy5} in the concentrate (Figure S11). The CAT-DNA_{Cy5} production yield was determined to be 91.7% using the above calculation method. Zeta potential analysis further validated the synthesis of enzyme-DNA, which showed values of -18.8 ± 0.34 mV and -22.6 ± 2.6 mV for GOx and CAT, and -30.1 ± 1.8 mV and -32.6 ± 2.6 mV for GOx-DNA and CAT-DNA, respectively, due to the conjugation of negatively charged DNA strands (Figure S12).

To synthesize the activity boosting module IL-NPs-DNA, cytokine IL-21 was assembled into nanoparticles (IL-NPs) using NHS-SS-NHS and subsequently conjugated with NH₂-modified DNA_{IL} (Scheme 1A, activity-boosting module). The elevated GSH levels in tumor microenvironments cleave NHS-SS-NHS to release IL-21. The particle size of IL-NPs affects their responsiveness to GSH. With a decrease in the NHS-SS-NHS/IL-21 reaction mass ratio from 2:1 to 1:10, the hydrodynamic diameter of the as-obtained IL-NPs decreased from 340.1 ± 26.6 nm to 20.3 ± 3.0 nm (Figure S13A). Different sizes of as-obtained IL-NPs were challenged with 5 mM GSH to mimic the tumor microenvironment^{41,42} (Figure S13B), and the hydrodynamic diameter decreased to 11.1 ± 2.7 nm for IL-NPs prepared with a 1:5 reaction ratio of NHS-SS-NHS/IL-21 (34.2 ± 5.8 nm hydrodynamic diameter) (Figure S13, IL-NPs_{1:5}), indicating almost complete degradation of IL-NPs. The transmission electron microscopy (TEM) image revealed that the as-optimized IL-NPs_{1:5} showed uniform distribution and an average diameter of 22 ± 3.3 nm (Figure 1C) and a zeta potential of -3.1 ± 0.3 mV (Figure S14A, IL-NPs). TAMRA-labeled IL-21 (IL-21_{TAMRA}) was used to prepare IL_{TAMRA}-NPs, and the conjugation efficiency was determined to be 82.3% by calculating the ratio of the fluorescence intensity of the ultrafiltered IL_{TAMRA}-NPs to that of IL-21_{TAMRA} before conjugation (Figure S15). NH₂-DNA_{IL} was then covalently conjugated to the residual NHS groups of IL-NPs, and this reduced the zeta potential to -18.3 ± 1.0 mV (Figure S14A, IL-NPs-DNA) and increased the hydrodynamic diameter to 44.8 ± 1.9 nm (Figure S14B), indicating the successful modification of negatively charged DNA strands onto IL-NPs. FAM-labeled DNA_{IL} (DNA_{IL/FAM}) was incubated with IL-NPs and NHS-PEG₄-Mal, and the production yield was determined to be 84.4% by dividing the FAM

intensity of the concentrate (IL-NPs-DNA_{FAM}) by the fluorescence intensity of the reactant (DNA_{IL/FAM}) (Figure S16). Both the hydrodynamic size and zeta potential of the as-obtained IL-NPs-DNA remained stable over 7 days of incubation in serum (Figure S17), confirming its structural integrity in systemic circulation and guaranteeing *in vivo* applications. GSH exposure disassembled IL-NPs by reducing disulfide groups into thiol groups, thereby releasing IL-21 (Figure S18). GSH-responsive disassembly of the IL-NPs-DNA module was further verified by challenging IL-TAMRA-NPs-DNA, which were synthesized using TAMRA-labeled IL-21 (IL-21-TAMRA) with 5 mM GSH, and the filtrate fluorescence intensity was measured over time (Figure 1D) and compared with that of IL-21-TAMRA used for preparing IL-TAMRA-NPs-DNA to obtain the proportion of released IL-21-TAMRA (Figure 1E). The filtrate fluorescence demonstrated a gradual increase according to treatment time, and 80.7% IL-21-TAMRA release was obtained by 12 h (Figure 1E). On the contrary, IL-TAMRA-NPs-DNA was incubated for 12 h in the absence of GSH, which showed barely any TAMRA fluorescence in the filtrate (Figure S19B, 24 h w/o GSH) with 14.4% IL-21-TAMRA release (Figure S19A, w/o GSH). These results demonstrate the GSH-responsive specificity of IL-NPs-DNA for mimicking the tumor microenvironment.

The efficiency-reporting module IEPD-hCy-DNA was synthesized by clicking IEPD-hCy-N₃ and DBCO-modified DNA_{Rep}. To prepare IEPD-hCy-N₃, NH₂-hCy-N₃ was first prepared by introducing amine and azide functional groups into hCy. The structure was confirmed by mass spectrometry at *m/z* 494.2962 (Figure S20A) and by NMR characterization (Figure S20B). NH₂-hCy-N₃ was then covalently conjugated to the C-terminus of the GzmB-specific tetrapeptide (IEPD) via an amide bond. The product was confirmed by mass spectrometry at *m/z* 990.5091 (Figure S21A). HPLC showed a single, sharp, and symmetrical peak at a retention time of 18.03 min, indicating the high purity of the as-obtained IEPD-hCy-N₃ (Figure S21B). Conjugation of the IEPD peptide to the donor site of hCy disrupts the intrinsic D- π -A conjugation, suppressing the ICT process of hCy, thus quenching its fluorescence.⁴³ IEPD-hCy-DNA was synthesized by clicking DBCO-functionalized DNA_{Rep} to IEPD-hCy-N₃, as demonstrated by HPLC analysis showing substantial area shrinkage of the IEPD-hCy-N₃ reactant peak with a conversion efficiency of 98.8% and the appearance of a new peak corresponding to the IEPD-hCy-DNA product (Figure S22). The as-obtained IEPD-hCy-DNA showed significantly reduced mobility in PAGE compared with DNA_{Rep} (Figure S23A). Upon GzmB-cleavage of the peptide IEPD, the ICT process was restored with hCy fluorescence recovery at 715 nm (Figure 1F), which showed a gradual increase in intensity with increasing GzmB concentration (Figure 1G) and a linear concentration–response profile from 5 to 50 ng/mL (Figure 1H) with the signal saturating at 80.2% compared with the intensity of NH₂-hCy-N₃, indicating the sensitive response of the efficiency reporting module. On the contrary, IEPD-hCy-DNA that was challenged with the nonspecific protease caspase-3 and untreated IEPD-hCy-DNA barely showed fluorescence recovery (Figure S23B). The GzmB-mediated hCy fluorescence recovery confirmed the capability of the efficiency reporting module IEPD-hCy-DNA for early reporting of NK cell function.

Synthesis of Multimodular Intelligent NK Cell

By hybridization with their corresponding linker DNA, the above-obtained barrier-penetration module, activity-boosting module, and efficiency-reporting module were anchored to NK-DNA scaffold cells to obtain multimodular intelligent NK cells (MI-NK). To visualize the module anchoring process, the three modules were labeled with three different dyes. The TAMRA dye-labeled barrier-penetration modules, GOx-DNA_{TAMRA} and CAT-DNA_{TAMRA} were prepared with TAMRA-labeled DNA_{GOx/TAMRA} and unlabeled DNA_{CAT/TAMRA} as components, respectively. The FAM labeled activity-boosting module, IL-NPs-DNA_{FAM}, was prepared with FAM-labeled DNA_{IL/FAM} as a component, and the Cy5-labeled efficiency-reporting module IEPD-hCy-DNA_{Cy5} was prepared with Cy5-labeled DNA_{Rep/Cy5} as component. NK-DNA scaffold was made with a 1:1:1:1 ratio of poly-DNA_{GOx}, poly-DNA_{CAT}, poly-DNA_{IL}, and poly-DNA_{Rep}, and was anchored with the above dye-labeled modules to obtain MI-NK-TAMRA/FAM/Cy5, which showed strong colocalization of the three fluorescence signals, indicating efficient membrane localization of all modules (Figure 1I). To further quantify the membrane-immobilized amounts of each module per cell, MI-NK-TAMRA/FAM/Cy5 was prepared with GOx-DNA_{TAMRA}, CAT-DNA_{TAMRA}, IL-NPs-DNA_{FAM}, and IEPD-hCy-DNA_{Cy5}; the fluorescence intensities of TAMRA, FAM, and Cy5 on MI-NK-TAMRA/FAM/Cy5 were measured and correlated with the corresponding calibration curves of GOx-DNA_{TAMRA}, IL-NPs-DNA_{FAM}, and IEPD-hCy-DNA_{Cy5} (Figure S24). The loading amounts were calculated as approximately 4.91×10^5 barrier-penetration modules (GOx-DNA_{TAMRA} and CAT-DNA_{TAMRA}) per cell; 1.93×10^5 activity-boosting modules (IL-NPs-DNA) per cell (each IL-NPs-DNA_{FAM} was estimated to contain ~ 200 IL-21 molecules); and 2.77×10^5 efficiency-reporting modules (IEPD-hCy-DNA_{Cy5}) per cell. These quantitative results further demonstrated the efficient and well-defined immobilization of multiple functional modules on NK cells. SEM images demonstrated much higher surface roughness for MI-NK cells compared with naked NK cells. These results confirmed the efficient anchoring of modules on the NK cell membrane and revealed the irregular, asymmetrical distribution of modules on the cell membrane (Figure 1J). TAMRA, FAM, and Cy5 fluorescence signals were well maintained with good overlap on the cell membrane and barely showed intracellular fluorescence after 4 h of incubation, indicating little module internalization (Figure 1K, MI-NK). On the contrary, cholesterol-modified linker DNAs (c-linker-DNA_{GOx}, c-linker-DNA_{CAT}, c-linker-DNA_{IL}, c-linker-DNA_{Rep}), as direct membrane anchors, were inserted into the NK cell membrane and hybridized with GOx-DNA_{TAMRA}, CAT-DNA_{TAMRA}, IL-NPs-DNA_{FAM}, and IEPD-hCy-DNA_{Cy5} at a 1:1:1:1 ratio. The as-obtained direct anchored multimodular intelligent NK cells (DA-MI-NK) showed substantial membrane fluorescence loss and obvious intracellular fluorescence after 4 h of incubation (Figure 1K, DA-MI-NK). MI-NK-TAMRA/FAM/Cy5 was continuously incubated for 24 h, and confocal images showed that TAMRA, FAM, and Cy5 fluorescence signals remained in good colocalization on the MI-NK cell membrane, and intracellular fluorescence was barely observed (Figure S25). These results indicated that the DNA scaffold could stably retain multiple modules on the cell membrane, which would be beneficial for adoptive cell therapy.

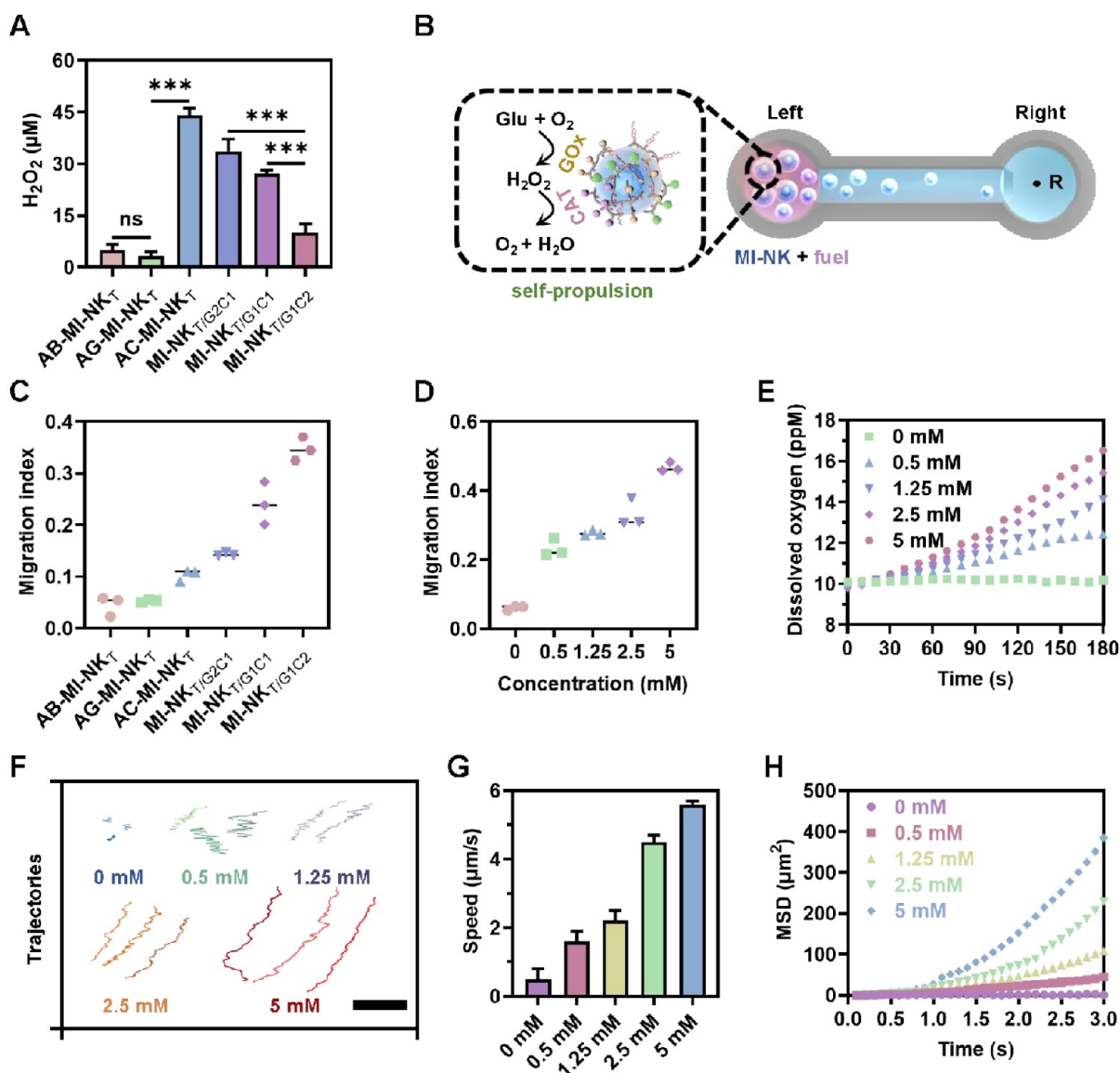


Figure 2. Motion capability characterization of MI-NK cells. (A) H₂O₂ production levels from AB-MI-NK_T, AG-MI-NK_T, AC-MI-NK_T, and MI-NK_T with different GOx-DNA/CAT-DNA ratios after 3 min at 5 mM glucose. Data are presented as mean $\pm$ SD ($n = 3$ independent samples), $P < 0.05$ was considered statistically significant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; ns, not significant. (B) Schematic illustration of self-propulsion of MI-NK cells in a homemade polydimethylsiloxane (PDMS) device. MI of (C) AB-MI-NK_T, AG-MI-NK_T, AC-MI-NK_T, and MI-NK_T with different GOx-DNA/CAT-DNA ratios at 20 min and (D) MI-NK_T corresponding to different Glu concentrations. Data in (C–D) are presented as mean $\pm$ SD ($n = 3$ independent samples). (E) Time-dependent dissolved oxygen levels of MI-NK_T at different Glu concentrations. (F) Trajectory pathways, (G) motion velocity, and (H) the corresponding MSD plots of MI-NK cells at different Glu concentrations. Scale bar in (F): 10 μ m; Data in (G) are presented as mean $\pm$ SD ($n = 10$ independent samples).

In Vitro Barrier Penetration Capability Characterization of Multimodular Intelligent NK Cell

The MI-NK cell has self-propulsion capability, which endows it with barrier-penetration capability in solid tumors. To rationally optimize the enzymatic cascade reactions on MI-NK cells, we quantitatively evaluated H₂O₂ production from MI-NK cells in the presence of glucose using the TMB colorimetric reaction by measuring its characteristic absorbance at 450 nm (Figure S26). The amount of H₂O₂ generated by MI-NK_{T/G1C2}, MI-NK_{T/G1C1}, and MI-NK_{T/G2C1} that prepared by anchoring GOx-DNA, CAT-DNA, IL-NPs-DNA, and IEPD-hCy-DNA to NK_{DNA scaffold/TAMRA} cells with GOx-DNA/CAT-DNA ratios of 1:2, 1:1, and 2:1, respectively, was quantified after 3 min of reaction at 5 mM glucose.³⁸ Control

groups were set with AB-MI-NK_T that prepared in the absence of the barrier penetration modules GOx-DNA and CAT-DNA, AG-MI-NK_T prepared in the absence of GOx-DNA, and AC-MI-NK_T prepared in the absence of CAT-DNA.

Notably, varying the GOx-DNA/CAT-DNA ratio resulted in distinct H₂O₂ output levels, which showed the lowest value for MI-NK_{T/G1C2} (Figure 2A). To further verify the self-propulsion capability of multimodular NK cells, a polydimethylsiloxane (PDMS) device was prepared with 400 μ m-high, 0.8 cm-diameter double-inlet chambers that were connected by a 1.6 cm channel (Figure S27, Figure 2B).⁴⁴ AB-MI-NK_T, AG-MI-NK_T, AC-MI-NK_T, and MI-NK_T with different GOx-DNA/CAT-DNA ratios were placed into the left chamber together with 5 mM glucose to mimic the tumor microenvironment. To

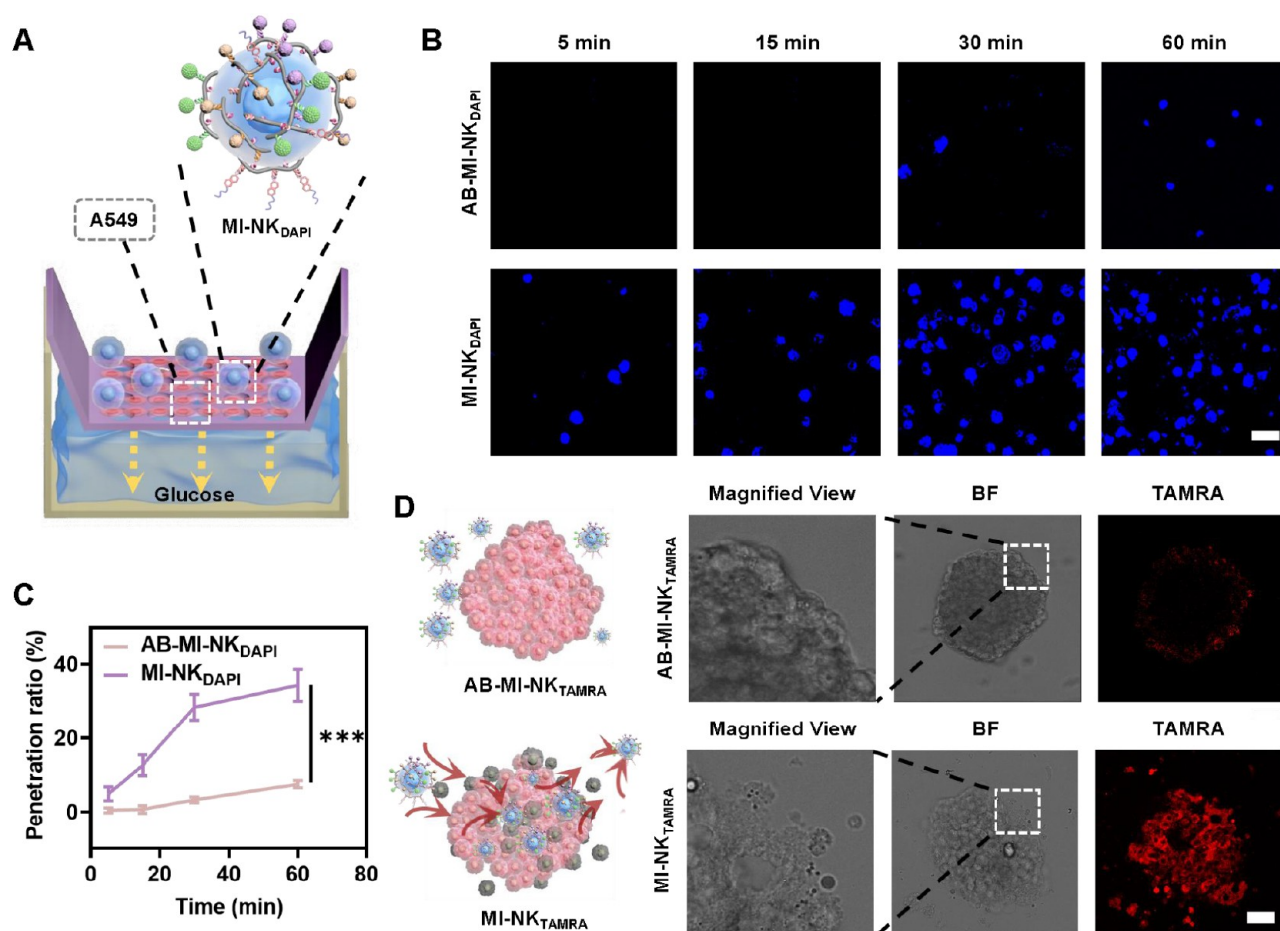


Figure 3. *In vitro* barrier penetration capability characterization of MI-NK cells. (A) Schematic illustration of a Transwell experiment by incubating MI-NK_{DAPI} with A549 cells in the upper chamber and Glu in the bottom chamber, and the corresponding (B) confocal images collected from the bottom chamber of the Transwell plate that were incubated with MI-NK_{DAPI} and AB-MI-NK_{DAPI} cells in the upper chamber, respectively (scale bar: 25 μ m), and (C) penetration ratios. Data are presented as mean $\pm$ SD ($n = 3$ independent samples), $P < 0.05$ was considered statistically significant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; ns, not significant. (D) Schematic illustration and corresponding confocal images of an A549 cell 3D MTS model incubated with MI-NK_{TAMRA} and AB-MI-NK_{TAMRA}. Scale bar: 50 μ m.

quantify the migration capability of MI-NK_T, the migration index (MI) was calculated by measuring TAMRA fluorescence intensity at the fixed sampling point R of the double-inlet chamber and comparing it to the total TAMRA fluorescence intensity of MI-NK_T. MI values at 20 min for AB-MI-NK_T, AG-MI-NK_T, AC-MI-NK_T, and MI-NK_T with various ratios of GOx-DNA/CAT-DNA were evaluated (Figure S28, Figure 2C). AB-MI-NK_T and AG-MI-NK_T barely migrated in the channel with MI values of 0.045 and 0.053, respectively, due to the absence of GOx; AC-MI-NK_T had a slightly higher MI of 0.10, while the coexistence of GOx and CAT effectively enhanced the migration distance of MI-NK_T, indicating it performed self-movement rather than Brownian motion. The migration index gradually increased with the CAT-DNA/GOx-DNA ratio, with optimal migration performance achieved on MI-NK_{T/G1C2} with a migration index of 0.35 (Figure 2C). Therefore, the cascade enzyme anchoring ratio was fixed at GOx-DNA:CAT-DNA of 1:2 for the as-obtained MI-NK cells. The time-corresponding fluorescence intensity at point R for MI-NK cells was also recorded under a series of Glu concentrations, which increased rapidly over 0–30 min and saturated within 30–60 min (Figure S29). The migration index value of MI-NK cells increased with Glu concentration, and a maximum MI value of 0.47 was achieved at 5 mM Glu at

1 h (Figure 2D). Oxygen generation from the barrier penetration module via Glu-activated cascade reactions was further verified by measuring dissolved oxygen, which showed a time-corresponding increase over 180 s of measurement, and enhanced values were achieved with increasing Glu concentrations (Figure 2E).

To visualize the movement of MI-NK cells, Glu solutions at different concentrations and MI-NK cells were dropped onto a glass slide, and movies of MI-NK cell motion and the corresponding movement trajectories were captured and recorded. Enhanced motion velocities were achieved at Glu concentrations of 0, 0.5, 1.25, 2.5, and 5 mM (Movie S1, Figure 2F), and the maximum velocity reached 15.3 μ m/s at 5 mM Glu (Figure 2G). The mean square displacement (MSD) of MI-NK cells under different Glu concentrations all demonstrated quadratic time dependence (Figure 2H), indicating that the motion was induced by enzyme cascade-powered self-propulsion rather than Brownian diffusion. On the contrary, AB-MI-NK cells that were prepared in the absence of the barrier penetration module showed Brownian motion at 5 mM Glu (Movie S2, Figure S30).

A Transwell experiment was performed to evaluate the barrier-penetration capability of MI-NK cells. A double-layer chamber was established, where A549 cells were seeded as a

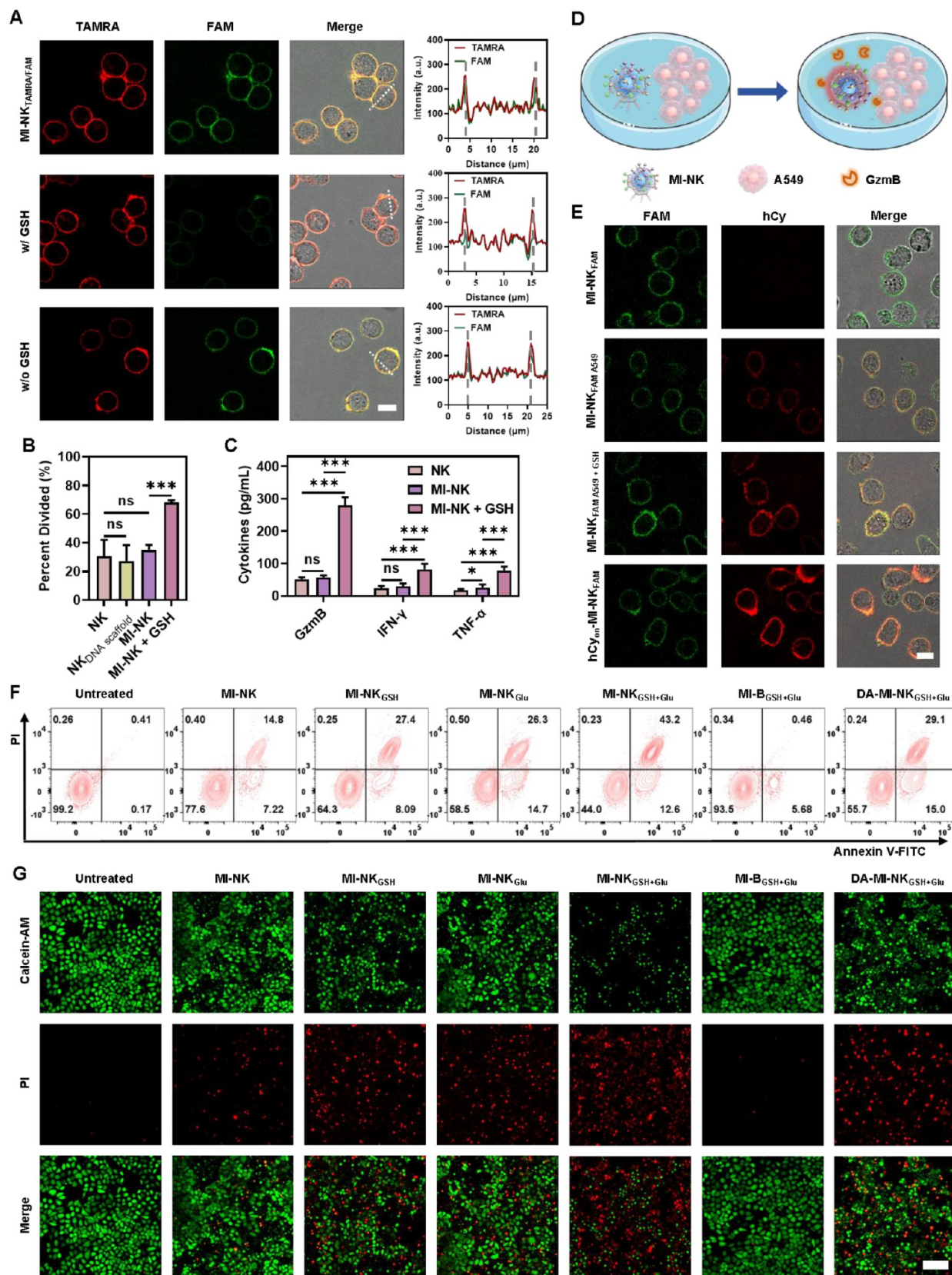


Figure 4. *In vitro* activity-boosting module and efficiency-reporting module characterization of MI-NK cells. (A) Confocal images of MI-NK_{TAMRA/FAM} and MI-NK_{TAMRA/FAM} cultured for 24 h in the presence (w/GSH) and absence of GSH (w/o GSH). Scale bar: 10 μ m. (B) Cell proliferation of NK cells, DNA-scaffolded NK cells (NK_{DNA scaffold}), MI-NK cells, and MI-NK cells in the presence of GSH (MI-NK + GSH). (C) GzmB, IFN- γ , and TNF- α secretion by NK cells, MI-NK cells, and MI-NK cells in the presence of GSH (MI-NK + GSH). Data are presented as mean $\pm$ SD ($n = 5$ independent samples), and $P < 0.05$ was considered statistically significant; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; ns, not significant. (D) Schematic illustration of MI-NK cell coculturing with A549 cells and real-time imaging of GzmB secretion,

Figure 4. continued

and (E) corresponding CLSM images of hCy fluorescence from hCy_{on}-MI-NK_{FAM} and hCy fluorescence recovery from MI-NK_{FAM}, MI-NK_{FAM} cocultured with A549 cells in presence (MI-NK_{FAM} A549+GSH) and absence of GSH (MI-NK_{FAM} A549). Scale bar: 10 μ m. (F) Flow cytometry and (G) live/dead cell assay of untreated A549 cells; A549 cells incubated with MI-NK; MI-NK in the presence of GSH (MI-NK_{GSH}), Glu (MI-NK_{Glu}), GSH and Glu (MI-NK_{GSH+Glu}); MI-B cells in the presence of GSH and Glu (MI-B_{GSH+Glu}); and with DA-MI-NK cells in the presence of GSH and Glu (DA-MI-NK_{GSH+Glu}), respectively. The scale bar in (G) is 100 μ m.

monolayer in the upper chamber, while Glu was added to the bottom chamber (Figure 3A). DAPI-stained MI-NK cells (MI-NK_{DAPI}) were added to the upper chamber, and their penetration percentage was evaluated by measuring DAPI fluorescence collected from the bottom chamber relative to the overall DAPI fluorescence from all incubated MI-NK_{DAPI}. Confocal images demonstrated time corresponding enhanced DAPI signals from the bottom chamber for MI-NK_{DAPI} (Figure 3B, MI-NK_{DAPI}) with a penetration percentage of 34.3% at 60 min (Figure 3C, MI-NK_{DAPI}). By contrast, AB-MI-NK_{DAPI} cells, in the absence of a barrier-penetration module, added to the upper chamber showed little DAPI fluorescence from the bottom chamber (Figure 3B, AB-MI-NK_{DAPI}) with a penetration percentage of 7.3% at 60 min (Figure 3C, AB-MI-NK_{DAPI}). DAPI fluorescence collected from the bottom chamber also showed enhanced intensity corresponding to the Glu concentration in the bottom chamber (Figure S31), indicating enhanced penetration capability of MI-NK cells. These results indicate that the tumor-penetration capability of MI-NK cells is attributable to self-propulsion effect instead of gravity.

A three-dimensional (3D) multicellular tumor spheroid (MTS) model was constructed using A549 cells to mimic the spatial complexity of tumor tissue and to further investigate the tissue penetration and infiltration capacity of MI-NK cells. MI-NK_{TAMRA} cells were prepared by anchoring GOx-DNA, CAT-DNA, IL-NPs-DNA, and IEPD-hCy-DNA to NK_{DNA scaffold/TAMRA} cells and then incubated with A549 MTSs, which showed widely distributed TAMRA fluorescence throughout the entire spheroid from the periphery to the central regions, indicating efficient penetration capability (Figure S32, MI-NK_{TAMRA}). In contrast, AB-MI-NK_{TAMRA} cells demonstrated limited infiltration capability, with TAMRA fluorescence remaining predominantly at the spheroid periphery (Figure S32, AB-MI-NK_{TAMRA}). The distribution of different functionalized NK cells in the tumor spheroids is shown in Figure 3D. MI-NK_{TAMRA} cells showed TAMRA fluorescence evenly distributed throughout the entire 3D MTS model, with a TAMRA fluorescence intensity of 162.4, while AB-MI-NK_{TAMRA} cells only showed TAMRA fluorescence within approximately 20–30 μ m from the spheroid boundary, with a TAMRA fluorescence intensity of 73.8 (Figure S33). As expected, MI-NK_{TAMRA} cells demonstrated superior MTS penetration and enrichment across all groups due to their self-propulsion capability. Bright-field images also showed an obvious difference: MI-NK_{TAMRA}-treated A549 MTS exhibited blurred edges and structural disintegration, while AB-MI-NK_{TAMRA}-treated A549 MTS maintained well-defined borders as well as a compact architecture (Figure 3D). The enzyme cascade reaction of the barrier penetration module efficiently generated substantial oxygen to enhance the tissue penetration capacity of MI-NK cells, which loosened the MTS structure and delivered NK cells through dense tumor barriers to reach the central region.

In Vitro Activity-Boosting Capability and Efficiency-Reporting Capability Characterizations of Multimodular Intelligent NK Cell

To visually validate the capability of the activity-boosting module of MI-NK cells, GSH-responsive IL-21 release was measured at the cellular level. MI-NK_{TAMRA/FAM} cells were prepared with TAMRA-labeled DNA scaffold_{TAMRA} and FAM-labeled IL-NPs-DNA_{FAM}, which showed good overlap of TAMRA and FAM fluorescence on the cell membrane (Figure 4A, MI-NK_{TAMRA/FAM}). GSH treatment led to significant attenuation of FAM fluorescence from IL-NPs-DNA_{FAM} while preserving TAMRA fluorescence from the DNA scaffold on the cell membrane (Figure 4A, w/GSH), indicating efficient GSH-triggered IL-NPs-DNA dissociation from DNA scaffolds and IL-21 release into the extracellular environment. In the absence of GSH treatment, TAMRA and FAM fluorescence exhibited good overlap throughout the 24-h incubation (Figure 4A, w/o GSH), indicating that the activity-boosting module IL-NPs-DNA maintained structural integrity in its normal positions.

The contribution of IL-21 release to NK cell activity boosting was then evaluated by investigating *in vitro* NK cell expansion using a carboxyfluorescein succinimidyl ester (CFSE) assay over 48 h. The cells were stained with CFSE, cultured for 48 h, and analyzed by flow cytometry (Figure S34). The dilution percentage of CFSE fluorescence was used to evaluate NK cell proliferation. The expansion of untreated NK cells served as the control. Both the NK_{DNA scaffold} group and the MI-NK cell group in the absence of GSH treatment showed no significant difference in proliferation compared with the untreated NK group, confirming that DNA scaffold encapsulation did not impair proliferative capacity. GSH-treated MI-NK cells exhibited a 2.24-fold increase in cell proliferation compared with untreated NK cells (Figure 4B). These results confirmed the capability of the activity boosting module to enhance NK cell proliferation in the tumor microenvironment. Furthermore, different sizes of IL-NPs were prepared with varying reaction ratios of NHS-SS-NHS and IL-21, were conjugated to MI-NK cells, and proliferation was evaluated using the CFSE assay. Cell proliferation was enhanced as IL-21 NP particle size decreased, which was probably due to the higher loading capacity and more efficient GSH-responsive IL-21 release for smaller-sized IL-NP-loaded NK cells. The highest proliferation of NK cells was achieved with 34.2 nm IL-NPs (Figure S35). Cytokine secretion corresponding to immune activation was further evaluated. Untreated NK cells, MI-NK cells, and MI-NK cells with GSH treatment were cultured for 24 h, and the levels of interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α), and granzyme B (GzmB) were measured in the supernatant using an ELISA kit and quantified based on corresponding standard curves (Figure S36). Due to IL-21 release upon GSH treatment, the MI-NK cell group showed 5.4-fold higher GzmB, 3.4-fold higher IFN- γ , and 4.3-fold higher TNF- α secretion compared with the untreated NK cell group (Figure 4C, MI-NK + GSH).

GzmB is an essential cytotoxic granule component whose release directly correlates with NK-cell cytotoxic potency, making it a quantitative functional marker of NK activity.⁴⁵ Therefore, GzmB-responsive fluorescence recovery from the efficiency reporting module reflected NK cell activity in real time (Figure 4D). MI-NK_{FAM} with a FAM-labeled DNA scaffold_{FAM} was challenged with GzmB and showed efficient hCy fluorescence recovery on the cell membrane corresponding to the GzmB concentration (Figure S37). The recovered hCy fluorescence intensity was ratioed to the internal standard dye FAM intensity and compared with that of hCy_{on}-MI-NK_{FAM} which had “always-on” hCy fluorescence, to quantify the extent of hCy fluorescence recovery (Figure S38A, Figure 4E, hCy_{on}-MI-NK_{FAM}). MI-NK_{FAM} was then cocultured with A549 tumor cells in the presence of GSH and showed 62.1% hCy fluorescence recovery after 6 h of incubation (Figure 4E, Figure S38B, MI-NK_{FAM} A549+GSH), indicating good activity of MI-NK cells. On the contrary, limited hCy fluorescence was observed from MI-NK_{FAM} cocultured with A549 cells in the absence of GSH treatment, with 41.4% hCy fluorescence recovery (Figure 4E, Figure S38B, MI-NK_{FAM} A549), and low hCy fluorescence was observed from MI-NK_{FAM} cells alone, with 6.44% hCy fluorescence recovery (Figure 4E, Figure S38B, MI-NK_{FAM}). GzmB generation in all three groups was further confirmed by an ELISA kit, which demonstrated a tendency similar to that of hCy fluorescence intensity recovery (Figure S38C). These results confirmed the capability of the efficiency reporting module for *in situ* reporting of NK cell function.

In Vitro Cancer Cell-Killing Capability Characterization of Multimodular Intelligent NK Cell

To evaluate the effects of module assembly on NK-cell viability, NK cells, NK_{DNA scaffold} cell, MI-NK cells, and MI-NK cells treated with Glu and GSH were cultured in medium for 24 h and subjected to CCK-8 cytotoxicity assays, all of which showed cell viability above 86% (Figure S39), indicating that neither DNA scaffold encapsulation nor the assembly of the three functional modules impaired NK-cell viability.

CD16 mediates target recognition and immune synapse formation, and its extracellular domain is highly sensitive to potential steric hindrance;⁴⁶ therefore, it was chosen as a representative cell membrane protein to evaluate whether the coating process affects the accessibility of key membrane receptors. NK cells, anchor-DNA-modified NK cell (NK_{anchor-DNA}), NK_{DNA scaffold}, and MI-NK cells were stained with anti-CD16_{FITC}, which demonstrated slight decreases in membrane FITC fluorescence (4.6% for NK_{anchor-DNA}, 8.2% for NK_{DNA scaffold}, and 9.4% for the respective groups) compared with that of NK cells (Figure S40), indicating that membrane proteins remained largely exposed on the cell membrane and retained its antibody recognition capability. To verify whether the assembly of functional modules affects the interaction between NK cells and tumor cells, an effector-target (E:T) engagement assay was performed by using flow cytometry. In this assay, NK cells were labeled with DiD and A549 cells were labeled with CFSE, and NK-tumor conjugates were identified as DiD⁺/CFSE⁺ double-positive events (Figure S41A). The results showed that the percentage of NK-tumor cell conjugates in the MI-NK group was slightly reduced by approximately 8.56% compared to the NK group (Figure S41B). Taking the AKT pathway, which is a well-established central regulator of NK cell activation and effector functions, as

a representative intracellular signaling pathway, the effect of membrane coating on intracellular signaling was also analyzed by Western blot. Total AKT expression levels were comparable across all groups (Figure S42, AKT), indicating that the coating process did not affect the overall expression of key signaling proteins. p-AKT levels were low in both unactivated NK and MI-NK cells, with relative p-AKT/AKT ratios of 0.27 and 0.30, respectively (Figure S42, p-AKT, G1, G3), while IL-2 stimulation showed similar increases in p-AKT levels for NK cells and MI-NK cells, with relative p-AKT/AKT ratios of 0.69 and 0.64, respectively (Figure S42, p-AKT, G2, G4), indicating that the AKT signaling pathway remained functionally responsive after the membrane coating process. These results further confirm that membrane modification preserved the functional integrity of NK cells at the signaling level.

To evaluate the cancer cell-killing capability and to indicate the contributions of different modules to MI-NK cells, A549 cells were cocultured with MI-NK cells, MI-NK cells in the presence of GSH, MI-NK cells in the presence of Glu, and MI-NK cells in the presence of GSH and Glu, and their apoptosis was verified via flow cytometry. Coculturing with MI-NK cells for 4 h resulted in A549 apoptosis of approximately 22.0% (Figure 4F, MI-NK), and coculturing with MI-NK cells in the presence of GSH, in the presence of Glu, and in the presence of GSH and Glu enhanced A549 cell apoptosis to 35.5%, 41.0%, and 55.8%, respectively (Figure 4F, MI-NK_{GSH}, MI-NK_{Glu}, MI-NK_{GSH+Glu}), indicating both the barrier penetration module and the activity boosting module effectively enhanced NK cell function. In addition, a DNA scaffold was constructed on B cells, which could not secrete GzmB, and assembled with three modules to prepare a multimodular intelligent B cell (MI-B), which barely generated A549 cell apoptosis (Figure 4F, MI-B_{GSH+Glu}). This result indicated that the multiple modules functioned through NK cells and lacked tumor-killing activity on their own. To demonstrate the contribution of DNA-scaffold encapsulation, DA-MI-NK cells were prepared by directly anchoring three modules to NK cells via a cholesterol functional group, and incubated with A549 cells in the presence of GSH and Glu, which caused cell apoptosis of 44.1% due to modules falling off or internalization during the incubation period (Figure 4F, DA-MI-NK_{GSH+Glu}).

The tumor cell-killing capability of MI-NK cells was further confirmed by performing Calcein-AM/PI dual staining after 6 h of incubation, which demonstrated a similar trend of gradually increased A549 dead-cell ratios of 9.4%, 28.1%, 21.8%, and 65.5%, respectively, by incubating with MI-NK cells, MI-NK cells in the presence of GSH, Glu, and in the presence of GSH and Glu (Figure 4G, MI-NK, MI-NK_{GSH}, MI-NK_{Glu}, MI-NK_{GSH+Glu}; Figure S43A, MI-NK, MI-NK_{GSH}, MI-NK_{Glu}, MI-NK_{GSH+Glu}). A CCK-8 assay was also performed after 12 h of co-incubation of A549 cells with various functionalized NK cells (Figure S43B). The killing capability of MI-NK cells (48.5% A549 cell viability) (Figure S43B, MI-NK) was comparable to that of NK cells (43.3%) (Figure S43B, NK), indicating that DNA scaffolding did not markedly impair the intrinsic cytotoxicity of NK cells. Though coculturing with GSH and Glu contributed little to NK-cell killing capability (Figure S43B, NK_{GSH+Glu}), it substantially reduced A549 cell viability to 7.4% (Figure S43B, MI-NK_{GSH+Glu}) due to the contribution of the barrier penetration module and the activity boosting module. MI-B cells, which lack GzmB secretion, resulted in a very low A549 dead-cell ratio of 1.8% in the presence of GSH and Glu after 6 h of

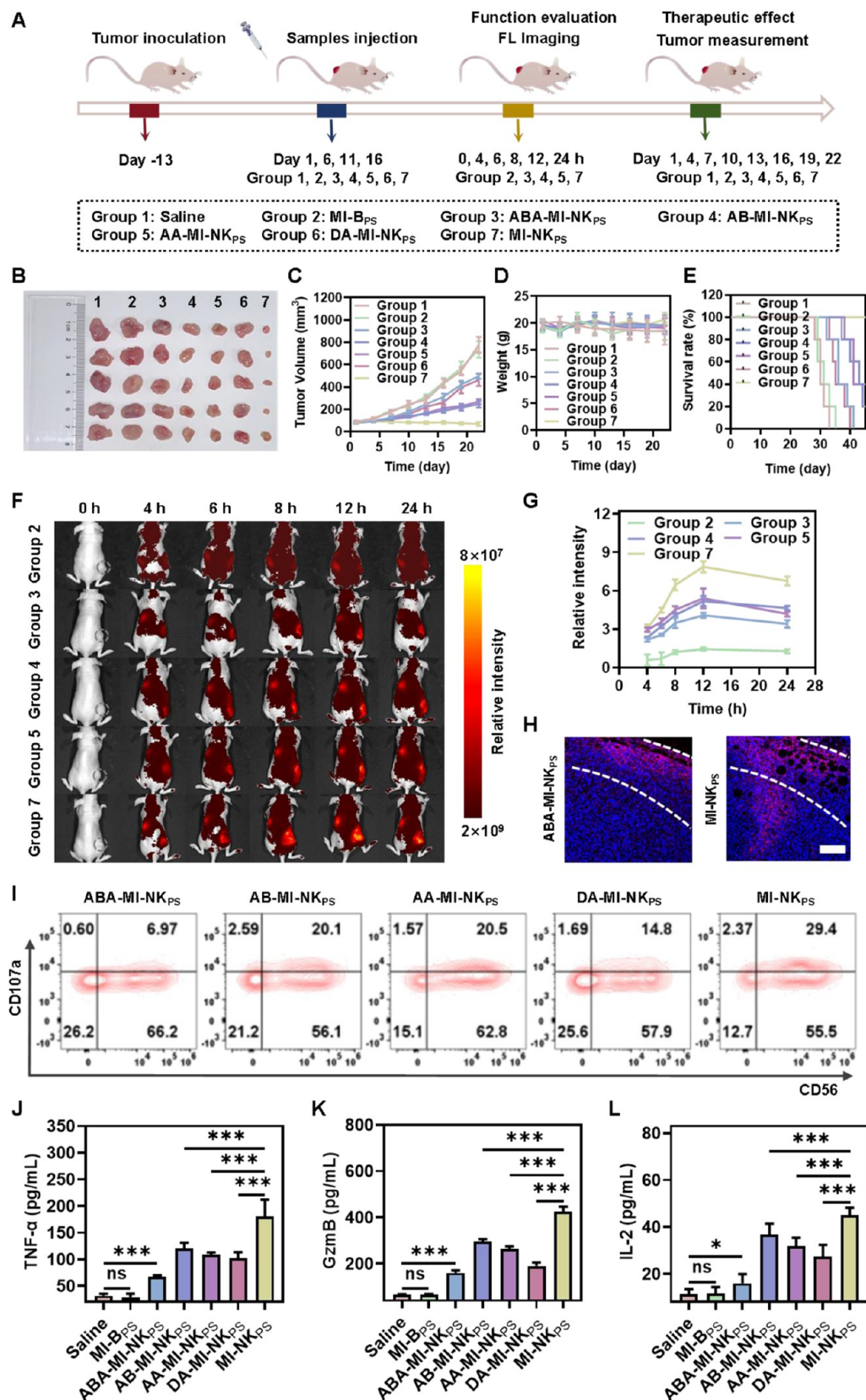


Figure 5. Real-time efficiency monitoring and antitumor efficacy of multimodule intelligent NK cells *in vivo*. (A) Schematic illustration of adoptive MI-NK cell treatment and its therapeutic efficiency evaluation in subcutaneous A549 tumor mouse models. (B) Photographs of tumor tissues on day 22, (C) tumor growth curves in mice, (D) weight curves, and (E) survival curves of tumor-bearing mice receiving different treatments with saline, MI-B_{PS}, ABA-MI-NK_{PS}, AB-MI-NK_{PS}, AA-MI-NK_{PS}, DA-MI-NK_{PS}, and MI-NK_{PS} respectively; data in (C–D) are presented as mean ± SD (*n* = 5 independent samples). (F) *In vivo* hCy fluorescence recovery images and (G) corresponding intensities at the tumor site for mouse groups that were treated with MI-B_{PS}, ABA-MI-NK_{PS}, AB-MI-NK_{PS}, AA-MI-NK_{PS} and MI-NK_{PS} respectively; data

Figure 5. continued

in (G) are presented as mean $\pm$ SD ($n = 3$ independent samples). (H) hCy fluorescence in tumor sections for ABA-MI-NK_{PS} and MI-NK_{PS} administrated mice group, respectively; scale bar: 50 μ m. (I) Flow cytometric analysis of adoptive NK cell activity, and secretion of (J) TNF- α , (K) GzmB, and (L) IL-2 from tumor-bearing mice receiving different treatments with saline, MI-B_{PS}, ABA-MI-NK_{PS}, AB-MI-NK_{PS}, AA-MI-NK_{PS}, DA-MI-NK_{PS} and MI-NK_{PS} respectively; data in (J-L) are presented as mean $\pm$ SD ($n = 5$ independent samples), and $P < 0.05$ was considered statistically significant; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; ns, not significant.

incubation (Figure 4G, MI-B_{GSH+Glu}; Figure S43A, MI-B_{GSH+Glu}), and they barely suppressed A549 cell viability after 12 h of incubation (Figure S43B, MI-B_{GSH+Glu}). DA-MI-NK cells resulted in an A549 dead-cell ratio of 31.3% in the presence of GSH and Glu after 6 h of incubation (Figure 4G, DA-MI-NK_{GSH+Glu}; Figure S43A, DA-MI-NK_{GSH+Glu}) and suppressed A549 cell viability to 33.4% after 12 h of incubation (Figure S43B, DA-MI-NK_{GSH+Glu}). These results indicated that the barrier-penetration module and the activity boosting module could effectively enhance the antitumor capacity of engineered NK cells, guaranteeing their *in vivo* application potential.

In Vivo Application of Multimodular Intelligent NK Cell with Enhanced Immunotherapy

A subcutaneous A549 lung cancer model was established in BALB/c nude mice for *in vivo* evaluation of the immunotherapeutic effect of MI-NK cells. Phosphorothioate-modified DNA strands were used as components to synthesize MI-NK cells (MI-NK_{PS}) to avoid DNA scaffold degradation in a complex *in vivo* environment. To verify the tumor accumulation of adoptive NK cells, NK cells were stained with 4-chlorobenzenesulfonate salt (DiD), a far-red plasma membrane fluorescent probe, for *in vivo* visualization. To avoid interference of hCy fluorescence with cell-membrane DiD fluorescence, AE-MI-NK_{PS/DiD} and ABE-MI-NK_{PS/DiD} lacking the efficiency-reporting module IEPD-hCy-DNA were prepared as comparison groups. The three cell groups were intravenously administered to the tumor-bearing mice. DiD fluorescence started to appear at the tumor site from 0.5 h post-administration in all three groups and gradually increased during the circulation period, demonstrating comparable fluorescence intensities at 6 h post-administration and indicating the intrinsic tumor-homing capability of NK cells. At 12 and 24 h postinjection, AE-MI-NK_{PS/DiD} exhibited slightly stronger fluorescence at the tumor site than ABE-MI-NK_{PS/DiD} and NK_{DiD} due to the enhanced tumor infiltration and intratumoral retention of MI-NK_{PS} containing the barrier-penetration module (Figure S44). Additionally, *ex vivo* fluorescence imaging of major organs was performed at 12 h postinjection, which demonstrated a similar tendency, with efficient adoptive cell accumulation at the tumor site for all three cell groups and slightly higher DiD fluorescence in mice administered AE-MI-NK_{PS/DiD} (Figure S45).

To perform adoptive NK cell therapy, the A549 tumor-bearing mice were randomly divided into 7 groups and were intravenously administered with Group 1: saline; Group 2: MI-B_{PS} cells assembled with three modules onto DNA_{PS} scaffold-encapsulated B cells; Group 3: ABA-MI-NK_{PS} cells prepared in the absence of both the barrier-penetration module and the activity boosting module; Group 4: AB-MI-NK_{PS} cells prepared in the absence of the barrier penetration module; Group 5: AA-MI-NK_{PS} cells prepared in the absence of the activity boosting module; Group 6: DA-MI-NK_{PS} cells prepared by directly anchoring three modules via cholesterol

insertion into the membrane; and Group 7: MI-NK_{PS} cells prepared by assembling three modules onto DNA_{PS} scaffold-encapsulated NK cells on the first, sixth, 11th, and 16th days. The tumor size and body weight of all mouse groups were monitored every 3 days until day 22. The hCy fluorescence recovery in Group 2: MI-B_{PS} cells, Group 3: ABA-MI-NK_{PS} cells, Group 4: AB-MI-NK_{PS} cells, Group 5: AA-MI-NK_{PS} cells, and Group 7: MI-NK_{PS} cells was recorded at 4, 6, 8, 12, and 24 h after intravenous administration of adoptive NK cells for therapeutic effect evaluation (Figure 5A). The *in vivo* therapeutic effect of noncoated native NK cells was also evaluated, and they demonstrated limited tumor inhibition (Figure S46).

Compared with saline-administered, tumor-bearing mice, ABA-MI-NK_{PS} showed little inhibition of tumor growth (Figure 5B, C, groups 1 and 3) due to the absence of both the barrier penetration module and the activity boosting module. The presence of the barrier penetration module or the activity boosting module enhanced the therapeutic effect of adoptive NK cells and resulted in $\sim 68.5\%$ and $\sim 65.6\%$ tumor-volume suppression, respectively (Figure 5B,C, groups 4 and 5). MI-NK_{PS} cells demonstrated the best antitumor effect with $\sim 91.2\%$ tumor-volume suppression (Figure 5B, C, group 7), indicating efficient therapeutic enhancement from the combination of the barrier penetration and activity-boosting modules. On the contrary, MI-B_{PS} demonstrated little therapeutic effect, with only 5.3% tumor-size suppression due to its inability to secrete GzmB (Figure 5B,C, group 2). DA-MI-NK_{PS} demonstrated similar tumor-growth suppression to ABA-MI-NK_{PS} due to the modules dropping off during the delivery process (Figure 5B,C, group 6). H&E staining and TUNEL experiments demonstrated similar trends across the seven mouse groups; ABA-MI-NK_{PS}, DA-MI-NK_{PS} administrated mice groups demonstrated some extent of tumor-cell apoptosis, while obvious necrotic cell death and green fluorescence indicating DNA fragmentation were observed for AB-MI-NK_{PS}, AA-MI-NK_{PS}, and MI-NK_{PS} administrated mice groups, and MI-NK_{PS} administrated group demonstrating the highest extent (Figures S47 and S48). To further evaluate the off-target cytotoxicity of MI-NK_{PS} at the cellular level, AML-12 mouse hepatocytes were chosen as a normal cell model and cocultured with MI-NK cells for 12 h both in the absence and presence of GSH and glucose, which remained at 97.6%, and 91.2% viability compared with AML-12 cells cultured alone (Figure S49), indicating very low off-target cytotoxicity of MI-NK cells toward normal cells. Body weights remained stable for all groups throughout the therapeutic process (Figure 5D). Consistent with the above results, the group 7 mice that were administered MI-NK_{PS} cells showed apparently prolonged survival compared with the other groups (Figure 5E). H&E-stained histopathological analysis of major organs (heart, liver, spleen, lung, and kidney) harvested from mice on Day 22 from all treatment groups exhibited minimal lesions (Figure S50), indicating satisfactory biocompatibility of MI-NK_{PS} cells.

In vivo hCy fluorescence recovery was recorded for early reporting of therapeutic efficacy. Group 2: MI-B_{PS}, Group 3: ABA-MI-NK_{PS} cell, Group 4: AB-MI-NK_{PS} cell, Group 5: AA-MI-NK_{PS} cell, and Group 7: MI-NK_{PS} cell-administrated mice all showed a time-dependent increase in hCy fluorescence as early as 4 h post adoptive NK cell administration, which gradually increased over time and reached maximum intensity at 12 h post-administration (Figure 5F,G, groups 3, 4, 5, 7). The strongest hCy fluorescence recovery was observed for Group 7 MI-NK_{PS} cell-administrated mice (Figure 5F,G, group 7). Group 2 MI-B_{PS} cells barely showed *in vivo* hCy fluorescence recovery during the experimental period due to the inability of B cells to secrete GzmB (Figure 5F,G, group 2). The *ex vivo* organ imaging also showed a similar tendency; ABA-MI-NK_{PS}, AB-MI-NK_{PS}, AA-MI-NK_{PS}, and MI-NK_{PS} groups of mice all demonstrated hCy fluorescence recovery, and the strongest hCy fluorescence recovery was observed in the MI-NK_{PS} cell-administered mice (Figure S51). The tendency of recovered hCy fluorescence intensities for MI-B_{PS} cell-, ABA-MI-NK_{PS} cell-, AB-MI-NK_{PS} cell-, AA-MI-NK_{PS} cell-, and MI-NK_{PS} cell-treated mice groups was similar to their therapeutic effects measured by tumor size changes (Figure 5C), indicating the capability of hCy efficiency-reporting module for early reporting of therapeutic effect. *In vivo* hCy fluorescence recovery monitoring indicated an intensity difference as early as 6 h after adoptive cell administration, while tumor size measurement started to show a difference on the 10th day of treatment. These results confirmed the feasibility of the efficiency-reporting module for early therapeutic effect reporting of adoptive NK cells. The hCy fluorescence was further imaged in tumor tissue sections to show the tumor-site penetration of the adoptive NK cells. The MI-NK_{PS} cell-administered mice group showed more efficient tumor-site penetration, with stronger hCy fluorescence intensity observed at both the edge and center of the tumor tissue. On the contrary, the ABA-MI-NK_{PS} cell-administrated mice group only showed hCy fluorescence at the edge of the tumor tissue (Figure 5H). Quantitative analysis showed about 1.76-fold higher hCy fluorescence for the MI-NK_{PS} group compared with the ABA-MI-NK_{PS} group (Figure S52), confirming the enhanced tumor infiltration and penetration of MI-NK cells.

To compare the immune activation of adoptive NK cells, the tumors were digested for cell collection on the sixth day, 12 h after adoptive NK cell administration. CD45 is a pan-leukocyte antigen widely used in flow cytometry to identify immune cells and to separate them from tumor or stromal cells in mixed samples.⁴⁷ CD56 is a canonical surface marker of human NK cells.⁴⁸ CD107a is a well-established functional marker of NK cell degranulation, and its surface up-regulation correlates tightly with NK cytotoxic activity.^{49,50} Therefore, the collected cells from mice were labeled with anti-CD45-FITC, anti-CD56-APC, and anti-CD107a-PE, respectively, and were sorted via flow cytometry (Figure S53). Compared with the saline treated mouse group, the MI-B_{PS} cell-, AB-MI-NK_{PS} cell-, AA-MI-NK_{PS} cell-, and MI-NK_{PS} cell-treated mouse groups all showed an increase in human cells in tumors. The proportion of human CD45⁺ cells in tumor tissues increased, with values of 2.08% for the AB-MI-NK_{PS} cell-treated group and 1.21% for the ABA-MI-NK_{PS} cell-treated group to 3.17% for the MI-NK_{PS} cell-treated group, indicating the highest level of tumor infiltration from MI-NK_{PS} cells (Figure S54, ABA-MI-NK_{PS}, AB-MI-NK_{PS} and MI-NK_{PS}). To further compare

adoptive NK cell activation efficiency, the proportion of CD56⁺ CD107a⁺ for different mouse groups was evaluated, showing 20.1% and 20.5% for AB-MI-NK_{PS} cell and AA-MI-NK_{PS} cell-treated groups (Figure S1, AB-MI-NK_{PS} and AA-MI-NK_{PS}), and reaching a maximum level of 29.4% for the MI-NK_{PS} cell-treated group (Figure S1, MI-NK_{PS}). These results indicated that MI-NK_{PS} cells exhibited superior efficacy and greater potential in tumor infiltration and immune activation, which led to enhanced antitumor immunity. ABA-MI-NK_{PS} cells and DA-MI-NK_{PS} cells showed less efficient tumor infiltration (Figure S54, ABA-MI-NK_{PS} and DA-MI-NK_{PS}) and NK cell activation (Figure S1, ABA-MI-NK_{PS} and DA-MI-NK_{PS}) due to of 6.97% and 14.8%. The extent of NK cell activation was further confirmed by measuring the secretion of immune-activation-related components GzmB, the cytotoxic lymphokine TNF- α , and the inflammatory cytokine IL-2 in the tumor environment post-treatment using ELISAs for different mouse groups and quantifying based on corresponding standard curves (Figures S36, S55). AB-MI-NK_{PS}, AA-MI-NK_{PS}, and MI-NK_{PS} all demonstrated enhanced secretion of TNF- α , GzmB, and IL-2 compared with the saline- and MI-B_{PS} administrated mice group, and MI-NK_{PS} administrated mice group showed the highest secretion levels, which were 5.7-fold for TNF- α (Figure S1, MI-NK_{PS}), 6.6-fold for GzmB (Figure S1, MI-NK_{PS}), and 4.1-fold for IL-2 (Figure S1, MI-NK_{PS}) compared with the saline-administered mouse group. ABA-MI-NK_{PS} cells and DA-MI-NK_{PS} cells showed lower levels of TNF- α , GzmB, and IL-2 secretion due to inadequate infiltration into tumor sites and insufficient immune activation (Figure S1–L, ABA-MI-NK_{PS}, MI-NK_{PS}), while MI-B_{PS} cells showed little difference compared with the saline-administered mouse group (Figure S1–L, MI-B_{PS}). The trends of cytokine secretion levels for ABA-MI-NK_{PS}, AB-MI-NK_{PS}, AA-MI-NK_{PS} and MI-NK_{PS} treated mouse groups were consistent with the *in vivo* hCy fluorescence recovery intensity and with the extent of tumor growth suppression. These results further confirmed the feasibility of the efficiency reporting module for *in vivo* reporting of NK cell functions and therapeutic efficacy.

CONCLUSIONS

To summarize, we report a multimodule intelligent NK cell (MI-NK) platform that enables simultaneous functional enhancement and early therapeutic efficacy reporting for adoptive cell therapy (ACT). Conventional functional ACT strategies typically rely on single-task modules that are inserted directly into the cell membrane or covalently bound to membrane receptors. These approaches are often limited by module detachment or endocytosis due to membrane dynamics. In contrast, our strategy uses a DNA scaffold providing abundant and stable attachment sites to anchor multiple functional modules on NK cells. The engineered MI-NK cells were equipped with a barrier penetration module, an activity boosting module, and a real-time efficacy-reporting module. The barrier-penetration module, consisting of GOx and CAT, promotes deep tumor infiltration by generating oxygen through a cascade of enzymatic reactions using tumor-abundant glucose and H₂O₂. The activity-boosting module releases IL-21 in response to elevated GSH levels to enhance NK cell activation. Meanwhile, the efficacy-reporting module enables early readout of cytotoxic activity by sensing granzyme B release and restoring hCy fluorescence. As demonstrated *in vivo*, MI-NK cells exhibited the highest levels of GzmB, IL-2, and TNF- α secretion and achieved superior therapeutic

efficacy compared with all control groups lacking individual modules or using direct membrane insertion strategies. Notably, hCy fluorescence recovery enabled therapeutic efficacy reporting as early as 6 h postadministration, preceding detectable tumor volume differences by several days. While low hCy fluorescence indicates insufficient NK cell activation, a personalized treatment strategy, such as additional adjuvant administration, could be employed to boost immune activity. In addition, the DNA-scaffold-mediated assembly allows flexible control over the type, proportion, and programming style of functional modules. Intermittent or reversible immune activation modes that are regulated by external stimuli, such as light or heat, can also be achieved for personalized treatment tailored to individual patient profiles. Overall, this DNA-scaffolded strategy provides a versatile and expandable platform for adoptive cell functionalization, offering opportunities to integrate additional programmable modules for enhanced immunotherapy and precision treatment optimization.

EXPERIMENTAL SECTION

Materials

Acrylamide (Acr), ammonium persulfate (APS), and N,N,N',N'-Tetramethylethylenediamine (TEMED) were purchased from Sigma-Aldrich (USA). Biomedical-grade dimethyl sulfoxide (DMSO), N-hydroxysuccinimide-SS-N-hydroxysuccinimide (NHS-SS-NHS), N-hydroxysuccinimide-PEG4-maleimide (NHS-PEG₄-Mal) and 5-(and-6)-Carboxytetramethylrhodamine N-hydroxysuccinimide ester (TAMRA-NHS) were purchased from Xi'an Ruixi Biotechnology Co., Ltd. (Xi'an, China). Human interleukin-21 (IL-21) was purchased from Nanjing Gonglu Biotechnology Co., Ltd. (Nanjing, China). Human Granzyme B, glucose oxidase, catalase, and Glu were purchased from Shanghai Haoyuan Biochemical Technology Co., Ltd. (Shanghai, China). Ac-Ile-Glu-Pro-Asp was purchased from Nanjing Taigu Biotechnology Co., Ltd. (Nanjing, China). Phenol red-free DMEM medium was purchased from Nantong Feiyu Biotechnology Co., Ltd. (Nantong, China). Phosphate buffer solution (PBS), trypsin-EDTA solution, fetal bovine serum, RPMI 1640 medium, Calcein-AM/PI apoptosis detection kit, Annexin V-FITC/PI apoptosis detection kit, CCK-8 cell proliferation and cytotoxicity detection kit, interferon- γ (IFN- γ), Granzyme B (GzmB), Interleukin-2 (IL-2) and tumor necrosis factor- α (TNF- α) ELISA kits, Carboxyfluorescein succinimidyl ester (CFSE), 3,3',5,5'-Tetramethylbenzidine (TMB) chromogenic substrate, and the human lung adenocarcinoma cell line A549 were purchased from Jiangsu KeygenBioTECH Co., Ltd. (Nanjing, China). Human interleukin-2 (IL-2), AKT antibody, and Phospho-AKT (Ser473) antibody were purchased from MedChemExpress (Monmouth Junction, NJ, USA). Transwell cell culture inserts and specially customized double-arrow fluidic channels were purchased from Nanjing Weiwo Biotechnology Co., Ltd. (Nanjing, China). The DiD cell membrane far-red fluorescent probe was purchased from Shanghai Beyotime Biotechnology

Co., Ltd. (Shanghai, China). Human natural killer cell line NK-92MI was purchased from Wuhan Procell Life Technology Co., Ltd. (Wuhan, China). Normal B cells were obtained from IASO Biotherapeutics and used immediately upon receipt. The antihuman CD16-FITC antibody (anti-CD16_{FITC}) was purchased from Biosin Biotechnology Co., Ltd. (Beijing, China). Horseradish peroxidase (HRP), methyl orange, antihuman CD45-FITC antibody, and antihuman CD56-APC antibody were purchased from Aladdin Biochemical Co., Ltd. (Shanghai, China). The antihuman CD107a-PE antibody was purchased from Absin Biochemical Co., Ltd. (Shanghai, China). All DNA strands were synthesized and purified by Sangon Biotech (Shanghai, China). The sequences for all DNA strands are listed in [Supplementary Table 1](#).

Apparatus

A JEM-2100 transmission electron microscope (JEOL, Japan) was used to acquire transmission electron microscope (TEM) images, while a JSM-7800F thermal field emission scanning electron microscope (JEOL, Japan) was employed to obtain scanning electron microscope (SEM) images. A SCIENTZ-IIID ultrasonic homogenizer (Ningbo, China) was utilized for ultrasonic dispersion. A Nano-Z zeta potential analyzer (Malvern Panalytical, UK) was used for zeta potential determination, and a ZetaPlus 90 Plus/BI-MAS (Brookhaven, USA) was employed for dynamic light scattering (DLS) analysis. Polyacrylamide gel electrophoresis (PAGE) experiments were conducted using a PowerPac Basic electrophoresis apparatus (Bio-Rad, USA), and DNA gel images were captured with a ChemDoc XRS gel imaging system (Bio-Rad, USA). Fluorescence spectra in the visible (Vis) region were collected using a FluoroMax-4 fluorescence spectrophotometer (Hitachi, Japan). A PL-GPC 50 Integrated GPC System (Agilent Technologies, USA) was used to acquire gel permeation chromatography (GPC) data. Cellular confocal imaging was performed on a TCS SP8 STED 3X confocal microscope (Leica, Germany). A Countess II FL automated cell counter (Thermo Fisher Scientific, USA) was used for cell counting. A microplate reader (Biotek CBM, USA) was used for CCK-8 cell viability assays and for detecting ELISA results. Flow cytometric analysis was carried out on a CytoFlex S flow cytometer (Beckman Coulter, USA). The oxygen content in solutions was measured using a portable dissolved oxygen meter (Aikeshi, China). The motility of NK cell motors was recorded with an inverted microscope (Leica DMI 3000B, Germany), and *in vivo* mouse imaging was performed on an IVIS Lumina XR III *in vivo* imaging system (PerkinElmer, USA) and an NIROPTICS/DaVinci YR-1000 near-infrared region II *in vivo* analyzer (Suzhou Yingrui Optics Technology Co., Ltd., China).

Preparation of Poly-DNAs

Poly-DNAs were prepared by copolymerization of acrylamide-modified DNA strands. Acrylamide-modified DNA strands—com-DNAs (100 μ M, 80 μ L) and linker-DNAs (100 μ M, 80 μ L)—were mixed at a 1:1 molar ratio and diluted with Tris-HCl buffer (10 mM, pH 8.0) to a final volume of 800 μ L. Subsequently, 160 μ L of 10% acrylamide was added. Different strand sequences were designed for linker-DNA_{GOx}, linker-DNA_{CAT}, linker-DNA_{IL}, linker-DNA_{Rep} respectively to prepare poly-DNA_{GOx}, poly-DNA_{CAT}, poly-DNA_{IL}, and poly-DNA_{Rep}. The mixture was bubbled with nitrogen in a 1.5 mL microcentrifuge tube and subsequently added with 10 μ L of 10% APS solution and 5 μ L TEMED to initiate polymerization. After polymerization at room temperature for 5 min, the mixture was transferred to a 4 $^{\circ}$ C refrigerator for further polymerization for 12 h. The resulting copolymers were purified by ultrafiltration using an ultrafiltration tube (30 kDa MWCO; Millipore) at 10,000 rpm for 5 min, and the as-obtained concentrate was washed three times with PBS to remove unreacted monomer units, salts, and initiators. The purified poly-DNAs were then dissolved in 800 μ L PBS for subsequent use. The concentration of poly-DNAs was set to 4 μ M.

Critical Micelle Concentration (CMC) Measurement

The CMCs of anchor-DNA and poly-DNA were evaluated using methyl orange (MO) as a probe,^{51,52} with poly-DNA_{GOx} selected as representative of poly-DNA strands. Briefly, 20 μ M MO was added to a series of DNA solutions with concentrations ranging from 0 to 5 μ M. The absorbance was recorded using a UV-vis spectrophotometer. The CMC was assessed based on the presence or absence of an inflection point in the absorbance-concentration curve.

Preparation of Barrier Penetration Module DNA-Modified Enzymes

The barrier-penetration module consisted of DNA-modified glucose oxidase (GOx-DNA) and DNA-modified catalase (CAT-DNA). Thiol group-functionalized DNA_{GOx} with the complementary sequence to linker DNA_{GOx} was conjugated to the amine groups of GOx via the linker molecule NHS-PEG₄-Mal. DNA_{GOx} (100 μ M, 25 μ L) was mixed with TCEP solution (5 mM) at room temperature, incubated

on a shaker for 30 min, and then diluted to 10 μM in TE buffer (50 μL , pH 8.0). The as-prepared DNA_{GOx} was mixed with glucose oxidase (10 μM , 50 μL , PBS) and the linker molecule, NHS-PEG₄-Mal (1 mg/mL, 1 μL , DMF). The resulting mixture was adjusted to a final volume of 1 mL with HEPES buffer (10 mM, pH 7.4, 150 mM NaCl) and vigorously shaken at room temperature overnight. The resulting mixture was ultrafiltered at 10,000 rpm for 5 min using a 30 kDa MWCO filter (Millipore) and washed three times with PBS, followed by reconstitution to 1 mL to obtain DNA_{GOx} modified Glucose Oxidase (GOx-DNA). The control group was performed according to the same procedure in the absence of linker molecule NHS-PEG₄-Mal. CAT-DNA was prepared by conjugating DNA_{CAT} that has the complementary sequence to linker DNA_{CAT} to catalase according to the same procedure.

Preparation of Activity-Boosting Module DNA-Modified IL-21 Nanoparticles

IL-21 nanoparticles (IL-NPs) were prepared by the self-assembling IL-21 protein. IL-21 (0.1 mg/mL, 50 μL) was dissolved in a mixture of sterile water and 5% trehalose in PBS, mixed with the small-molecule cross-linker NHS-SS-NHS (0.1 mg/mL, 10 μL) dissolved in DMF, and adjusted to a final volume of 1 mL with PBS. The mixture was then shaken at room temperature for 30 min, subsequently ultrafiltered at 8000 rpm for 5 min using a 30 kDa MWCO filter (Millipore), and washed three times with PBS to obtain IL-NPs.

To monitor the conjugation efficiency of IL-NPs, TAMRA-labeled IL-21 (IL-21_{TAMRA}) was prepared by mixing NHS-TAMRA (1 μM) with IL-21 (5 $\mu\text{g/mL}$ in 0.1 M HEPES buffer, pH 7.4). After 1 h of gentle shaking at 25 $^{\circ}\text{C}$ in the dark, the reaction was quenched by adding 50 mM Tris-HCl (pH 8.0) for 15 min. The mixture was then ultrafiltered at 8000 rpm for 5 min using a 10 kDa MWCO filter (Millipore) and washed three times with PBS, followed by reconstitution to 1 mL to obtain IL-21_{TAMRA}. IL_{TAMRA}-NPs were subsequently prepared using the same procedure described above. The fluorescence intensities of 200 μL (5 $\mu\text{g/mL}$) IL-21_{TAMRA} and the ultrafiltered IL_{TAMRA}-NP solution were measured and compared to determine the conjugation efficiency of IL-NPs.

Amine group-functionalized DNA_{IL} that has a complementary sequence with linker-DNA_{IL} was conjugated to the above-prepared IL-NPs using the remaining NHS groups. DNA_{IL} (10 μM , 50 μL , TE buffer, pH 8.0) was mixed with IL-NPs (2 μM , 50 μL), shaken at room temperature for 30 min, then ultrafiltered at 8000 rpm for 5 min using a 30 kDa MWCO filter (Millipore), washed three times with PBS, and reconstituted to 1 mL to obtain DNA-modified IL-NPs (IL-NPs-DNA).

In Vitro Characterization of IL-NP-DNA Decomposition in Response to GSH

To monitor the GSH-responsive decomposition efficiency of IL-NPs-DNA, IL-21_{TAMRA} was prepared as described above. IL_{TAMRA}-NPs-DNA of different sizes were prepared by reacting NHS-SS-NHS with IL-21 at different ratios. The as-obtained IL_{TAMRA}-NPs-DNA were dispersed in 1 mL of PBS, placed in a dialysis bag (MWCO 35,000), and incubated in PBS containing 5 mM GSH for 12 h. 500 μL of the PBS outside the dialysis bag was collected to measure TAMRA fluorescence intensity at 580 nm. The hydrodynamic size of IL_{TAMRA}-NPs-DNA decomposed product left in the dialysis bag was measured after 12 h.

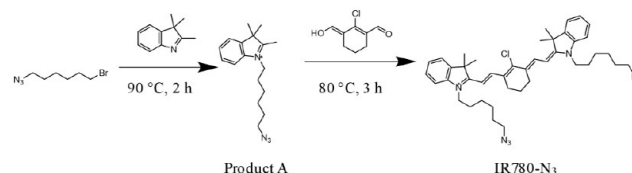
The long-term stability of IL_{TAMRA}-NPs-DNA in a complex system was assessed by incubating IL-NPs-DNA in RPMI 1640 medium supplemented with fetal bovine serum. The hydrodynamic size and ζ -potential of IL-NPs-DNA were measured using dynamic light scattering and ζ -potential analysis on days 0, 1, 2, 3, 4, 5, 6, and 7.

Synthesis of Amine/Azide-Functionalized Hemicyanine Dye NH₂-hCy-N₃

To synthesize IR780-N₃, as the precursor for NH₂-hCy-N₃, 2,3,3-trimethyl-3H-indole (3.0 mmol) and azidoethyl bromide (3.0 mmol) were placed in a 50 mL three-necked flask and the mixture was stirred at 90 $^{\circ}\text{C}$ for 2 h. After cooling, 40 mL of petroleum ether was added to induce precipitation of the product, and the precipitated material

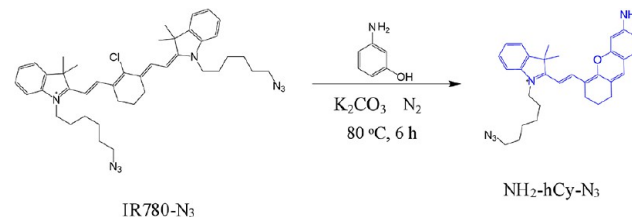
was collected by centrifugation at 3000 rpm for 3 min. The as-obtained precipitate was then dissolved in dichloromethane, and the solvent was removed by rotary evaporation in a round-bottom flask to obtain product A quantitatively. Product A (5.0 mmol), (E)-2-chloro-3-(hydroxymethylene)cyclohex-1-ene-1-carbaldehyde (2.5 mmol), and anhydrous sodium acetate (7.5 mmol) were refluxed in 25 mL of acetic anhydride at 80 $^{\circ}\text{C}$ for 3 h. After cooling, the reaction mixture was concentrated under reduced pressure, and the acetic anhydride was removed by rotary evaporation at 60–70 $^{\circ}\text{C}$ using an oil pump. Subsequently, 20 mL of distilled water was added, and the mixture was extracted with dichloromethane (20 mL) to obtain the crude product, which was purified by silica gel column chromatography and concentrated by rotary evaporation to yield IR780-N₃ as a uniform green solid (synthesis route of IR780-N₃, eq 1)

Synthesis route of IR780-N₃.



3-Aminophenol (0.4 mmol) and potassium carbonate (0.4 mmol) were vigorously stirred in CH₃CN (20 mL) under N₂ protection for 30 min, followed by addition of the above-obtained IR780-N₃ (5 mL, 0.1 mmol, dissolved in acetonitrile), and the mixture was stirred at 76 $^{\circ}\text{C}$ for an additional 6 h. The solvent was then removed under vacuum, and the residue was dissolved in dichloromethane and washed three times with H₂O. The as-obtained product was purified by column chromatography (dichloromethane:MeOH = 20:1), and the solvent was removed under reduced pressure to obtain the amine/azide functionalized hemicyanine dye NH₂-hCy-N₃ as a uniform blue solid (Synthesis route of NH₂-hCy-N₃, eq 2). After further

Synthesis route of NH₂-hCy-N₃.



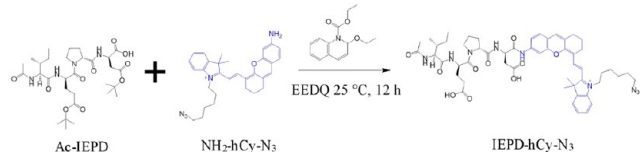
purification by HPLC, the product was obtained with a MW of 494.2962. ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.48 (d, *J* = 14.3 Hz, 1H), 7.74–7.68 (m, 2H), 7.54–7.44 (m, 3H), 7.35 (t, *J* = 7.2 Hz, 1H), 6.78 (dd, *J* = 8.5, 2.0 Hz, 1H), 6.71 (d, *J* = 2.0 Hz, 1H), 6.29 (d, *J* = 14.4 Hz, 1H), 4.26 (t, *J* = 7.4 Hz, 2H), 3.35 (t, *J* = 6.8 Hz, 2H), 2.75 (t, *J* = 6.0 Hz, 2H), 2.70 (t, *J* = 6.2 Hz, 2H), 1.89–1.83 (m, 2H), 1.81–1.77 (m, 2H), 1.75 (s, 6H), 1.56 (p, *J* = 6.8 Hz, 2H), 1.44 (dt, *J* = 9.6, 6.2 Hz, 4H), 1.35–1.25 (m, 2H).

Synthesis of Efficiency-Reporting Module IEPD-hCy-N₃

N-Acetyl-functionalized peptide Ac-Ile-Glu-Pro-Asp (Ac-IEPD) (0.02 mmol), as the substrate of Granzyme B, was dissolved in tetrahydrofuran (THF, 10 mL), to which previously obtained NH₂-hCy-N₃ (0.08 mmol) and N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) (0.08 mmol) were added, and the mixture was stirred at 25 $^{\circ}\text{C}$ for 12 h. Afterward, the solvent was removed by rotary evaporation, and the mixture was subjected to deprotection by reacting with 1 mL trifluoroacetic acid (TFA) and 2 mL dichloromethane at 25 $^{\circ}\text{C}$ for 90 min. Finally, the volatile solvents were removed by rotary evaporation, and the residue was purified by chromatographic separation to obtain the IEPD-hCy-N₃ as a

uniform blue solid (synthesis route of IEPD-hCy-N₃, eq 3) with a MW of 990.5091.

Synthesis route of IEPD-hCy-N₃.



Preparation of Efficiency-Reporting Module IEPD-hCy-DNA

The above-obtained IEPD-hCy-N₃ (50 μ L, 1 mM) was reacted with DBCO-functionalized DNA_{Rep} (50 μ L, 1 mM) in HEPES buffer (400 μ L) at room temperature on a shaker for 4 h. The reaction mixture was then purified by chromatographic separation to obtain IEPD-hCy-DNA.

In Vitro Validation of Efficiency-Reporting Module IEPD-hCy-DNA

To *in vitro* validate the NIR fluorescence of IEPD-hCy-DNA by Granzyme B (GzmB) cleavage *in vitro*, it was dissolved in PBS (50 μ M, 200 μ L) and treated with various concentrations of preactivated GzmB. The mixture was adjusted to a final volume of 500 μ L with Tris-HCl buffer (50 mM, pH 8.0) and then incubated at 37 $^{\circ}$ C in a shaking incubator at 120 rpm for 6 h. NIR fluorescence recovery from hCy was detected at 715 nm upon excitation at 670 nm.

Cell Culture

NK-92MI cells were cultured in MEM- α medium supplemented with 12.5% horse serum, 0.2 mM inositol, 0.02 mM folic acid, 0.1 mM β -mercaptoethanol, 12.5% fetal bovine serum, and 100 mg/mL streptomycin and penicillin. A549 cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 100 μ g/mL streptomycin and penicillin. All cells were cultured at 37 $^{\circ}$ C in a humidified atmosphere containing 5% CO₂. Cell numbers were counted using a Countess II FL Automated Cell Counter (Thermo Fisher Scientific).

Preparation of DNA-Scaffolded NK Cells

The Poly-DNA scaffold was constructed on the NK-92MI cell surface via insertion of a cholesterol-modified DNA strand into the cell membrane and DNA self-assembly. Cultured NK-92MI cells (1×10^6 in 1 mL medium) were transferred to a centrifuge tube and centrifuged at 800 rpm for 5 min to remove serum proteins and cellular metabolic products from the culture medium, and were gently resuspended by pipetting. To determine the appropriate anchor-DNA concentration, we performed a flow cytometry-based optimization prior to the reported experiments. 1×10^6 NK-92MI cells were incubated with 200 μ L of cholesterol-modified anchor-DNA_{TAMRA} at three different concentrations (0.5, 1, and 1.5 μ M), with a PBS-treated group as a control. Based on the flow cytometry results, 1 μ M was chosen as the optimal incubation concentration for anchor-DNA. Under this condition, the cells were incubated at room temperature for 5 min and then centrifuged again at 800 rpm for 5 min. To optimize the poly-DNA coating on NK_{anchor-DNA} cells, the as-treated cells were incubated with 800 μ L of poly-DNA_{FAM} at three concentrations (40, 100, and 200 nM). A PBS-treated group was included as a control. Based on the flow cytometry results, 100 nM was chosen as the optimal concentration of poly-DNA for NK cell coating. Under this condition, the as-treated NK_{anchor-DNA} cells were incubated with 200 μ L each of 100 nM poly-DNA_{GOx}, poly-DNA_{CAT}, poly-DNA_{IL}, and a poly-DNA_{Rep} mixture at 37 $^{\circ}$ C for 30 min to allow for base-pairing assembly. The as-treated cells were then centrifuged at 800 rpm for 5 min, followed by being resuspended and washed three times with RPMI 1640 medium to remove unreacted poly-DNA, yielding DNA-scaffolded NK cells.

To visualize the assembly of the DNA scaffold on NK cells, a TAMRA-modified anchor strand anchor-DNA_{TAMRA} and an FAM-labeled linker strand poly-DNA_{GOx/FAM} were employed to label the membrane-anchored DNA network. The resulting DNA-scaffolded NK cells were imaged using a confocal laser scanning microscope (CLSM). FAM fluorescence was collected at 500–530 nm upon 488 nm excitation. TAMRA fluorescence was collected at 570–600 nm upon 550 nm excitation.

Preparation of Multimodular Intelligent NK (MI-NK) Cell

To prepare MI-NK cells, the 1×10^6 as-obtained DNA-scaffolded NK cells were incubated with 200 μ L of the above-prepared barrier-penetration module (GOx-DNA and CAT-DNA each at 250 nM), 200 μ L of the 250 nM activity-boosting module IL-NPs-DNA, and 200 μ L of the 250 nM efficiency-reporting module IEPD-hCy-DNA, with a certain proportion in phenol red-free DMEM at 37 $^{\circ}$ C, 5% CO₂ for 30 min, washed three times with 200 μ L RPMI 1640 medium, and centrifuged at 800 rpm for 5 min to remove any remaining unreacted materials. To visualize modifications of different modules on NK cells, DNA_{GOx/TAMRA}, DNA_{CAT/TAMRA}, DNA_{IL/FAM}, and DNA_{Rep/Cy5} were reacted to prepare GOx-DNA_{TAMRA}, CAT-DNA_{TAMRA}, IL-NPs-DNA_{FAM} and IEPD-hCy-DNA_{Cy5}. The 1×10^6 DNA-scaffolded NK cells were coincubated with 250 nM GOx-DNA_{TAMRA}, CAT-DNA_{TAMRA}, IL-NPs-DNA_{FAM}, and IEPD-hCy-DNA_{Cy5} (200 μ L each), and the as-obtained MI-NK_{TAMRA/FAM/Cy5} cells were observed under a confocal laser scanning microscope (CLSM). TAMRA fluorescence was collected at 570–600 nm under 550 nm excitation. FAM fluorescence was collected at 500–530 nm under 488 nm excitation. Cy5 fluorescence was collected at 655–695 nm under 633 nm excitation.

To prepare the control-group NK modules that directly anchor modules to NK cells in the absence of a DNA scaffold, membrane-direct anchors—cholesterol-modified linker-DNA_{GOx} (c-linker-DNA_{GOx}), cholesterol-modified linker-DNA_{CAT} (c-linker-DNA_{CAT}), cholesterol-modified linker-DNA_{IL} (c-linker-DNA_{IL}), and cholesterol-modified linker-DNA_{Rep} (c-linker-DNA_{Rep})—were anchored to the NK cell membrane. The c-linker-DNA_{GOx}, c-linker-DNA_{CAT}, c-linker-DNA_{IL}, and c-linker-DNA_{Rep} are complementary to the DNA_{GOx}, DNA_{CAT}, DNA_{IL}, and DNA_{Rep}. The above-obtained anchor-DNA modified NK cells were gently resuspended by pipetting and incubating at room temperature for 5 min, and subsequently incubated with GOx-DNA_{TAMRA}, CAT-DNA_{TAMRA}, IL-NPs-DNA_{FAM}, and IEPD-hCy-DNA_{Cy5} at a 1:1:1:1 ratio in phenol red-free DMEM at 37 $^{\circ}$ C, 5% CO₂ incubator for 30 min, washed three times with RPMI 1640 medium, and centrifuged at 800 rpm for 5 min to remove any remaining unreacted materials to obtain directly anchored multimodular intelligent NK cells (DA-MI-NK). DA-MI-NK cells were observed under a confocal laser scanning microscope (CLSM). TAMRA fluorescence was collected at 570–600 nm under 550 nm excitation. FAM fluorescence was collected at 500–530 nm under 488 nm excitation. Cy5 fluorescence was collected at 655–695 nm under 633 nm excitation.

SEM Characterization of MI-NK Cell

The as-obtained MI-NK cell was fixed in 2.5% glutaraldehyde for 4 h and subsequently subjected to gradient dehydration with 30%, 50%, 70%, 80%, and 90% ethanol solutions for 5 min each, followed by centrifugation at 800 rpm for 5 min. The as-obtained sample was dehydrated twice in 100% ethanol for 5 min each, and centrifuged at 800 rpm for 5 min after each step. The as-treated sample was then resuspended in 10 μ L of 100% ethanol, dropped onto a silicon wafer, and allowed to dry naturally at room temperature for 5 min. The naked NK cell was dehydrated and prepared using the same procedure.

In Vitro Motility Characterization of MI-NK Cell

The self-propulsion capabilities of MI-NK cells were characterized in a customized double-arrow PDMS mold that was oxygen-plasma-treated prior to use. Glucose and MI-NK cells labeled with TAMRA (MI-NK_T) were added to the left end of the mold. A series of MI-NK_T cells were prepared with different ratios of GOx and CAT, and

TAMRA fluorescence intensity was measured at 580 nm at fixed sampling points at the right end of the PDMS mold to observe self-propulsion. Different concentrations of fuel (Glu) were added to the left end of the mold to explore the effect of fuel concentration on the self-propulsion capability of MI-NK_T. Control group AB-MI-NK_T was prepared in the absence of the barrier penetration module for DNA-scaffolded NK cells. AC-MI-NK_T was prepared only with GOx-DNA in the absence of CAT-DNA.

The motion of MI-NK cells was also captured using an inverted optical microscope equipped with a 40× objective lens. Aliquots of 10 μL of Glu solutions at different concentrations were dropped onto a glass slide. Subsequently, 5 μL of MI-NK cell suspension was added. Movies of MI-NK cell motion and the corresponding movement trajectories were captured and recorded. Movement trajectories were traced and analyzed using the Leica MM AF 1.5 system and GraphPad Prism software. Based on the extracted trajectories, the motion speed of MI-NK cells was calculated using the equation:

$$\text{Speed} = \chi / t \quad (4)$$

where χ and t represent the travel distance and duration of the MI-NK cell, respectively.

The corresponding mean squared displacement (MSD) was determined according to the equation:⁵³

$$\text{MSD} = (x_{\Delta t} - x_0)^2 + (y_{\Delta t} - y_0)^2 \quad (5)$$

where (x_0, y_0) and $(x_{\Delta t}, y_{\Delta t})$ refer to the positions of the MI-NK cell at time point of t_0 and after time interval of Δt , respectively.

The dissolved oxygen levels during MI-NK cell movement were also monitored in real time. A total of 1×10^5 MI-NK cells were added to 1 mL of PBS in a 1.5 mL centrifuge tube, along with different concentrations of Glu. A portable dissolved oxygen meter was used to monitor the dissolved oxygen content in PBS in real time, thereby capturing fluctuations in dissolved oxygen during the enzymatic cascade reactions and MI-NK cell movement.

In Vitro Barrier Penetration Capability Characterization of MI-NK Cell

Penetration capability of MI-NK cells in tumor tissue was characterized with a Transwell assay. 1×10^5 A549 cells were seeded in the upper chambers of each insert (polycarbonate filter, 8 μm pore size, Corning) in a 12-well Transwell plate filled with RPMI 1640 medium devoid of Glu and serum to mimic the interior of tumor tissue. A total of 1×10^5 MI-NK cells, prestained with DAPI, were introduced into the upper chamber, while varying concentrations of Glu solution were added to the lower chamber. The setup was then incubated at 37 °C with 5% CO₂. At different time points, MI-NK cells that had migrated through the membrane to the lower chamber were imaged using a confocal microscope to evaluate their migration and penetration ability. AB-MI-NK cells were used as the control group and characterized using the Transwell plate.

The penetration capability of MI-NK cells was further characterized using a 3D multicellular tumor spheroid (MTS) model. To establish an A549 MTS model, poly(2-hydroxyethyl methacrylate) (pHEMA) gel solution was evenly spread at the bottom of a T25 cell culture flask and then placed in a 45 °C oven to allow the alcohol to evaporate and form a gel.⁵⁴ The flask was then transferred to a laminar flow hood and sterilized by UV light irradiation for 2 h. A total of 5×10^5 A549 cells suspended in 5 mL RPMI 1640 medium containing 10% FBS were seeded into the pHEMA-coated T25 cell culture flask prepared above and incubated at 37 °C in a humidified incubator with 5% CO₂. The medium was changed on days 3, 5, and 7 of culture, and the morphology and growth of the tumor spheroids were observed using an inverted microscope, and A549 3D MTSs spontaneously formed within 7 days. The as-obtained A549 3D MTS model was then aspirated and seeded into confocal culture dishes and washed three times with PBS, then coincubated with MI-NK_{TAMRA} cells and AB-MI-NK_{TAMRA} cells, respectively, at 37 °C in a humidified incubator with 5% CO₂ for 6 h. The as-treated 3D MTSs were

then washed with PBS, resuspended, and imaged using a CLSM imaging system.

In Vitro Activity-Boosting Capability Characterization of MI-NK Cell

The activity-boosting capability of MI-NK cells was achieved by GSH-responsive IL-21 release. To visualize GSH-responsive IL-21 release, A549 cells were seeded at a cell density of 1×10^5 per well in confocal dishes and incubated at 37 °C in a 5% CO₂ incubator for 24 h. The as-prepared A549 cells were cocultured with 1×10^5 MI-NK_{TAMRA/FAM} cells that were prepared with a TAMRA labeled DNA scaffold_{TAMRA} and FAM-labeled IL-NPs-DNA_{FAM} for 12 h in 1640 culture medium with 5 mM GSH. The supernatant containing MI-NK_{TAMRA/FAM} was collected and centrifuged at 800 rpm for 5 min, and washed with PBS. GSH-responsive release of IL-21 from MI-NK_{TAMRA/FAM} was visualized by measuring FAM and TAMRA fluorescence under CLSM. TAMRA fluorescence was collected at 570–600 nm with 550 nm excitation. FAM fluorescence was collected at 500–530 nm with 488 nm excitation. A control experiment was performed with the same procedure in the absence of GSH.

To verify NK cell proliferation induced by IL-21 release, MI-NK cells were labeled with carboxyfluorescein succinimidyl ester (CFSE; 1 μg/mL). The cells were then resuspended and cultured in RPMI 1640 medium with or without 5 mM GSH. After 48 h of culture, CFSE fluorescence intensity in MI-NK cells was analyzed by flow cytometry. The dilution of CFSE fluorescence was used to evaluate NK cell proliferation. Control experiments were performed using MI-NK cells and the NK-DNA scaffold in the absence of GSH.

To verify NK cell activation induced by IL-21 release, MI-NK cells were treated with 5 mM GSH and cultured in an incubator at 37 °C with 5% CO₂ for 12 h. Secretion of the effector molecules interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and Granzyme B (GzmB) was measured using ELISA kits.

In Vitro Efficiency-Reporting Capability Characterization of MI-NK Cell

The efficiency of MI-NK cells was characterized by Granzyme B (GzmB) secretion, which cleaved the substrate peptide IEPD and restored the hCy fluorescence of the efficiency reporting module. A549 cells were seeded in confocal dishes and cultured in RPMI 1640 medium at 37 °C in a 5% CO₂ incubator for 24 h, then MI-NK cells were added and treated with GSH for 6 h. The suspended MI-NK cells were collected and resuspended in 200 μL PBS. The recovery of the NIR fluorescence of hCy was recorded on a confocal microscope at 715 nm with 670 nm excitation. Control groups were untreated MI-NK cells and MI-NK cells co-cultured with A549 cells but without GSH treatment.

In Vitro Tumor Cell-Killing Capability Characterization of MI-NK Cell

NK-Tumor Cell Conjugation Assay. To verify the effector-target (E:T) engagement between MI-NK cells and tumor cells, three experimental groups were included: unmodified NK cells, NK_{anchor-DNA} cells, NK_{DNA scaffold} cells, MI-NK cells. 1×10^6 A549 cells were labeled with CFSE (1 μg/mL) at 37 °C for 10 min and washed twice with PBS to remove excess dye. Meanwhile, 1×10^6 NK cells were stained with DiD (5 μM) at 37 °C for 10 min and washed twice with medium. The labeled NK cells were then coincubated with A549 cells at an E:T ratio of 1:1 in RPMI 1640 medium at 37 °C for 45 min. After incubation, the cell mixtures were fixed in 1% PFA and analyzed by flow cytometry. NK-tumor conjugates were identified as DiD⁺/CFSE⁺ double-positive events, and the percentage of conjugated NK-tumor cell pairs was quantified based on the proportion of DiD⁺/CFSE⁺ double-positive events among all analyzed cells.

Western Blot Assay. NK cells and MI-NK cells were divided into four groups: G1: NK cells; G2: IL-2-activated NK cells; G3: MI-NK cells; and G4: IL-2-activated MI-NK cells. For activation, 1×10^6 cells were resuspended in 200 μL of culture medium and treated with recombinant human IL-2 (1000 IU/mL) for 15 min. After stimulation, cells were immediately placed on ice to terminate

activation and lysed in RIPA buffer containing protease and phosphatase inhibitors for 40 min. The total protein concentration was determined using a BCA protein assay. Equal amounts of protein were separated by SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with 5% BSA and incubated with primary antibodies against AKT and phosphorylated AKT (p-AKT, Ser473) overnight at 4 °C, followed by incubation with HRP-conjugated secondary antibodies. Protein bands were visualized using an enhanced chemiluminescence (ECL) detection system.

CKK-8 Assay. 100 μ L of A549 cell suspension was seeded in a 96-well plate at a density of 5×10^4 cells mL^{-1} per well, and cultured at 37 °C for 24 h, subsequently, 100 μ L of 5×10^4 NK cells, NK cells with Glu and GSH treatment, MI-NK cells, MI-NK cells with GSH treatment, MI-NK cells with Glu treatment, DA-MI-NK cells MI-B cells, and MI-NK cells with both Glu and GSH treatment were added, and the cultures were continuously cocultured for 12 h. NK cells were then aspirated from the 96-well plate. The seeded A549 cells were then washed with PBS three times, 10 μ L of WST-8 solution was added per well, and the cells were incubated for 3 h to test cell viability. A549 cells seeded in a 96-well plate without any treatment were set as the control group. The absorbance was measured at 450 nm with a microplate reader.

Cytotoxicity Evaluation. A549 cells were seeded in confocal dishes and cultured in RPMI 1640 medium at 37 °C in a 5% CO_2 incubator for 24 h, and were subsequently treated with multimodule B (MI-B) cells pretreated with GSH and Glu, MI-NK cells, MI-NK cells pretreated with GSH, MI-NK cells pretreated with Glu, DA-MI-NK cells pretreated with GSH and Glu, and MI-NK cells pretreated with GSH and Glu, and cocultured for 6 h. After cocubation, the culture medium containing suspended NK cells was carefully removed, and only the adherent A549 cells were retained for subsequent analysis. The as-treated A549 cells were gently washed three times with PBS, stained with the live/dead staining reagents Calcein AM and PI, and observed under a confocal microscope.

The apoptosis of A549 cells in different treatment groups was evaluated by flow cytometry. After incubation, the culture medium containing a suspension of NK cells was carefully removed, and the adherent A549 cells were gently washed three times with PBS to eliminate residual effector cells. A549 cells that were treated with MI-B cells pretreated with GSH and Glu, MI-NK cells, MI-NK cells pretreated with GSH, MI-NK cells pretreated with Glu, DA-MI-NK cells pretreated with GSH and Glu, and MI-NK cells pretreated with GSH and Glu were washed three times with PBS, digested with EDTA-free trypsin, and stained with an Annexin V-FITC/PI assay kit for cell apoptosis analysis.

In Vivo Fluorescence Characterization of MI-NK Cell

Tumor Accumulation. Female BALB/c nude mice (4–6 weeks old) were used to establish a subcutaneous lung tumor model by injecting A549 cells (1×10^7 cells) into the lateral side of the right hind limb. Tumor volumes were calculated using the formula $V = (L \times W^2)/2$, where L and W represent the length and width of the tumor, respectively. When the tumor volume reached approximately 80 mm^3 , the mice were randomly assigned to different groups for *in vivo* experiments. All *in vivo* procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23 Rev. 1985) and approved by the Science and Technology Ethics Committee of Nanjing University under approval number IACUC-2506004.

To visualize the accumulation of MI-NK cells at the tumor site after intravenous injection, the cells were stained with the cell membrane dye DiD. The as-obtained AE-MI-NK_{PS/DiD} cells (100 μ L, 1×10^7 cells) were injected into the tail vein of mice, with NK_{DiD} cells and ABE-MI-NK_{PS/DiD} cells serving as control groups, and the biodistribution of DiD fluorescence was monitored at different time points at 700 nm under 650-nm excitation using an *in vivo* imaging system.

Biosafety Evaluation. 100 μ L AML-12 cell suspension was seeded in a 96-well plate at a density of 5×10^4 cells mL^{-1} per well and cultured at 37 °C for 24 h to allow cell attachment, subsequently,

100 μ L of 5×10^4 MI-NK cells and MI-NK cells, both with Glu and GSH treatment, were added, and the cells were continuously cocultured for 12 h. NK cells were then aspirated from the 96-well plate. The seeded AML-12 cells were washed three times with PBS, 10 μ L WST-8 solution was added to each well, and the plate was incubated for 3 h to test cell viability. AML-12 cells seeded in a 96-well plate without any treatment were set as the control group. The absorbance was measured at 450 nm with a microplate reader.

In Vivo Therapeutic Effect of MI-NK Cell

A549 tumor-bearing mice were randomly divided into seven groups ($n = 16$; five for tumor measurement, five for survival monitoring, three for FL imaging, three for flow cytometry and intravenously injected as follows: Group 1, saline; Group 2, 1×10^7 MI-B_{PS} cells that assembled three modules onto B cells via a DNA scaffold; Group 3, 1×10^7 ABA-MI-NK_{PS} cells that were prepared in the absence of the barrier penetration module and the activity boosting module; Group 4, 1×10^7 AB-MI-NK_{PS} cells that were prepared in the absence of the barrier penetration module; Group 5, 1×10^7 AA-MI-NK_{PS} cells that were prepared in the absence of the activity boosting module; Group 6, 1×10^7 DA-MI-NK_{PS} cells that were directly modified with the three modules via cholesterol insertion into the membrane; and Group 7, 1×10^7 MI-NK_{PS} cells. The administration was performed on days 1, 6, 11, and 16, respectively. The body weight of the mice as well as the tumor size were measured every 3 days. On the sixth day, 12 h after injection, subcutaneous tumor tissues from three mice were collected and placed in precooled PBS. The tumors were minced and digested in a dissociation buffer containing 1 mg/mL collagenase I, 0.5 mg/mL DNase I, and 0.5 mg/mL hyaluronidase at 37 °C with shaking for 40 min. The resulting cell suspension was filtered, centrifuged, and treated with red blood cell lysis buffer, and then resuspended in FACS buffer. Approximately 1×10^6 cells were used for staining. After blocking with human Fc-blocking reagent for 10 min, cells were incubated with antihuman CD45-FITC, CD56-APC, and CD107a-PE antibodies for 30 min in the dark. After two washes with FACS buffer, the cells were resuspended and analyzed by flow cytometry to assess the infiltration of human CD45⁺ and CD56⁺ NK cells in the tumor and the percentage of CD107a⁺ cells, reflecting NK cell degranulation activity. On day 22, five mice were sacrificed for tumor assessment and TUNEL and H&E staining to assess the therapeutic effect. In addition, major organs such as the heart, liver, spleen, lungs, and kidneys were dissected and collected for histopathological analysis. To evaluate the level of NK cell activation, mice were sacrificed and their blood was collected at the end of the treatments, the expression levels of IL-2, GzmB, and TNF- α in the mouse serum were measured with ELISA kits. The remaining five mice were used for survival monitoring.

Function Evaluation. Time-corresponding NIR fluorescence was recorded *in vivo* to evaluate adoptive cell function. *In vivo* fluorescence imaging was first performed after MI-NK administration. NIR fluorescence recovery was recorded at 715 nm under 670 nm excitation using an *in vivo* imaging system at 0, 4, 6, 8, 12, and 24 h after adoptive NK cell administration for Group 2: MI-B_{PS} cell, Group 3: ABA-MI-NK_{PS} cell, Group 4: AB-MI-NK_{PS} cell, Group 5: AA-MI-NK_{PS} cell, and Group 7: MI-NK_{PS} cell administrated mice, respectively.

Statistical Analysis. Statistical analyses and illustrations were conducted using GraphPad Prism (Version 9.5.0). Sample sizes are specified in the figure captions, where n refers to the number of distinct samples. All values are expressed as the mean $\pm$ standard deviation. Comparisons between two groups were performed using an unpaired, two-tailed Student's *t*-test. For comparisons involving multiple groups or time points, two-way ANOVA with appropriate multiple comparisons was used. A *P*-value <0.05 was considered statistically significant.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsnano.6c10559>.

Supporting Movie S1: MI-NK cell motion under different Glu concentrations (MP4)

Supporting Movie S2: Brownian motion of AB-MI-NK cells in 5 mM Glu (MP4)

Supporting Figures S1–S10: Schematic of poly-DNA synthesis and NK_{DNA scaffold} construction, PAGE gels, GPC characterization of poly-DNAs, optimization and characterization of anchor-DNA insertion into the NK cell membrane, optimization and characterization of DNA scaffold assembly and stability on NK cells. Supporting Figures S11–S25: Synthesis and characterization of functional modules, CLSM imaging, fluorescence spectroscopy, zeta potential, DLS, mass spectrometry, HPLC characterization, and stability and functional validation of DNA-based modules on NK cells. Supporting Figures S26–S33: PDMS device construction, fluorescence imaging and motion analysis, glucose-responsive self-propulsion, and barrier penetration validation in Transwell and 3D tumor spheroid models. Supporting Figures S34–S43: Evaluation of NK cell proliferation, cytokine secretion, efficiency reporting, cell viability, CD16 accessibility, tumor cell conjugation, AKT signaling, and tumor cell killing capability. Supporting Figures S44–S55: In vivo tracking and biodistribution, antitumor efficacy, histological and biosafety evaluation, ex vivo fluorescence imaging, tumor infiltration, and ELISA analysis. Supporting Tables S1–S2: DNA sequences, material names, and corresponding abbreviations (PDF)

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Author Contributions

C.C. designed and performed all experiments, collected the experimental data, and performed data analysis. C.C. wrote the original draft. J.S. and R.W. helped with data analysis and cell experiments. Y.Z. and Z.C. helped with organic synthesis experiments. Prof. Y.L. supervised the project and revised the manuscript. Prof. H.J. provided advice and suggestions regarding the experimental data. All the authors discussed the results.

Notes

The authors declare no competing financial interest.

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